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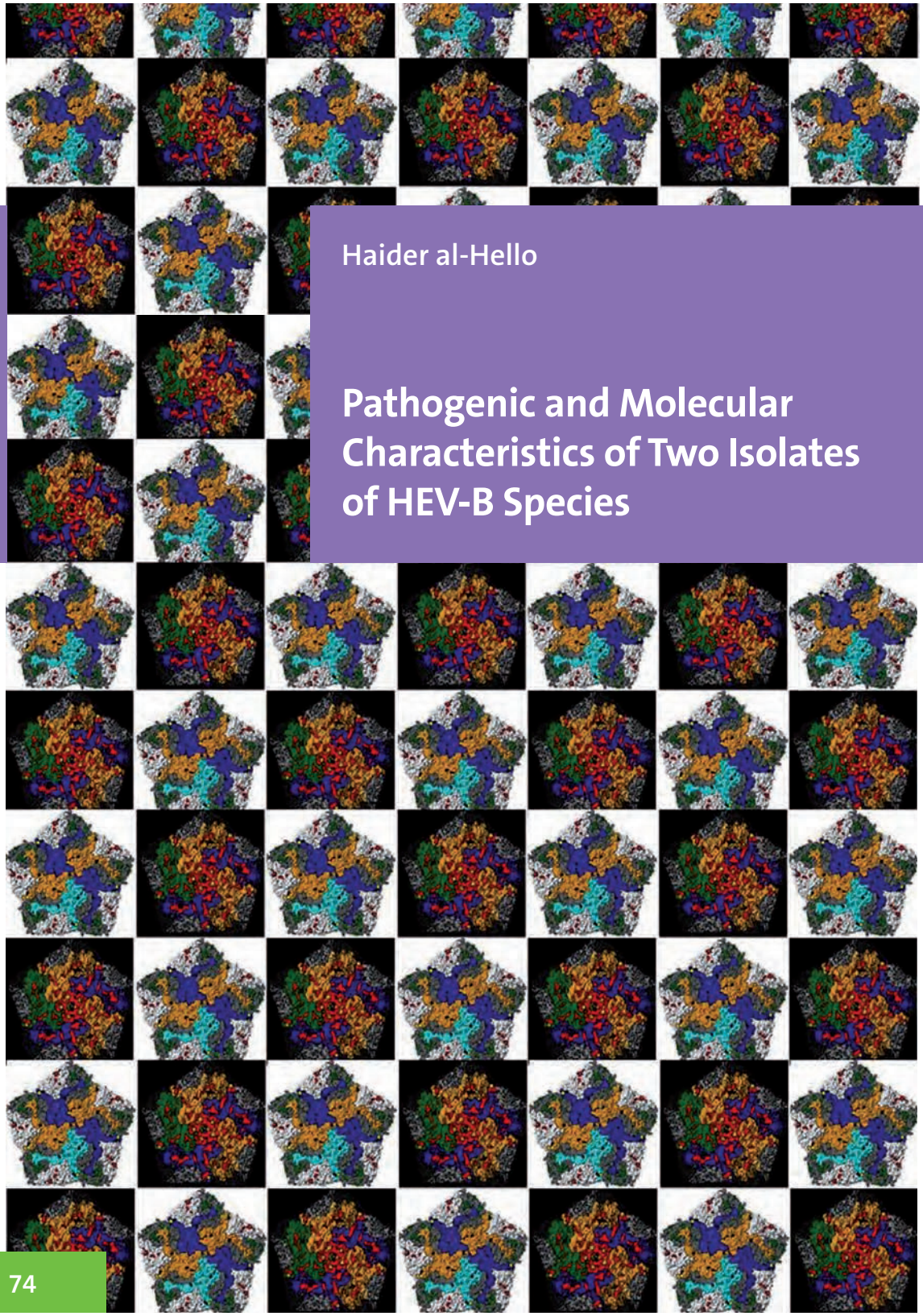

RESEARCH

Haider al-Hello

Pathogenic and Molecular Characteristics of Two Isolates of HEV-B Species

RESEARCH 74

Haider al-Hello

PATHOGENIC AND MOLECULAR CHARACTERISTICS OF TWO ISOLATES OF HEV-B SPECIES

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki for public examination in the auditorium 1041 of Biocenter 2, Viikinkaari 5, Helsinki on March 16th, 2012, at 12 o'clock noon

National Institute for Health and Welfare
Department of Infectious Disease Surveillance and Control
Virology Unit
Helsinki, Finland

and

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Faculty of Biological and Environmental Sciences
Department of Biosciences
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To the only one .....

Abstract

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Enteroviruses (EVs) are small non-enveloped RNA viruses forming a large group of different serotypes. EVs belong to the family *Picornaviridae*. The primary replication site of an enterovirus is typically the epithelium of the respiratory tract and the gastrointestinal mucosa. Virus replication in the gastrointestinal mucosa may continue, often asymptomatically, for several weeks occasionally causing viremia. During the viremia the virus spreads through the lymphatic system and circulation. Organ-specific symptoms rise after viral replication in the secondary target tissues. Occasionally, cellular adaptation is required for a virus to initiate replication in the secondary target tissue(s). Adaptation is linked to mutation(s) which may lead to alteration in cellular tropism, e.g., recognition of new surface receptor molecules or other host cell constituents essential for virus entry and replication. However, the critical step may also occur later during the interaction of the host cell and the replicating virus.

In the present study, genetic changes responsible for altered phenotypic features were sought using two strains of *Human enterovirus B* (HEV-B) species. Firstly, a laboratory isolate of coxsackievirus B5 (CV-B5), strain DS, was passaged 15 times in mouse pancreas *in vivo*, which resulted in a diabetogenic mouse pancreas passaged virus strain (MPP). The concept of "diabetogenic" means the ability of the MPP strain to replicate, cause insulitis and dysregulation of the glucose metabolism in the mouse pancreas *in vivo*. The interaction between the MPP virus strain and insulin producing β-cells was further studied in cell culture using a mouse-derived insulinoma cell line, MIN-6 cells, as an experimental model. The replication of the MPP virus strain was clearly slower in the MIN-6 cells compared to the other tested cell lines. After three days of incubation, extensive replication of MPP was evident in MIN-6 cells and resulted in a MIN-6 cell-adapted virus strain (MCA). Secondly, the ability of the D207 virus strain, isolated from a type 1 diabetic patient, to replicate in a primary human β-cell culture was tested. D207 was initially serotyped as coxsackievirus A9 (CV-A9) in a virus-specific neutralization assay. The D207 virus strain was found to cause cytolysis in the primary human β-cells and, simultaneously, severe functional damage of the surviving β-cells.

The genomes of the four virus strains DS, MPP, MCA and D207 were cloned and sequenced. The sequence comparison of three CV-B5 strains (DS, MPP, and MCA) revealed only limited changes, three capsid and two non-structural (NS) amino acid substitutions between MPP and DS, and two capsid and six NS amino acid substitutions between MCA and MPP. In order to determine which of the amino

acid substitutions were responsible for the changed phenotype *in vivo* and *in vitro*, full-length infectious clones were constructed from the MPP virus and its parental DS virus. By using reverse mutagenesis and chimeric viruses (MPP/DS and DS/MPP), it was shown that a change from MPP to the MCA phenotype in MIN-6 cells was mediated by only a single amino acid at position 94 in VP1, while the *in vivo* adaptation of the DS virus strain to the inflammation-inducing MPP virus strain may require multiple genetic determinants in the virus capsid and probably also in the NS proteins.

Sequence analyses of D207 revealed that the virus belonged to a genogroup D of E-11, but was also neutralized with monotypic antisera to CV-A9. The isolate D207 was found to be closely related to a specific E-11 strains known to cause uveitis. Uveitis-causing E-11 strains were also found to be well neutralized with both CV-A9- and E-11-specific antisera. In a further study, a wide range of E-11 isolates were included to test the observed dual neutralizibility among isolates belonging to the D genogroup. Five of the six studied strains belonging to genogroup D were also neutralized with antisera against coxsackievirus A9 Griggs. The peptide scanning technique was utilized to identify antigenic regions of the capsid proteins of the D207 strain responsible for the observed dual neutralization. Several regions in the capsid of D207 were found to cross-react with an antiserum raised against CV-A9. However, epitopes responsible for the cross-neutralization remained unidentified.

In conclusion, these studies indicate that the specific location of mutation may affect the phenotype of an enterovirus more than the overall quantity of changes. In the experimental settings, radical changes in the viral phenotypic features occurred only after a few amino acid substitutions. The majority of the studied viruses in the genogroup D of E-11 maintained exceptional phenotypic property, the cross-neutralization with CV-A9 specific antiserum, despite their genetic divergence.

Keywords: infection, viremia, secondary target tissue, adaptation, virus receptor, passaged virus strain, *in vivo*, coxsackievirus B5 strains MPP and MCA, *in vitro*, reverse mutagenesis, genetic determinant, antisera, coxsackievirus A9, E-11, dual neutralization, epitope.

Tiivistelmä

Haider al-Hello. Pathogenic and Molecular Characteristics of Two Isolates of HEV-B Species. [Kahden HEV-B-lajiin kuuluvan viruskannan patogeeniset ja molekulaariset ominaisuudet]. Terveyden ja hyvinvoinnin laitos. Tutkimus 74. XXX sivua. Helsinki, Suomi 2012. ISBN 978-952-245-583-3 (painettu); ISBN 978-952-245-584-0 (pdf)

Pikornaviruksiin kuuluvat ihmisen enterovirukset ovat pieniä, pyöreitä ja vaipattomia RNA-viruksia, joista tunnetaan suuri määrä erilaisia serotyyppejä. Enterovirusinfektiot alkavat yleensä hengitystien ja suoliston epiteelissä, jossa suurin osa viruksista kulkeutuu mahalaukun läpi suolistoon. Enterovirukset aiheuttavat pääasiallisesti systeemisiä yleisinfektioita, joiden oirekirjo määräytyy virusten toissijaisen lisääntymispaikan mukaan. Joskus enteroviruksen lisääntyminen toissijaisessa lisääntymispaikassa edellyttää sopeutumisprosessia. Sopeutuminen johtuu geneettisistä mutaatioista, jotka voivat johtaa uuden solunpintareseptorimolekyylin tai muun isäntäsolun molekyylin tunnistamiseen ja jotka ovat välttämättömiä virusten pääsylle soluun. On myös mahdollista, että sopeutumiselle kriittinen vaihe on myöhempi vuorovaikutus isäntäsolun ja lisääntyvän viruksen välillä.

Tutkimuksen tarkoituksena oli tunnistaa fenotyyppisistä ominaisuuksista vastaavia virusgenomin osia kahdesta ihmisen enterovirus B -lajiin kuuluvasta viruksesta. Coxsackie B5 -viruskanta (DS) saatiin muuntumaan kasvattamalla sitä viisitoista kertaa hiiren haimassa *in vivo*. Muuntuneiden virusten joukosta löytyi ns. diabetogeeninen virus (diabetogeeninen CV-B, CV-B5-MPP), joka sai aikaan kroonisen tulehdusreaktion hiiren haimassa. Osalla hiiristä todettiin myös häiriötä sokerimetaboliassa. Seuraavaksi MPP-infektiota tutkittiin yksityiskohtaisemmin hiiren insulinomasolulinjassa (MIN-6). Tutkimuksessa havaittiin, että edellä kuvatun MPP-viruksen lisääntyminen on hyvin hidasta. Replikaatio kiihtyi vasta kolmen vuorokauden kuluttua. Tutkimuksessa tarkasteltiin myös tyypin 1 diabeetikolta (lapsi) eristetyn coxsackievirus A9:n (CV-A9) molekyyligeneettisiä ja biologisia ominaisuuksia. Kyseinen kanta (D207) oli eristetty Slovakiassa, jossa se tyypitettiin serologisin testein coxsackievirus A9:ksi. D207-kanta infektoi ihmisen primäärisiä insuliinia tuottavia β-soluja tehokkaasti aiheuttaen solutuhon sekä vakavan toiminnallisen häiriön eloonjääneissä β-soluissa.

Neljän viruksen DS, MPP, MCA ja D207 genomit kloonattiin ja sekvensoitiin. Ensimmäisten kolmen viruksen sekvenssit analysoitiin ja havaittiin, että DS- ja MPP-kantojen välillä löytyi vain viisi aminohappoeroa, joista kolme sijaitsi kapsid- ja kaksi ei-rakenneproteiinissa. Vertailtaessa MPP- ja MCA-kantoja niissä havaittiin kahdeksan aminohappoeroa, joista kaksi kapsid- ja kuusi ei-rakenneproteiineissa. Kapsidialueelta löydettyjen aminohappomuutosten merkitys selvitettiin ns. kohdennetun mutageneesin avulla. Hiiren haimassa *in vivo* kasvatetun CV-B5-MPP-

viruksen infektiivisen genomin mutaatiokohdat muutettiin yksitellen alkuperäiseen DS- tai MCA-viruskannan kaltaisiksi. Tutkimuksessa tehtiin myös kaksi rekombinanttivirusta liittämällä MPP:n viruskannan 5'-UTR-kapsidi-alue DS viruskannan ei-rakenneproteiinin 3'UTR-alueeseen ja vastaavasti päinvastoin (MPP/DS ja DS/MPP). Tulokset mutanteilla tehdyistä MIN-6-solujen infektiokokeista osoittivat selvästi, että kapsidiproteiinin VP1 aminohappo 94 (Ile) oli vastuussa viruksen lisääntymisestä insuliinia tuottavissa MIN-6-soluissa. Sen sijaan in vivo infektio oli monimutkaisempi ja vaati useampia aminohappomuutoksia kapsidissa ja todennäköisesti myös ei-rakenneproteiini-alueella.

D207-viruksen sekvenssien analyysi muihin virussekvensseihin verrattuna osoitti, että D207 kuuluu echovirus 11:n (E-11) alaryhmään D. Tästä huolimatta viruksen havaittiin neutraloituvan hyvin CV-A9 spesifisellä neutraloivalla vasta-aineella. Kaksoisneutraloituvuus osoittautui olevan yhteinen ominaisuus suuremmalla osalla E-11-virusten D-alaryhmään kuuluvilla viruksilla. Jatkotutkimuksen tulokset osoittivat, että kaikki tutkitut kannat neutraloituivat odotetusti E-11:n prototyyppikannan Gregoryn antiseerumilla. Yllättävää oli se, että viisi kuudesta D-genoryhmään kuuluvasta viruskannasta neutraloitui myös CV-A9:n Griggs-prototyypin antiseerumilla. Tämä osoitti, että tutkituilla echoviruskannoilla oli yhteisiä antigeenisiä alueita CV-A9:n kanssa. Vaikka monia ristiinreagoivia antigeenisiä peptidejä on löydetty, tässä tutkimuksessa neutraloituvuudesta vastaavia epitooppeja ei yrityksistä huolimatta kyetty tunnistamaan.

Nämä tutkimukset osoittivat, että mutaation sijainnilla on enemmän vaikutusta enteroviruksen fenotyyppiin kuin kokonaismutaatiomäärällä. Koejärjestelyissä vain muutamat aminohapposubstituutiot johtivat radikaaleihin muutoksiin virusfenotyypissä. Suuralla osalla E-11 D -alaryhmän viruksia oli poikkeuksellinen fenotyyppi omaisuus, ristiin neutraloituminen CV-A9 -spesifisellä antiseerumilla, vaikka virukset olivat geneettisesti hyvin erilaisia.

Avainsanat: infektio, toissijainen lisääntymispaikka, reseptorimolekyyli, muokattu viruskanta, coxsackievirus B5 kannat MPP ja MCA, kohdennettu mutageneesi, geneettinen determinantti, vasta-aine, coxsackievirus A9.

Contents

List of original papers

I al-Hello Haider, Davydova B., Smura T., Kaialainen S., Ylipaasto P., Saario E., Hovi T., Rieder E., Roivainen M. (2005). Phenotypic and genetic changes in coxsackievirus B5 following repeated passage in mouse pancreas *in vivo*. J Med Virol 75, 566-74.

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Abbreviations

ATCC	American Type Culture Collection
CAR	coxsackievirus and adenovirus receptor
CL	cloverleaf
CRE	cis-acting replication element
CV-A	coxsackievirus A
CV-B	coxsackievirus B
DAF	decay-accelerating factor
DiMe	Diabetes Intervention and Management with Excellence
DIPP	Diabetes Prediction and Prevention
HEV	human enterovirus
HRV	human rhinovirus
ICAM-1	intracellular adhesion molecule-1
IF	immunofluorescence
IFN	interferon
MSD	multisystem disease
NOD	non-obese diabetic
PV	poliovirus
PVR	poliovirus receptor
RdRp	RNA-dependent RNA polymerase
RGD	arginine-glycine-asparagine
SOCS-1	the suppressor of cytokine signalling-1
SVDV	swine vesicular disease virus
TCID	tissue culture infectious dose
T1D	type 1 diabetes
TRIGR	the Trial to Reduce IDDM in the Genetically at Risk
UMP	uridine monophosphate
UTR	untranslated region
VLA-2	very-late antigen-2
VLDL	very-low-density lipoprotein

1 Introduction

The *Enterovirus* (EV) genus of the family *Picornaviridae* contains a large number of human viral pathogens and is comprised of four *Human enterovirus* (HEV A-D, HEVs) and three *Human rhinovirus* (HRV A-C, HRVs) species. HEVs exhibit a wide range of clinical manifestations including meningitis, myocarditis, encephalitis and paralysis, common cold like symptoms, eye infections and skin disease. Increasing evidence suggests that HEV infections may have a role in the pathogenesis of certain chronic diseases, such as type 1 diabetes (T1D) (Hyöty et al., 1995, Clements et al., 1995, Hiltunen et al., 1997, Andreoletti et al., 1997, Roivainen et al., 1998a Roivainen et al., 2000, Roivainen et al., 2002), atherosclerotic disease, myocardial infarction, and childhood asthma (Andreoletti et al., 1997, Roivainen et al., 1998b, Reunanen et al., 2002, Jartti et al., 2004, Kwon et al., 2004, Reunanen et al., 2005).

During the recent decades, HEV types associated with T1D have been studied by cross-sectional and prospective studies on diabetic patients and/or pre-diabetic individuals. The results of the first prospective cohort study, carried out in Finland, revealed that infections caused by CV-A9, CV-B1, 3, and 5 were associated with increases in islet cell autoantibodies or the onset of clinical T1D or both (Roivainen et al., 1998a). More recently, corresponding results were obtained from infections caused by several echoviruses (reviewed by Roivainen, 2006).

Several studies also indicated that virus types that are classified as coxsackieviruses B (CV-Bs) are found at the manifestation of clinical T1D (Gamble et al., 1969, Yoon et al., 1979, Champsaur et al., 1982, Dotta et al., 2007). To date, the pathogenic mechanisms CV-Bs use to cause T1D are mainly studied in animal models, whether the results correspond to humans disease remain to be determined. In mouse, CV-Bs have a strong affinity for the exocrine pancreas. The pathogenicity of CV-Bs can be enhanced by repeated passaging of the virus either in mouse pancreas *in vivo* or in cultured mouse β-cells (Toniolo et al., 1982). However, the genetic determinants leading to increased pathogenicity have remained undetermined.

2 Review of the Literature

2.1 Enteroviruses

The genus *Enterovirus* (EV) belongs to the family *Picornaviridae*. Previously, sixty-six serotypes were identified based on the antibody neutralization assay. In the 1990s, a novel genetic typing method was developed. This method relied on the complete sequencing of the VP1 gene of the capsid region and showed good correlation with the serotype detected by antibody neutralization assay (Oberste et al., 1999). Genetic typing made sequencing easier, and therefore, sequence data has increased rapidly. Consistently, the number of members of the genus *Enterovirus* has increased as the new phylogenetic type identification reveals new types among previously 'non-typeable' strains. In this thesis, a 'type' refers to the previous enteroviruses assigned as serotypes and also to the genetically typed enteroviruses.

On the basis of their pathogenesis in humans and animals, and replication in cell cultures, enteroviruses were classified into four groups, polioviruses (PV), coxsackieviruses A (CV-A), coxsackieviruses B (CV-B) and echoviruses. Echoviruses (**e**nteric **h**uman **c**ytopathogenic **o**rphan **v**iruses) which were identified in the late 1940s to 1950s (Melnick, 1996, Modlin, 1996, Muir et al., 1998), are so named, because at the time of their discovery they could not be associated with any apparent disease (Pallanch and Roos, 2007). At present, classification is based solely on sequence data. Therefore, new species in the EV genus and new genera in the family *Picornaviridae* have been reclassified in accordance with their genetic similarities (King et al., 2000, Stanway et al., 2005). The reclassified EV genus is composed of four species of *Human enterovirus* (HEV A-D), three species of *Human rhinovirus* (HRV A-C), three species of non-human enterovirus, and six unassigned enteroviruses (Table 1). The three poliovirus types were moved to the HEV-C species, and the three HRV species were recently reclassified under the genus *Enterovirus* (http://www.picornaviridae.com/) in accordance with their sequence similarities (Ledford et al., 2004, Laine et al., 2005). The genus *Enterovirus* now covers at least 200 enterovirus types (http://www.ictvonline.org). Seven types of non-human virus pathogen are included in the species HEV–A and –B (Oberste et al., 2007), thus, making the word "human" debatable. In this thesis, EV refers to all members within the recently classified *Enterovirus* genus, while HEV refers to the traditionally classified human enteroviruses (excluding HRVs and non-human species). A number of different methods are used in publications for naming enterovirus types. However, the names used in this thesis are in accordance with the recommendation given on the picornavirus website (http://www.picornaviridae.com/).

Table 1. The current classification of the members of the genus *enterovirus*.

Species (number of types)	Members	Clinical diseases in man
Human enterovirus A (22)	– Human coxsackievirus A 3-8, 10, 12, 14, 16 – Human enterovirus A 71, 76, 89-92, 114 – Simian enteroviruses SV-19, 43, 46 – Baboon enterovirus A 13	Meningitis, encephalitis paralysis, myocarditis, rash, HFMD, diarrhea neurologic disease of children
Human enterovirus B (60)	– Human coxsackieviruses B[§] 1-6 – Human coxsackievirus A 9 – Human echoviruses 1[Δ]-7, 9, 11-21, 24-27, 29-33 – Human enteroviruses B 69, 73-75, 77-88, 93, 97-98, 100, 101, 106, 107, 110 – Simian enterovirus SA5	Meningitis, encephalitis paralysis, myocarditis, respiratory infections pleurodynia, gatsoenteritis,
*Human enterovirus C (21)**	– Poliovirus 1-3 – Human coxsackievirus A 1, 11, 13, 17, 19-22, 24 – Human enterovirus C 95, 96, 99, 102, 104, 105, 109, 113, 116	Poliomyelitis, gastroenteritis, respiratory infection, conjunctivitis
Human enterovirus D (4)	– Human enterovirus D 68, 70, 94, 111	conjunctivitis, respiratory infections
Human rhinovirus A (77)	– Human rhinovirus A 1*, 2, 713, 15, 16, 18-25, 28-34, 36, 38-41, 43-47, 49-51, 53-68, 71, 73-78, 80-82, 85,88-90, 94-96, 98, 100, 101, 102,103	Common cold, otitis, chronic bronchitis, excerbation of asthma, sinusitis
Human rhinovirus B (25)	– Human rhinovirus B 3-6, 14, 17, 26, 27, 35, 37, 42, 48, 52, 69, 70, 72, 79, 83, 84, 86, 91, 92, 93, 97, 99	Common cold, otitis, chronic bronchitis, excerbation of asthma, sinusitis
Human rhinovirus C (49)	– Human rhinovirus C 1-49	Common cold, otitis, excerbation of asthma
Bovine enterovirus (2)	– Bovine enterovirus BEV-1 and BEV-2	
Simian enterovirus A (3)+	– Simian enterovirus SV4, SV28, SA4, SEV-A2	
Porcine enterovirus B (2)	– Porcine enterovirus PEV-9 and PEV-10	
Unassigned enteroviruses (6)	– EV-103, EV-108, EV-112, EV-115, SV-6, SV-47	

[+] All four viruses have closely genetic relationship and therefore they might belong to a single serotype, simian enterovirus A1 (SEV-A1).
[§] Include Swine vesicular disease virus (SVDV).
***** Enterovirus type species.
[Δ] Include echovirus 8

HEVs cause frequent infections in human and, particularly in children. Typical clinical manifestations of HEV infections are shown in Table 1. HEV infections are transmitted by the fecal-oral route and, less commonly, via the respiratory tracts.

The frequency of HEV infections peaks in the late summer and autumn, athough sporadic infections occur throughout the year.

Human enterovirus B (HEV-B) species represent a large group containing at least 60 human pathogens. HEV-B includes coxsackieviruses B1 – B6 (CV-B1 to CV-B6), coxsackievirus A9 (CV-A9), 28 types of echoviruses (E), and 23 types of newer enteroviruses, and the number of viruses in the HEV-B species is continuously increasing. Epidemiological studies from several countries suggest that the most common HEV strains belong to the HEV-B species. The two HEV-B virus strains studied in this thesis belong to the most common clinical isolates in Europe and USA. In Europe, the most common clinical isolates are echoviruses E-6, 7, 9, 11, 13, 19, 30, CV-A9, CV-B2 and B5 (Hovi et al., 1996, Maguire et al., 1999, Antona et al., 2007). The Centers for Disease Control and Prevention (CDC) has summarised the epidemiological data collected over 35 years in the USA and reported that the five most common HEV types are echoviruses E-6, 9, 11, 30 and CV-B5 (Khetsuriani et al., 2006). In addition to clinical samples, it has been shown in several reports that virus types belonging to HEV-B species are frequently found in environmental samples (Sellwood et al., 1981, Hovi et al., 1996, Sedmak et al., 2003, Klemola et al., 2008). However, the correlation between virus type detection from the environment and clinical disease is difficult to establish and remains under debate.

2.2 Enterovirus structure

Human enteroviruses are small non-enveloped particles with a diameter of about 30 nm. The RNA genome is surrounded by an icosahedral protein capsid containing 12 pentagon-shaped pentamers formed by five protomers (Fig 1). Several enterovirus capsid structures have been resolved including polioviruses (Hogle et al., 1985, Chow et al., 1987), coxsackievirus A9 (CV-A9) (Hendry et al., 1999), coxsackievirus B3 (CV-B3) (Muckelbauer et al., 1995), and echovirus 11 (E-11) (Stuart et al., 2002a). The capsid structure of coxsackievirus B5 (CV-B5), the other virus type besides E-11 analysed in this study, has not yet been determined. However, the structure of swine vesicular disease virus (SVDV), a genetic sublineage of CV-B5 that evolved between 1945 and 1965 after the transfer of CV-B5 from humans to pigs (Zhang et al., 1999), has been resolved (Verdaguer et al., 2003). Among all determined capsid structures PV remains one of the most thoroughly studied and best understood to date, and shows structural properties common to all enteroviruses.

All of the resolved atomic structures are highly similar in their overall capsid structure. Each of the three major capsid proteins (VP1, VP2, and VP3) consists of eight-stranded antiparallel β-barrel with two flanking helices, while VP4 has a less ordered structure and is situated inside the capsid close to the viral RNA genome. The β-barrels of VP1 are located around the five-fold axis, while VP2 and VP3 are around the three-fold axis. The sequences of the loops connecting the β-strands in

VP1 – VP3 are highly diverse, particularly at the top of the β-barrel domain. These loops serve as the major antigenic sites during infection.

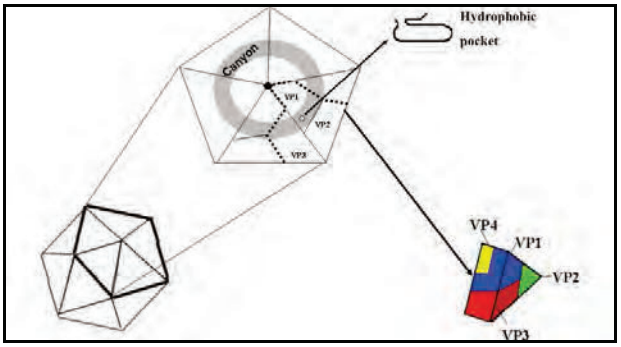


Figure 1. Schematic diagram of an enterovirus capsid (modified from Hadfield et al., 1995).

The enterovirus capsid morphology is characterized by narrow depression surrounding the five-fold axis termed the canyon. The canyon was hypothesized to be too narrow to accommodate antibodies, but large enough to harbour a receptor binding site, hence, allowing the virus to escape the immune system (Minor et al., 1986, Rossmann et al., 1985, Rossmann, 1989, Couch & Turner, 2007, Racaniello, 2007). However, studies of HRV-14 and a strongly neutralizing antibody Fabl7-IA which binds bivalently to the virion, revealed that Fabl7-IA penetrates deep within the canyon and overlap with binding site of surface cellular receptor for HRV-14 (Smith et al., 1996). These results show that the capsid depression does not merely exist to allow the virus to escape the immune system, hence, challenging the canyon hypothesis. Underneath the canyon lies a hydrophobic pocket that may be occupied by pocket factors which are believed to play a role in the stability of the viral particle and the uncoating process (Rossmann, 1994). The pocket factors possibly originate from the host cell. Pocket factor is either fatty acid or fatty acid-based molecule. The hydrophobic pocket of PV contains sphingosine (Filman et al., 1989), that of CV-B3 and E-1 contain palmitate (Muckelbauer et al., 1995, Filman et al., 1998), and the pocket of bovine enterovirus contains myristate (Smyth et al., 1995).

2.3 Genome structure

The positive-stranded RNA genome of enteroviruses consists of approximately 7,500 nucleotides and acts as messenger RNA (mRNA) in the host cell. It contains a single open reading frame which encodes three polyproteins required for viral replication and assembly. The open reading frame is flanked at both the 5’ and 3’ ends by an untranslated region (UTR) containing two cis-acting replication elements (CREs).

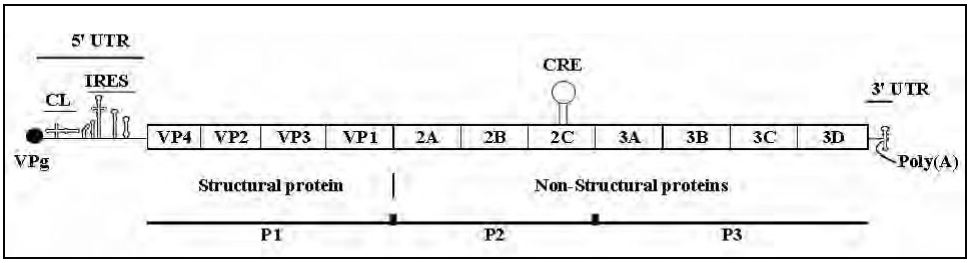


Figure 2. Schematic illustration of the genome of enteroviruses (modified from Yin et al., 2007).

The 5'UTR makes up to 10% of the genome, consisting of two independent domains, a cloverleaf-like structure (CL) and internal ribosomal entry site (IRES). CL functions in both synthesis of positive-strand RNA and the process of switching from translation to replication (Andino et al., 1990, Andino et al., 1993, Harris et al., 1994, Xiang et al., 1995, Gamarnik & Andino, 1998, Paul et al., 2000), while IRES to promotes the translation of a polyprotein (Harris et al., 1994, Pelletier et al., 1988a). The uridine monophosphate (UMP) of 5'UTR is covalently linked to the hydroxyl group of the virus-encoded VPg protein by a phosphodiester bond (Fig 2).

The viral genome is terminated with a poly(A) tail (Yogo & Wimmer, 1972). The poly(A) is bound to a stem-loop structure of the 3'UTR. The length of 3'UTR varies from 70 nucleotides to 100 nucleotides. Current findings regarding the significance of the 3'UTR are contradicting. Results from several studies suggest that it might be involved in the initiation of the negative-strand RNA synthesis (Pilipenko et al., 1992, Pierangeli et al., 1995, Pilipenko et al., 1996, Melchers et al., 1997, Agol et al., 1999), while other studies suggest that 3'UTR is not essential for replication (Rohll et al., 1995, Agol et al., 1999), and even deletion mutants lacking the 3'UTR have shown to be infectious in cell culture (Todd et al., 1997).

A third cis-acting replication element required for RNA replication (Agol et al., 1999) was first identified in the VP1 gene of human rhinovirus 14 (HRV-14) (McKnight & Lemon, 1996, McKnight & Lemon, 1998). Thereafter, similar replicating elements have been identified for poliovirus and coxsackievirus B3 within the 2C protein (Goodfellow et al., 2000, van Ooij et al., 2006).

2.4 Enterovirus life cycle

The infection cycle of an enterovirus starts upon the interaction of the virus with its cell surface receptor(s). Poliovirus is the most studied enterovirus and, for this reason, it is selected here as a model to review the entry and genome delivery of non-enveloped viruses. Conformational changes are essential for viral entry and RNA release which are triggered by the virus binding to its cellular receptor (Rossmann et al., 2002). Upon binding to the receptors, poliovirus loses the majority

of the 60 copies of the capsid protein VP4 (a virion that has lost its VP4 is called a 135S or A particle), exposing the N terminus of VP1, and altering the antigenicity and protease sensitivity in comparison to the intact virions (called 160S). This process, thus, initiates uncoating to release the viral genome into the cell.

2.4.1 Translation of enteroviral genome

All enteroviral genomes contain an internal ribosomal entry site that promotes the initiation of translation (Jang et al., 1988, Pelletier & Sonenberg, 1988, Pelletier et al., 1988b), and facilitates cap-independent ribosomal binding to the viral mRNA (Hellen & Sarnow, 2001). The cellular machinery lacks RNA-dependent RNA polymerase (RdRp), a protein essential in the synthesis of a complimentary negative-strand using the viral RNA genome as a template. Therefore, the synthesis of viral RdRp and other viral proteins are required for virus replication. Viral RNA is translated by a cap-independent mechanism (Pause et al., 1994, Ohlmann et al., 1996, Pestova et al., 1996, Pestova et al., 2007). An additional poly(A)-binding protein (PABP) significantly enhances IRES-mediated translation of an enterovirus by interacting with eIF4G (Imataka et al., 1998, Bergamini et al., 2000, Hellen & Sarnow, 2001), thus mediating IRES-dependent translation.

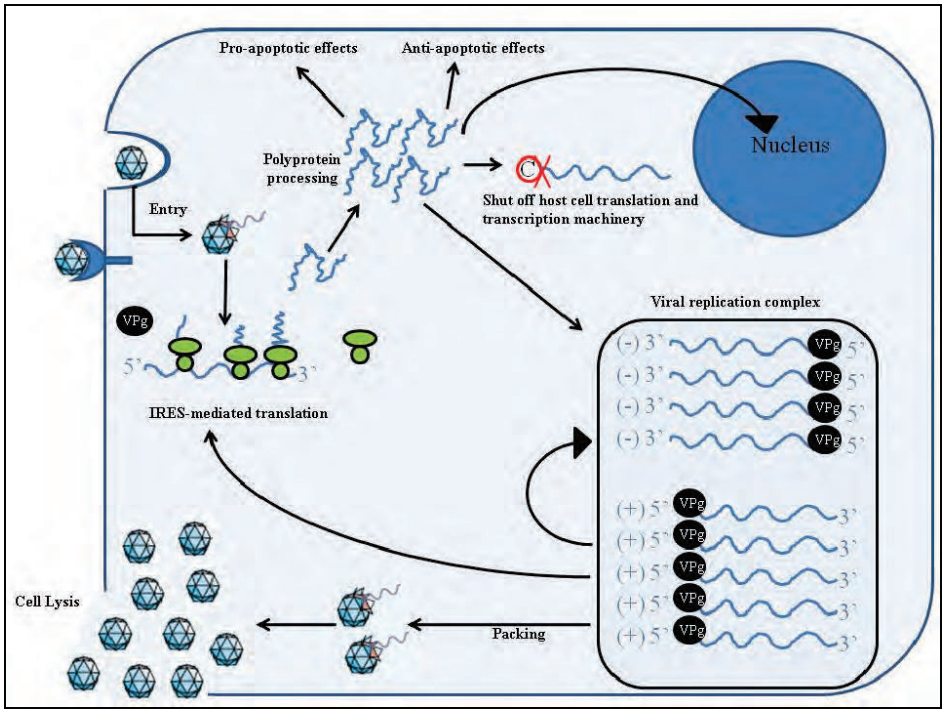


Figure 3. Schematic image of the enterovirus life cycle (modified from Whitton et al., 2005).

2.4.2 Polyprotein processing

The complete viral protein-coding region is translated as a single, large polyprotein, which is proteolytically cleaved by the viral 2A protein into precursors P1 and P2P3. P2P3 is further cleaved by 3C and its precursor 3CD. P1 is cleaved into three capsid proteins VP0, VP3 and VP1 of which VP0 is further cleaved into VP2 and VP4 during capsid assembly. The proteins of the P2 domain are predominantly involved in inducing the biochemical and structural changes that occur in the infected cell (Bienz et al., 1990), but 2C is also essential for viral genome replication (Pfister & Wimmer, 1999). Most P3 proteins are directly involved in RNA synthesis. These include viral RNA polymerase (3D), which is both primer- and template-dependent. Other P3 proteins include the small membrane-bound protein 3A, the 22- amino-acid terminal protein VPg, proteinase 3C, and two multifunctional precursors 3AB and 3CD.

2.4.3 Replication of enteroviruses

After polyprotein processing, the same RNA molecule used in translation is copied and amplified into negative-sense RNA strands by the virus-encoded RdRp with the assistance of other non-structural proteins and cellular factors (Flanegan & Baltimore, 1977, Racaniello, 2007). These negative RNA strands, which carry VPg at their 5'terminus, are used in the synthesis of large amounts of positive-stranded RNA copies. Simultaneously, VPgs of some positive RNA strands are removed to allow their use in the translation of viral proteins. The cloverleaf-like structure interacts with viral and cellular proteins to form a ribonucleoprotein (RNP) complex that is required for the initiation of both negative- and positive-strand RNA synthesis (Andino et al., 1990, Herold & Andino, 2000).

2.4.4 Assembly and virus release

The assembly of a new virion begins when the P1 precursor protein is cleaved into capsid proteins, VP0, VP1 and VP3, which form protomers that carry one copy of each capsid protein. Five of these protomers associate into a pentameric structure. Twelve of these pentamers then associate to form procapsids ($VP013_{60}$) that are filled with a single copy of the genomic RNA (Racaniello, 2007). The RNA that still carries the VPg protein is then encapsidated into the assembling virions. The viral 2C protein is involved in the protein–nucleotide primer formation and is important for encapsidation (Vance et al., 1997, Goodfellow et al., 2003). At the end of RNA encapsidation, the VP0 is cleaved into VP2 and VP4. This is called maturation cleavage, which is thought to lock the assembled capsid into a stable, mature virion (Compton et al., 1990, Ansardi & Morrow, 1995). Release of enteroviruses occurs upon lysis of the cell. The viral 2B protein plays a main role in the release process. It induces the formation of numerous vesicles that are believed to be important for virion release; however, the mechanisms of vesicle formation and regulation are

unclear. The protein is a transmembrane pore-forming protein that participates in a range of viral functions (Gonzalez & Carrasco, 2003).

2.5 Cellular receptors for enteroviruses

Enterovirus infection starts by attachment of a virus to cell surface receptor(s). Several receptors are known to mediate enterovirus infection. These include cell surface molecules from the immunoglobulin (Ig) and integrin families as well as other molecules (Table 2). The binding site of these receptors may be the canyon (Olson et al., 1993) or a capsid region other than the canyon (Hewat et al., 2000). These interactions are, however, not well known. In the case of some enteroviruses, receptor binding to the canyon causes virus instability, followed by entry into the cell where the virus undergoes uncoating, which converts the infectious virion to an altered A-particle (135S), and the release of viral genome (Rieder & Wimmer, 2002). The non-canyon binding receptors do not cause virus instability (Hoover-Litty & Greve, 1993, Hewat et al., 2000). For many viruses, binding to the primary receptor does not mediate cell entry. For instance, the prototype strain of coxsackievirus A21 (CV-A21) displays dual-receptor specificity. For cell entry, CV-A21 requires the intracellular adhesion molecule 1 (ICAM-1) and a decay-accelerating factor (DAF, CD55) (Shafren et al., 1997). In addition to their primary receptor, several echoviruses and CV-A9 are likely to utilize co-receptor(s), such as β2-microglobulin, in the entry process (Triantafilou et al., 1999, Ward et al., 1999, Chevaliez et al., 2008, Heikkilä et al., 2010).

2.5.1 Members of immunoglobulin superfamily

Ig-superfamily members are membrane-bound glycoproteins, which consist of tandem repeats of two to five Ig-like domains, a transmembrane segment, and a cytoplasmic tail. The Ig-like domain 1 (D1) provides a virus attachment surface by binding to the canyon. Receptors belonging to the Ig superfamily include the poliovirus receptor (PVR) utilized by all three poliovirus types (Mandelsohn et al., 1989, He et al., 2000, Hogle., 2002), ICAM-1, which is the receptor for the major group of rhinoviruses (Greve et al., 1989, Tomassini et al., 1989) and several CV-As, as well as a coxsackievirus and adenovirus receptor (CAR) that is shared by two evolutionary distant virus groups. So far, the six CV-B types and SVDV are the only enteroviruses, which use human CAR as a binding and entry receptor (Martino et al., 2000).

CAR is expressed within intercellular tight junctions of the cells. Since it has only two Ig-like domains, CAR is shorter than other EV receptors belonging to Ig-superfamily (Bergelson et al., 1997a, Bergelson et al., 1998). The overall homology between the CAR gene from human, pig, mouse, rat, dog, and cow is over 90% (Fechner et al., 1999, Thoelen et al., 2001, Freimuth et al., 2008). CV-Bs are capable of infecting mice and pigs through attachment to CAR molecules (Tomko et al.,

1997b, Bergelson et al., 1998, Myers et al., 2004, Ahn et al., 2008). However, it remains to be determined whether enteroviruses can initiate infection in other species expressing these homologous CAR receptors.

2.5.2 Integrins as receptors

Cell-adhesion molecules, integrins, are expressed in all multicellular animals. They are crucial for cell invasion, mobility and survival (Hood & Cheresh, 2002). Integrins consist of two covalently linked subunits, α and β (Ruoslahti & Pierschbacher, 1987, Takagi & Springer, 2002). In humans, these 18 α and 8 β subunits can form 24 combinations of heterodimers. Integrins are involved in several cell development processes, immune response, chronic inflammation and invasive cancer (Hynes et al., 2002). The first integrin binding sequence that was identified was an arginine-glycine-aspartic acid (RGD) motif. This is shared by many, but not all integrin ligands. The RGD motif binds specifically to certain integrins known as vitronectin and fibronectin receptors such as α5β1, α8β1, αvβ3, αIIbβ3, αvβ5, αvβ6 and αvβ8 (Ruoslahti & Pierschbacher, 1987, Luo et al., 2007).

Several pathogens, including EVs, use integrins as attachment molecules (Bergelson et al., 1992, Roivainen et al., 1994, Berinstein et al., 1995). CV-A9 interacts with integrins αvβ3 and αvβ6 through the RGD motif (Roivainen et al., 1991, Roivainen et al., 1994, Williams et al., 2004). On the other hand, the RGD motif is not fully essential for infectivity of CV-A9, since the virus can efficiently infect cells by an RGD-independent attachment and internalization mechanisms (Roivainen et al., 1991, Hughes et al., 1995, Roivainen et al., 1996, Triantafilou et al., 1999, Heikkilä et al., 2009, Heikkilä et al., 2010,). Other HEV-B types, such as echovirus 9 strains Barty and Hill, also use αvβ3 as a cellular receptor in an RGD-dependent or -independent manner. E-9 Barty has the RGD motif in the VP1 capsid protein, while the E-9 strain Hill lacks the RGD motif. By using polyclonal antiserum, Nelsen-Salz et al., (1999) demonstrated that the E-9 strain Barty, but not Hill, was blocked by antibodies against αvβ3 (vitronectin receptor), suggesting that viruses may use a different mechanisms to bind to the receptor. This does not exclude the possibility that the E-9 strain Hill may use different cell surface receptor.

Paananen et al., (2003) used the RRRGDL-peptide to demonstrate that the RGD motif of the E-9 strain Barty, but not Hill, is involved in virus entry into GMK cells. The results were further confirmed by showing that infections of the E-9 strain Barty were inhibited by an in house produced high-titre polyclonal antiserum to the human integrin αvβ3; this antiserum was different to that used by Nelsen-Salz et al., (1999). Interestingly, infection of E-9 Hill was also blocked with the same high-titre polyclonal antiserum, but not with the RGD-containing peptide (Paananen et al., 2003). Furthermore, the infection and virus-induced cellular death of the RGD-lacking E-9 Hill strain was prevented in human islets by the same high-titre

polyclonal antiserum to integrin αvβ3 (Ylipaasto et al., 2004). More recently, it has been indicated by blocking experiments with polyclonal antiserum to integrin αvβ3 that both primary human islets and a continuous laboratory cell line of green monkey kidney origin (GMK) are protected similarly from the adverse effects of several non-RGD-containing echovirus (E-7, 11, 25, 30, and 32) infections (Ylipaasto et al., 2010). As such, αv integrins serve as receptors for several RGD motif containing viruses, but also for several echoviruses that lack the RGD motif.

2.5.3 Other enterovirus receptors

Non-canyon binding molecules include DAF, very-low-density lipoprotein (VLDL), very-late antigen-2 (VLA-2) and integrins. Binding to a region outside the canyon does not cause viral instability, thus, viruses are internalized by a different mechanism. Some echoviruses, such as E-11, utilise DAF as their primary receptor (Bergelson et al., 1994, Stuart et al., 2002b). The attachment site of DAF is around the two-fold axis of the capsid (He et al., 2002). DAF consists of several short consensus repeats (SCR). SCR binding specificity among EV strains is variable. Some of the clinical isolates of E-11 and CV-B3 bind to the first SCR in DAF, while CV-A21 binds to the third one (Bergelson et al., 1997b, Shafren et al., 1997, Lea et al., 1998). In humans, DAF was identified as a co-receptor for CV-B1, CV-B3 and CV-B5, although it is not involved in virus entry (Bergelson et al., 1995, Shafren et al., 1995, Tomko et al., 1997a, Carson, 2001). CV-Bs and certain echoviruses that use human DAF for attachment do not bind to the murine analogue (Spiller et al., 2000). Taken together, expression of certain receptors on the surface of target cells will constrict cell tropism, while variation in the sequence of given EV receptors among species limits the host range. EVs use multiple receptors to attach and enter host cells, possibly enhancing their probability of survival by infecting target cells also through an alternative gateway (Roivainen et al., 1991, Frisk et al., 2001). On the other hand, some EVs, such as PVs and the major group HRVs use only a single receptor to ensure efficient cell attachment, uncoating and entry. It is worth mentioning that most studies on virus-receptor interactions have been conducted by cell culture-passaged virus strains and/or laboratory virus strains in continuous (cancer) cell lines. These strains may have been adapted to use certain receptors and, therefore, may not be similar to clinical isolates. For example, clinical isolates of coxsackievirus B3 and some echoviruses exhibited differences in their interactions with cell surface-expressed decay accelerating factor (DAF) compared to their respective prototype strain (Bergelson et al., 1997b, Stuart et al., 2002c). Receptors are essential for viruses to initiate an infection cycle; however, pathogenesis is a broad concept that is not solely determined by receptor recognition. Other factors such as intracellular proteins, spreading of the virus between organs, host immune responses, etc. have an influence on the development of infection.

Table 2. Known cell surface receptors for enteroviruses.

Receptor	Virus	Refrences
Immunoglobulin superfamily:		
Poliovirusreceptor (PVR, CD155)	PV-1 to PV-3	Mendelsohn et al.,1989
Intracellular adhesion molecule 1 (ICAM-1, CD54)	CV-A13, 18, 21	Colonno et al.,1986a
	CV-A15, 20	Pulli et al.,1995
	Major group HRV	Greve et al.,1989
Coxsackievirus and adenovirus receptor (CAR, CXADR)	CV-B1 to CV-B6	Bergelson et al.,1997a
	SVDV	Martino et al., 2000
Integrins[§]:		
$\alpha_2\beta_1$ integrin (VLA-2)	E-1	Bergelson et al.,1992
$\alpha_v\beta_3$ and $\alpha_v\beta_6$ integrins	CV-A9	Roivainen et al.,1994, Williams et al.,2004
$\alpha_v\beta_3$ integrins	E-9 (Barty)	Nelsen-Salz et al.,1999,
	E-9 (Barty and Hill)	Paananen et al., 2003
	E-7, 11, 25, 30, 32	Ylipaasto et al.,2010
Other:		
Decay-accelarating factor (DAF, CD55)	CV-A13, 18	Colonno et al.,1986a, & 1986b
	CV-A21*	Shefren et al.,1997
	CV-B1*, 3*, 5*	Shafren et al.,1995, Bergelson et al.,1995
	E-3, 6, 7, 11-13, 20, 21, 24, 29, 33	Bergelson et al.,1994, Ward et al.,1994, 1998
	EV-D70	Karnauchow et al.,1996
Low-density-lipoprotein receptor (LDL-R, LDLR)	Minor group HRV	Hofer et al.,1994
Sialic acid (SAS, NANS)	HRV-87[Δ]	Uncapher et al.,1991
	EV-D70	Utagawa et al.,1982, Alexander et al.,2002
sialic acid-containing O-linked glycoconjugates	CV-A24v	Mistry et al., 2011
Heparan sulfate	E-6*	Goodfellow et al.,2001
	CV-B3*	Zautner et al.,2003
	SVDV*	Escribano-Romero et al.,2004
β2-microglobulin (B2-M)	E-1*, 6, 9,12, 13*-19, 21*, 24, 26, 27, 29*, 33*	Ward et al.,1998
	E-11	Chevaliez et al.,2008
	CV-A9*	Triantafilou et al.,1999
	E-7	Ward et al.,1998
Glucose-regulated protein (GRP78)	CV-A9	Triantafilou et al.,2002
PSGL-1	EV-A71	Nishimura et al.,2009
SCARB2	CV-A16, EV-A71	Yamayoshi et al.,2009

*The virus(es) use(s) this receptor as a coreceptor.
[§]The names of integrin genes are ITGA for alpha followed by numbers or letters, and ITGB for beta followed by numbers.
[Δ] This virus is included in EV-68.
PSGL-1 for Human P-selectin glycoprotein ligand-1 and SCARB2 for Human Scavenger receptor B2.

2.6 Entry route

The initial goal for a virus entering living cells is to deliver its genome to the cytoplasm and to utilize the cellular machinery for production of new progeny by

replication. To do so, most viruses use one of two suggested basic mechanisms of endocytosis, pinocytosis and phagocytosis. Pinocytosis is a receptor-dependent entry mechanism while phagocytosis is restricted to specialized cells, such as macrophage. Pinocytosis is further divided into several sub-mechanisms (Fig 4). These include at least the dynamin-dependent and dynamin-independent pathways followed by multiple adapter molecules that coat the vesicles and induce their internalization. Virus genomes may directly penetrate the plasma membrane, or the virus itself is delivered to the cytoplasm by receptor-mediated cellular endocytosis including lipid rafts, caveolae, macropinocytosis and non-caveolae and nonclathrin-dependent mechanisms (Johannes & Lamaze, 2002, Smith & Helenius, 2004, Marsh & Helenius, 2006, Mercer & Helenius, 2009). So far, it has been suggested that several uptake mechanisms are utilized by EVs, and more are likely to be identified in the future.

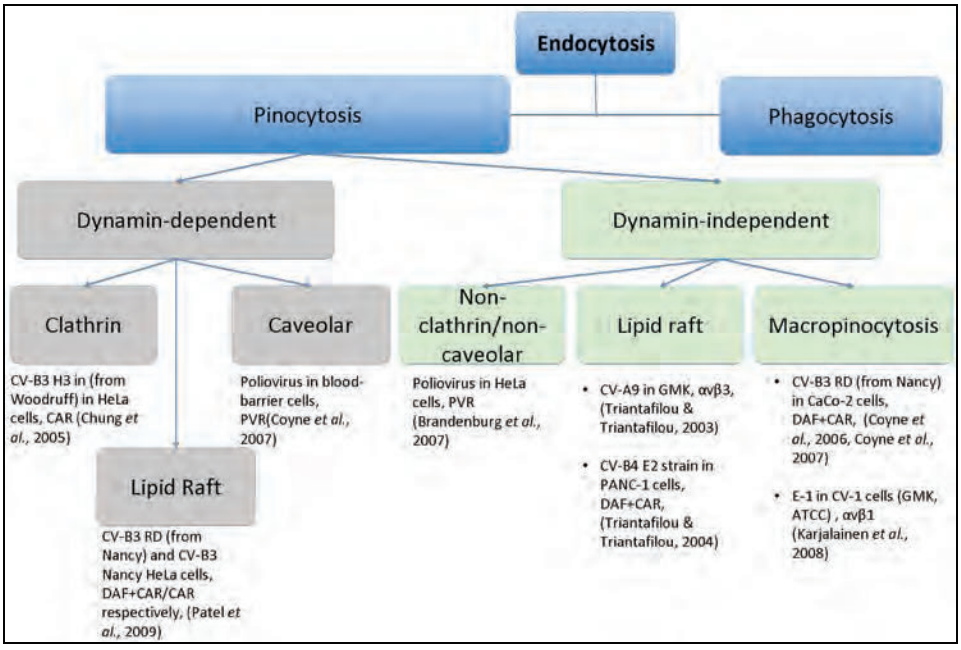


Figure 4. Endocytic pathways used by enteroviruses (modified from Mercer & Helenius 2009). CV-B3 RD is a virus strain derived from the Nancy strain which has the ability to utilize decay-accelerating factor (DAF) as a coreceptor. CV-B3 H3 is a virus strain derived from Woodruff which binds only to coxsackievirus and the adenovirus receptor (CAR). CV-B4 E2 is a virus strain isolated from the pancreas of a diabetic child, E stands for Edward.

Poliovirus (PV) is one of the most studied enterovirus. In HeLa cells, PVs release their genome into the cytoplasm through a pore formed at the plasma membrane

consecutive to receptor binding. The hydrophobic N-terminus of VP1 and possibly the myristate group of VP4, presumably combined with changes in Ca^{2+} concentrations, allow membrane binding and pore formation, which lead to the penetration of viral RNA into the cytoplasm. Alternatively, it is possible that the pore is formed in the membrane of a cytoplasmic vesicle (DeTulleo & Kirchhausen, 1998, Kronenberger et al., 1998, Hogle., 2002, Danthi & Chow, 2004, Bubeck et al., 2005a, Bubeck et al., 2005b, Tuthill et al., 2006). However, two recent studies re-addressed the question of PV entry (Brandenburg et al., 2007, Coyne et al., 2007a) (Fig 4). Both studies indicated that the entry mechanisms of PV depend on the cell line studied.

Likewise, studies carried out using three CV-B3 strains (Nancy, RD and H3) showed that the entry mechanism depends on the cell lines, CaCo-2 and HeLa (Coyne et al., 2007b, Coyne et al., 2006, Patel et al., 2008), and also on the virus strain used in the study (Chung et al., 2005, Patel et al., 2008). Other studies showed that different viruses exploit the same mechanism in different cells, for example CV-B3 and E-1, and CV-A9 and CV-B4 E2 (E for Edward) (Triantafilou & Triantafilou, 2003, Triantafilou & Triantafilou, 2004a, Coyne et al., 2006, Coyne et al., 2007b, Karjalainen et al., 2008). However, very little is known of the endocytic pathway during and after entry of HEV-B types, and the results obtained are controversial as might be cell type specific.

Taken together, these results describe that entry of nonenveloped viruses may switch from one uptake mechanism to another to ensure sufficient entry to the host. In the case of PV, it was shown in two separate studies (Brandenburg et al., 2007 and Coyne et al., 2007a) that PV binds to PVR, revealing that the entry mechanism is host cell dependent. In the case of the HEV-B types, it seems that different viruses may use the same pathway for host entry and, in addition, a single virus strain may switch between the pathways.

2.7 Evolution of human enteroviruses

Like other RNA viruses, enteroviruses are genetically diverse. Evolution of enteroviruses is driven by point mutations and recombination. The evolution of a single enterovirus type appears to occur through gradual changes to the viral genome by point mutations that affect genetic drift and selection during cellular infection. In recombination, larger fragments are switched to produce progeny with genomic parts from both parental strains infecting the same cell. These fragments may contain additional mutations beneficial to the recombined progeny viruses, and allow them rapidly to adapt to new environments. Moreover, recombination allows the progeny viruses to get rid of deleterious mutations.

Enteroviruses are known to circulate among populations. Two different infection patterns for the emergence of circulating EVs can be observed: epidemic and endemic. The epidemic pattern is shown by E-30. Like influenza viruses, new

variants of E-30 may take place after emergence of subtypes, which circulate globally for several years. These emerged subtypes then disappear and are replaced by new variants. On the other hand, the disappeared variants may re-emerge. Recently, it has been indicated that the re-emergence of E-30 and E-9 variants after a concealed phase is associated with the emergence of novel recombinant genetic lineages of the viruses (McWilliam Leitch et al., 2009, 2010). Conversely, the endemic pattern is geographically restricted, and typically several separate lineages of the same genotypes circulate at the same time, as shown for circulating polioviruses (Kew et al., 2005, Yang et al., 2005).

2.7.1 Mutation

The concept of quasispecies defines the nature of RNA virus populations as a group of viruses with related sequences rather than a single virus variant (Holland et al., 1982, Holland et al., 1992). Quasispecies is a result of activity of error-prone viral RdRp that mediates virus replication (Hughes et al., 1988, Domingo & Holland, 1997, Domingo et al., 1998a, Domingo., 1998, Domingo., 2000). Mutations occur due to the lack of proofreading and correction mechanisms in RdRp (Holland et al., 1992). RdRp has a mutation rate of one in every 2200 bases, or approximately four mutations per transcript (Holland et al., 1990, Domingo., 2000). A high mutation rate is not always beneficial to a RNA virus since it results in nonviable virus form. In contrast, high fidelity of RdRp also seems to attenuate the virus (Vignuzzi et al., 2005, Pfeiffer & Kirkegaard, 2005). There is, therefore, a balance between viable virus forms and proof-reading activity of RdRp. This activity ensures that accumulation of mutations generated during replication result in a pool of heterogeneous viruses containing potentially beneficial mutations (Domingo & Holland., 1997, Domingo et al., 1998b). These microvariant populations have a greater chance of adapting to new environments (Vignuzzi et al., 2006). The RNA of EVs is variable and, thus subjected to selection pressure by the environment. This influences the emergence of new viral pathogens and may even lead to development of zoonotic features (Zhang et al., 1999).

2.7.2 Recombination

HEVs are well known for their ability to undergo extensive recombination. Recombination is possible among viruses having sufficient similarities in their genomic sequences and the capability of infecting the same cell. Recombination has been shown to occur, for example, between vaccine and wild-type strains of PVs (Furione et al., 1993, Georgescu et al., 1994, Georgescu et al., 1995, Guillot et al., 2000, Liu et al., 2000, Georgopoulou & Markoulatos, 2001, Liu et al., 2003, Yang et al., 2003, Karakasiliotis et al., 2005). The current evidence supports a model in which homologous recombination occurs by strand switching (copy choice) between RNA molecules during minus-strand RNA synthesis and is mediated by the viral

RdRp (Kirkegaard & Baltimore, 1986, Jarvis & Kirkegaard, 1992). A non-replicative recombination model has also been suggested to occur before generation of the RdRp. In this process, recombining RNAs are cleaved and exposed termini are cross-ligated (Gmyl et al., 2003).

In most cases, PV recombination has been reported in the non-structural genome region, but intertypic recombinations in the structural genome region have also been documented (Liu et al., 2000, Martin et al., 2002, Blomqvist et al., 2003, Kyriakopoulou et al., 2006, Dedepsidis et al., 2008, Savolainen-Kopra et al., 2009). Intertypic recombination has also been reported in non-poliovirus HEVs, such as coxsackievirus B group and echoviruses, both among the prototype strains (Santti et al., 1999, Brown et al., 2003, Oberste et al., 2004a, Oberste et al., 2004b, Oberste et al., 2004c) and in circulating viruses (Santti et al., 2000, Oprisan et al., 2002, Lindberg et al., 2003, Lukashev et al., 2003a, Chevaliez et al., 2004, Lukashev et al., 2004, Oberste et al., 2004b,). Although interspecies recombination is rare among HEV species, signs of its occurrence in the past have been reported (Santti et al., 1999, Smura et al., 2007, Huang et al., 2009, Wisdom et al., 2009 McIntyre et al., 2010).

2.8 Enteroviral diseases

The enterovirus infectious cycle goes through multiple steps and usually results in cell death and tissue injury. The replication site defines the symptoms of an enterovirus infection. Rhinoviruses are acid labile, hence, their site of replication is restricted to the upper respiratory tract and their clinical symptoms range from common cold to exacerbation of asthma. In contrast, HEVs may pass through the digestive systems causing either symptomatic or asymptomatic infection. Some of the coxsackie A types belonging to the HEV-C species (CV-A) utilize the same receptor as the HRV major group, ICAM-1, and thus may induce similar symptoms to HRVs. However, CV-As are also known to pass through the digestive system, like other HEVs. In general, HEV replication occurs in the epithelia of the nasopharynx. However, most of the viral inoculum is swallowed and infects the gastrointestinal tract, where virus replication continues for several weeks. The acidic stability of HEVs allows them to cross the stomach and reach Peyer's patches in the lower gastrointestinal tract, where they are thought to replicate (Bodian, 1955, Wolf & Bye 1984, Sicinski et al., 1990). Occasionally, virus may cross the gastrointestinal tract and cause viremia. During viremia the virus spreads through circulation and may infect numerous organs systems, including the central nervous system, liver, lungs, heart, and pancreas. Further replication at these sites results in disease associated with clinical manifestations.

The clinical and pathological manifestations of enteroviruses are remarkably diverse, including poliomyelitis, aseptic meningitis, paralytic disease, encephalitis, postfatigue syndrome, congenital and neonatal infection, cardiac disease,

herpangina, hand-foot-mouth disease, fever, acute hemorrhagic conjunctivitis, respiratory disease, otitis media exanthema, epidemic pleurodynia, and myocarditis of the newborn, myocarditis in children, pericarditis, undifferentiated febrile illness, and type 1 diabetes. However, the majority of infections are asymptomatic (Minor et al., 1998). The same virus type can be associated with a large variety of different diseases, or the same clinical manifestation can be induced by different virus types. For example, the coxsackievirus B group has been linked to different diseases such as type 1 diabetes (reviewed by Hyöty, 2002, Roivainen, 2006, Richer & Horwitz, 2009, Jaïdane et al., 2010).

2.9 Enterovirus and type diabetes

2.9.1 Epidemiology of type 1 diabetes

Type 1 diabetes (T1D) is a multifactorial disease, which leads to β-cell destruction and, hence, insulin deficiency. It is an autoimmune disease characterized by inflammation of the β-cells in the islet of Langerhans known as insulitis. At the time of diagnosis, it is estimated that only 10–20% of insulin-producing cells are still functioning. Susceptibility to T1D is inherited. The key genes involved in the disease are located in the human leukocyte antigen (HLA) class II locus on the short arm of chromosome 6. However, these HLA genes are estimated to explain only 50% of cases of familial susceptibility to T1D. In addition, there are about 40 other non-HLA genes that are considered to account for the remaining genetic predisposition (Davies et al., 1994, Barret et al., 2009). However, only about 10% of those who develop T1D have a family history of T1D (Roche et al., 2005). Furthermore, the concordance between monozygotic twins for T1D is only about 40% (Redondo et al., 2001, Hyttinen et al., 2003). Thus, complex genetic predisposition alone does not explain the etiology of this disease. The incidence of T1D is increased in autumn, suggesting the involvement of an infectious agent in the etiology of the disease (Adams, 1926, Gamble & Taylor, 1969). Moreover, the incidence of T1D has increased worldwide by 3–5% per year, strongly suggesting involvement of an environmental agent in the etiology of T1D.

The epidemiology of T1D is also changing. T1D usually begins before the age of 40 with incidence peaks at age 2, 4–6 and 10–14 years (Norris et al., 1987). This has been reported in many countries (Joner & Sovik, 1989, Karvonen et al., 1993, Llanos & Libman, 1994, Tuomilehto et al., 1995, Gardner et al., 1997, Onkamo et al., 1999). A significant increase in the incidence of T1D over the past few decades has been reported worldwide (Onkamo et al., 1999, Green & Patterson, 2001, Gale, 2002). Worldwide incidence of onset T1D among children under 14 years of age varies by more than 350-fold (Karvonen et al., 2000), ranging from 0.1 per 100,000 per year in China to 36 per 100,000 per year in Finland in 1980. Worryingly, in 2005 the incidence increased to 64 per 100,000 per year in Finland (Tuomilehto et

al., 1999, Kondrashova et al., 2005, Lammi et al., 2007, Harjutsalo et al., 2008). Since the first report in 1954 when the numbers were 12/100,000/year (Somersalo, 1954) the incidence in Finland has increased more than five-fold. The worldwide increase in the incidence of T1D remains unexplained probably because the etiology of the disease is still poorly understood. Using more precise epidemiological studies, the variability in the incidence of T1D between countries, ethnic groups and seasons could be utilized to identify the etiological risk factors for the development of T1D.

2.9.2 Link between HEVs and T1D

Studies to find the links between environmental factors and the etiology of T1D have been ongoing for more than a century (Yoon et al., 2000). There are several reasons why environmental factors, such as HEVs, need to be considered as etiological agents of T1D. For example, whereas less than 10% of genetically predisposed individuals develop T1D, and the concordance rate between homozygotic twins is only about 30–50% (Redondo et al., 1999, Redondo et al., 2001), the incidence of T1D has largely increased, and there is a temporal correlation between the peaks in HEV infection (late summer/early autumn) and the metabolic disorder, which precedes T1D in late fall/early winter (Friman et al., 1985, Green & Patterson, 2001, Hyöty, 2002). This indicates that T1D cannot merely result from genetic susceptibility.

Several cross-sectional and prospective studies on diabetic patients and prediabetic individuals support the hypothesis that at least some cases of T1D are caused by HEVs (Table 3). The first evidence of a link between HEV and T1D came from a study that reported higher titres of serum antibodies against HEVs, particularly CV-B4, within three months of the clinical onset of diabetes compared with serum antibody titres from patients with diabetes of a longer duration (Gamble et al., 1969). In 1979, Yoon and co-workers reported a 10-year-old child presenting diabetic ketoacidosis (Yoon et al., 1979). A virus isolated from the pancreas was found to be CV-B4. When used in mouse experiments, this virus was found to induce diabetes. Several further case studies have since described the isolation of different HEV types from patients at disease onset (Champsaur et al., 1982, Hindersson et al., 2004, Dotta et al., 2007, Elshebani et al., 2007).

After the first observation made by the Gamble group, several seroepidemiologic studies have been carried out on T1D patients with a recent onset of the disease, revealing a unique diabetogenic variants of HEV, particularly CV-B. Table 3 shows the results of 38 studies including the site of study, number of subjects, diagnostic method employed, HEV types studied and odds ratio. The majority of the studies indicated at least one positive association with specific CV-B and/or a greater frequency of positive serology for several HEVs, however, no single HEV type was linked to T1D (Table 3).

However, also controversial results have been reported showing no association between HEV infection and T1D (reviewed by Filippi and Von Herrath, 2008). It is important to note that the majority of these studies indicating no association with diabetes are based on traditional serological assays and their results were not confirmed by more sensitive or specific methods (Table 3). Moreover, they focus on a limited range of HEVs and they do not take into account the correlation between the geographic area and the epidemic period. Recently, case control studies have focused on the increasing use of PCR technology to identify HEV RNA in serum or whole blood (Table 3). Direct detection of EV RNA increases sensitivity and specificity, thus, avoiding some of the problems associated with serological methods. Concurrently, PCR and sequencing allow the identification of possible diabetogenic variant(s) (Nairn et al., 1999, Yin et al., 2002a). The use of more advanced methods, such as *in situ* -hybridization, electron microscopy and immunohistochemistry, have revealed that HEV positive cells are exclusively localized to the pancreatic islets (Ylipaasto et al., 2004, Dotta et al., 2007). These findings have brought more insight into the causal relationship between HEV and T1D.

Prospective studies based on the follow-up of genetically predisposed children have provided information on the pathogenic processes that arise during the preclinical phase of T1D. The results of the first Finnish study (DiMe) suggest that the infections carried by CV-A9, CV-B1, 3, and 5 are associated either with seroconversion of islet cell autoantibodies or the onset of overt disease (Hyöty et al., 1995, Hiltunen et al., 1997, Roivainen et al., 1998, Lönnrot et al., 2000a,). Another Finnish prospective study (DIPP) found no relationship between any other viruses included in the DIPP and autoimmunity, except for HEVs (Lönnrot et al., 2000b, Kupila et al., 2001, Sadeharju et al., 2001). Another prospective study (TRIGR) found a relationship between HEVs and the induction of autoimmunity against β-cells (Sadeharju et al., 2003). Furthermore, Swedish investigators have shown that children whose mothers were exposed to HEV infections have a higher incidence of T1D than children whose mothers did not encountered HEV (Dahlquist et al., 1995a, Dahlquist et al., 1995b, Dahlquist et al., 1999). In contrast, no association was found in a large Finnish study of mothers whose children manifested T1D before the age of 15 during the period 1987–1995. Although the results from the vast majority of prospective and retrospective studies suggested a role of HEVs in the etiology of T1D, it should be emphasized that not all HEV infections lead to β-cell damage and T1D. Factors that determine susceptibility to HEV infection of the islets and the development of T1D remain unclear. It is likely that T1D results from several combined factors, such as virus strain, genetic susceptibility of the host, and time of infections.

Table 3. Systemic analysis of published cohort studies.

Study	Study site	Enterovirus tested	Total number of subjects Case/control	Case/controls EV+	Case/controls EV-	Assays	OR	Case studies
Gamble et al., 1969a	UK	CV-B1 to CV-B6	46/75	21/16	25/59	CF	3,1	
		CV-A2, 5, 10, 16	47/114	5/9	42/105		1,4	
Gamble et al., 1969b	UK	CV-B4	?	66%/40 %	34%/60%	NAb	2,9	
Hadden et al., 1972	Ireland	CV-B1 to CV-B6	58*/121	12/24	22/97	NAb	2,2	
Gamble et al., 1973#	UK	CV-B1 to CV-B5	162/319	114/186	48/133	NAb	1,7	
Huff et al., 1974 #	USA	CV-B1 to CV-B6	9/36	3/2	6/34	NAb	8,5	
Nelson et al., 1975	UK	CV-B1 to CV-B5	27/27	17/16	10/11	NAb	1,2	
Andersen et al., 1977	Danmark	CV-B4	41/239	20/129	21/110	NAb	0,8	
Schmidt et al., 1978	Germany	CV-B1 to CV-B5	81/80	8/11	73/69	HA for IgM and IgG	0,7	Yoon et al., 1979
Hazra et al., 1980	Asia	CV-B1 to CV-B5	20/20	12/3	8/17	?		
West et al., 1981	Greater Montreal	CV-B1 to CV-B6	72/72	6/0	66/72	NAb	NC	
Palmer et al., 1982	Seattle	CV-B3 to CV-B5	33/33	15/17	18/16	NAb	0,8	
Sakurami et al., 1982	Japan	CV-B1 to CV-B5	103/56	29/4	74/52	NAb	5,1	
			103/56	0/0	103/56	NAb	NC	
King et al., 1983	UK	CV-B1 to CV-B6	28/290	11/16	17/274	ELISA IgM	11,1	
Prince et al., 1983	France	CV-B1 to CV-B6	55/55	48/14	7/41	NAb	20,1	
Mertens et al., 1983	Germany	CV-B1 to CV-B6	166/0	80%/ ?	20%/ ?	NAb	NC	
Orchard et al., 1983	USA	CV-B1 to CV-B6	?	34%/42%	66%/58%	IgM, IgG IF	0,7	Champsaur et al., 1985
Frisk et al., 1985**a**	Sweden	CV-B1 to CV-B5	24/78	16/17	8/61	RIA IgM	7,6	

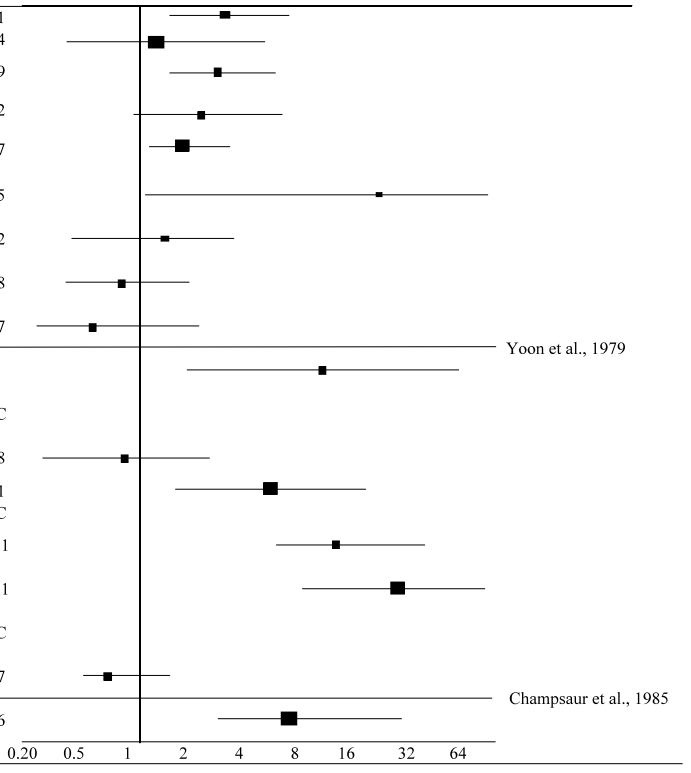


Table 3 continued

Study	Study site	Enterovirus tested	Total number of subjects Case/control	Case/controls EV+	Case/controls EV-	Assays	OR	Case studies
Banatvala et al., 1985[§]	England, Austria, Australia	CV-B1 to CV-B5	122/204	37/15	85/189	ELISA IgM	6,3	
Gamble et al., 1985	UK	CV-B	578/227	194/85	384/142	ELISA IgM	0,8	
Field et al., 1987	Canada	CV-B1 to CV-B6	113/87	28/4	85/83	NAb	6,8	
Tuvemo et al., 1988	Sweden	CV-B1 to CV-B5	24/24	16/3	8/21	RIA IgM	14	
Tuvemo et al., 1989	Sweden	CV-B1 to CV-B6	98/94	11/16	87/78	RIA IgM	0,6	
Tuszkiewic-Misztal, 1991[B]	Poland	CV-B4	66/42	28(42,4%) /6(14,3%)	38/36	NAb	4,4	
d'Alessio et al., 1992	USA	CV-B	39/71	10/7	29/80	NAb IgM	4,55	
Frisk et al., 1992a	Sweden	CV-A and echoviruses	35/47	19/29	16/18	RIA IgM	0,7	
Frisk et al., 1992b	Sweden	CV-B	23/46	14/3	9/43	RIA IgM	22,3	
Emekdas et al., 1992	Turkey	CV-B1 to CV-B5	37/100	13/44	24/56	NAb	0,7	
Helfand et al., 1995	USA	14 EV serotypes	128/120	98/69	30/51	IgM ELISA	2,4	
Clements et al., 1995	UK	All serotypes	14/45	9/2	5/43	EV RNA	38,7	Lönnrot et al., 1998
Nairn et al., 1999	UK	All serotypes	110/182	30/9	80/173	EV RNA	7,2	Otonkoski et al., 2000; Vreugdenhil et al., 2000
Juhela et al., 2000	Finland	CV-A16, CV-B4, PV-3	13/13 29/29	9/1 17/25	4/12 12/4	T-cell prolif. To EV ag	27 0,23	

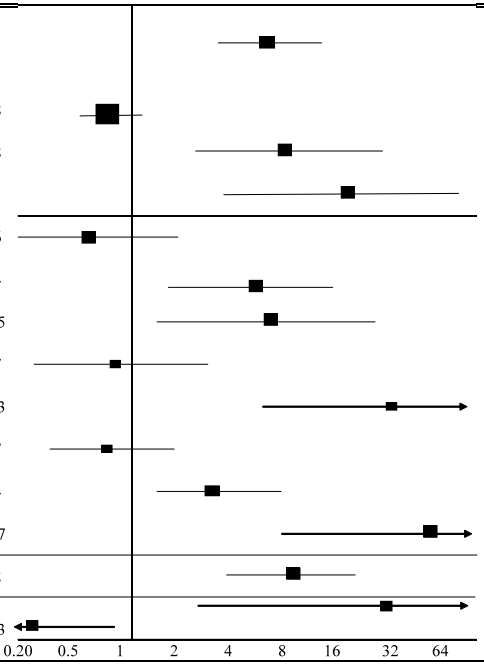
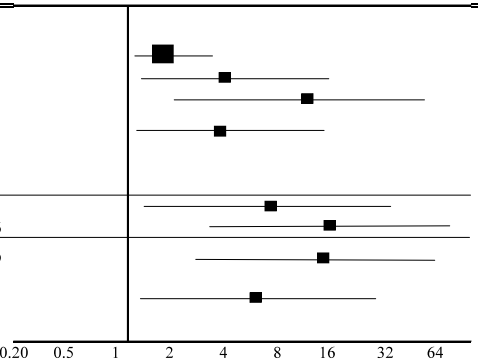


Table 3 continued

Study	Study site	Enterovirus tested	Total number of subjects Case/control	Case/controls EV+	Case/controls EV-	Assays	OR	OR & 95% CI	Case studies
Chehadeh et al., 2000	France	CV-B2 to CV-B4	56/37	21/0	35/37	EV RNA	NC		
Lonnrot et al., 2000	Finland	CV-B4, E-11, and immunodominant peptide ag.	125/567	33/103	92/464	EV infection	1,6		
			21/104	12/32	9/72	ab detection	3,7		
			100%/100%	29/6 %	71/94 %	EV RNA	8,4		
Yin et al., 2002	Sweden	All types	24/43	18/20	6/23	EV RNA	3,5		
Maha et al., 2003	Egypt	CV-B	40/30	19/0	21/30	NAb	NC		
			30/30	7/0	23/30		NC		
Moya-Suri et al., 2005	Germany	CV-B	50/50	10/2	40/48	EV RNA	6,0		Ylipaasto et al., 2004
			47/50	17/2	30/48		13,6		Dotta et al., 2007
Sarmiento et al., 2007	Cuba	All types	34/68	9/2	25/66	EV RNA	11,9		
Elfving et al., 2008	Sweden	CV-B3, E-11, CV-A16 ag. made broadly reactive to different EV	14/52	6/7	8/45	EIA IgM	4,8		



[#] The incident of CV-B 4 was considered the major for all viruses, as at least 114 of the patients had an enterovirus type. It is evident that multiple patients and controls have acquired multiple infections by different CV-B types.

[*] mixture of type 1 and type 2 patients

[§] Overall result of three contries.

[a] Overall result of the control group.

[B] percentage according to the abstract.

Animal models in T1D studies

Increasing data suggest that HEVs, especially CV-Bs, have an etiological role in human T1D. In order to elucidate the mechanism by which HEVs facilitate the onset of T1D and to develop therapeutic and preventive intervention, experiments on animal models are essential. The aim is to reproduce the situation of a predisposition to T1D. Animal studies have brought about more insight into the mechanisms by which CV-Bs modulate the diabetogenic processes. These models include animals developing T1D spontaneously, such as non-obese diabetic mouse (NOD) and several transgenic mice (Makino et al., 1980, Tracy et al., 2000, Tracy et al., 2002, Drescher et al., 2004, Lee et al., 2005, Kanno et al., 2006, Tracy et al., 2006). Unfortunately, CV-Bs do not replicate well in rats, which presents an obstacle for studying the effect of CV-Bs infection on spontaneously diabetic bio-breeding rats (BB rat) (Wong & Janeway, 1999, Roep & Atkinson, 2004, Roep et al., 2004).

NOD mice and other inbred mouse strains, such as C57BL/K, SJL/J, and Balb/C, have been used to investigate the mechanism of viral T1D. Several factors govern the choice of mouse strain, including physiological relationship to humans and the expression of viral receptors in mouse tissues. NOD mice develop T1D spontaneously and the development of the disease has several similarities to that of human T1D (Makino et al., 1980), including the presence of autoantibodies, T-cell mediated insulitis and various susceptibility genes. Controversies include the higher T1D incidence in female compared to male NOD mice, a phenomenon that is not replicated in humans (Leiter & von Herrath, 2004). Moreover, the nature of insulitis in NOD mice is characterized by a massive accumulation of leukocytes adjacent to and infiltrating the islets, whereas insulitis in human pancreas is characterized by massive leukocyte infiltration within the islets (Atkinson & Leiter, 1999).

CV-Bs isolated from two T1D patients have shown to induce diabetes (Yoon et al., 1979) and dysregulation of glucose metabolism (Champsaur et al., 1982) in several mice strains. These experiments clearly indicate that several CV-Bs are naturally diabetogenic. Moreover, the diabetogenicity of a virus strain can be enhanced by repeated passaging either *in vivo* in mice or in cultured mouse β-cells (Toniolo et al., 1982). However, in mice the infection is restricted to the exocrine tissue, in particular, while even in lethal cases the islets, ducts and connective tissue remain unaffected (Ross et al., 1974, Szopa et al., 1989, Vuorinen et al., 1989, Vella et al., 1992, Arola et al., 1995, See & Tilles, 1995, Ramsingh et al., 1997a, Ramsingh, 1997, Flodström et al., 2002b). In humans, HEV RNA has been found in numerous pancreatic islets and in some duct cells but not in the exocrine cells indicating an important host species difference between humans and mice in the cell type specificity of pancreatic HEV infection (Ylipaasto et al., 2004).

The islet protection of mouse islets from CV-B infection is thought to be mediated by cytokines. Virus-mediated damage in the target organs, such as pancreatic tissue, leads to the production of cytokines, specifically type 1 (IFN-α and IFN-β) and type 2 interferons (IFN-γ), which protect the islets from infection by

CV-B (Horwitz et al., 1998, Chehadeh et al., 2000, Horwitz et al., 2000). To determine the reason why CV-B4 infection in mice is exocrine tissue restricted, Flodström et al. (2002a) used transgenic mice that express the suppressor of cytokine signalling-1 (SOCS-1). They found that the β-cells of mice with the SOCS-1 gene were highly susceptible to CV-B4 infection, suggesting that IFN-α and IFN-γ are important in preventing viral infection of β-cells (Flodström et al., 2002b), thus shifting the focus towards the role of natural killer cells in regulating autoimmunity (Flodström et al., 2002a, Flodström et al., 2002b).

2.9.3 Potential mechanisms for induction of T1D by HEVs

Several mechanisms have been suggested for the role of HEVs in the induction of T1D (reviewed by Hyöty, 2002, Roivainen, 2006, Richer & Horwitz, 2009, Jaïdane et al., 2010), including direct β-cell cytolysis, bystander activation of autoreactive T-cells, viral persistency, and molecular mimicry. However, these mechanisms are not mutually exclusive.

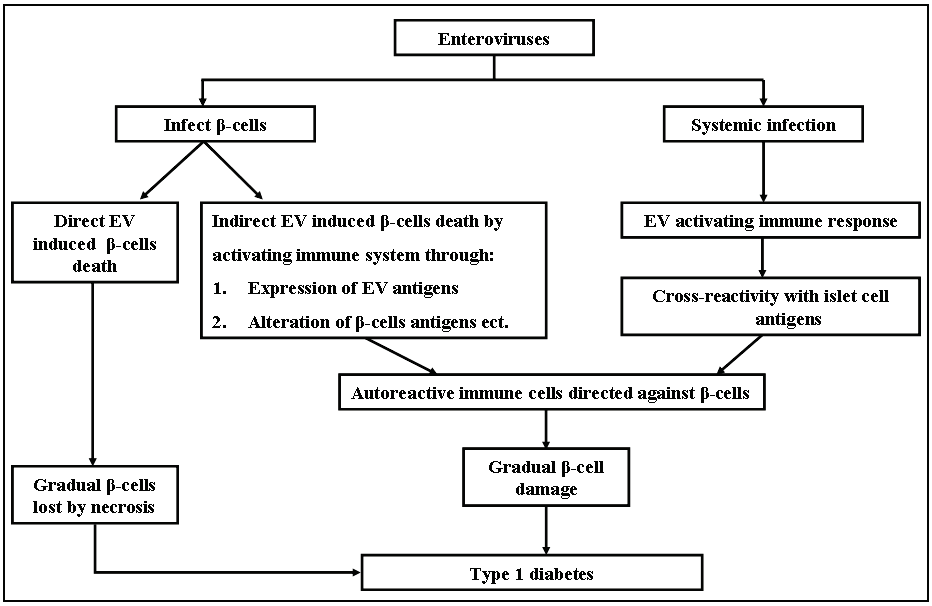


Figure 5. Schematic presentation of potential mechanism for HEV induced diabetes.

2.9.3.1 Direct cytolysis

The first documented report of enterovirus infection in the pancreatic islets of a T1D patient was by Ylipaasto et al. in 2004 and supportive results have been subsequently published by two separate teams (Oikarinen et al., 2008, Richardson et al., 2009). This suggests that during systemic infection, HEV may cross the gut

mucosa and reach the pancreatic islets and cause direct β-cell damage. Cellular experiments on human pancreatic islets have further supported the role of direct cytolysis induced by most HEV strains (Frisk & Diderholm, 2000, Roivainen et al., 2000). It has also been demonstrated that several HEV types frequently detected in the environment can damage human islet cells (Klemola et al., 2008). Based on the studies by Roivainen and co-workers, HEVs can be divided into those which directly destroy β-cells by virus-induced cytolysis, and less aggressive viruses (Roivainen et al., 2000). In both cases, β-cell damage may be mediated by inflammatory reactions.

Experiments on mice have verified the observations made in human primary islets concerning differences between types and strains, and regarding the natural predilection to the pancreas. The key difference in this regard between humans and mice is that in mice CV-Bs infect the exocrine pancreas, whereas in humans they infect islets. However, CV-B3 was detectable in the β-cells of NOD mice during the acute phase of infection (Kanno et al., 2006). Adaptation of CV-B4 JVB and CV-B4 P15 to mouse β-cells led to the induction of diabetes by viral replication in β-cells *in vivo* (Yoon et al., 1978, Vella et al., 1992). The diabetogenic CV-B4 E2 infects the islets of CD-1 mice and induces autoimmunity against β-cells. Viral RNA persistency in islets for several months was found to be a requirement for the induction of insulitis and autoantibodies (See & Tilles, 1995). These observations suggest that some CV-B strains preferentially infect islet cells.

2.9.3.2 Persistent infection

Enterovirus infections, CV-Bs in particular, have been associated with chronic immune disorders (Kandolf et al., 1999, Hyöty & Taylor, 2002, Maisch et al., 2003, Fujioka et al., 2004), but direct proof is lacking and a pathogenic mechanism has not yet been elucidated. Explanations are sought either in viral persistence in target organs (Kandolf et al., 1999, Ylipaasto et al., 2004, Bopegamage et al., 2005), blood (Clements et al., 1995, Andreoletti et al., 1997, Chehadeh et al., 2000), or in viral triggering of an autoimmune process by a virus (Rose, 2000, Fairweather & Rose, 2002,).

Several different HEV types have been shown to infect human islet cells and cause functional damage and destruction of pancreatic β-cells (Yoon et al., 1978, Vuorinen et al., 1992, Chehadeh et al., 2000, Frisk & Diderholm, 2000, Roivainen et al., 2000, Roivainen et al., 2002, Smura et al., 2010). HEVs are classically associated with lytic infections. The infections induce dramatic metabolic and morphologic changes in the host cell known as cytopathic effects (CPE). They can also establish non-cytolytic or chronic infections by persisting in various cell types including human pancreatic islet cells (Chehadeh et al., 2000, Roivainen et al., 2000, Yin et al., 2002b, Zanone et al., 2007). CV-B4 E2 infection in mouse pancreatic islets *in vivo* led to the development of chronic islet inflammation, β-cell destruction

and diabetes. This was considered a result of virus persistency in the islets for several months (See & Tilles, 1995).

CV-B persistency was suggested to have a possible role in β-cell death. HEV was detected within the islets of T1D patients in several independent studies in which different approaches were used, suggesting that HEV may persist within the islets (Ylipaasto et al., 2004, Dotta et al., 2007). Persistency can lead to the development of T1D through several mechanisms: continuous replication may disturb β-cell function, and persistency may lead to prolonged activation of the immune system due to continuous introduction of viral antigens and self antigens to T lymphocytes (reviewed by Hyöty, 2002, Richer & Horwitz, 2009). If the virus causes chronic infections by persisting in human β-cells, it may cause chronic inflammation leading to the gradual destruction of β-cells.

2.9.3.3 Bystander activation

Viral infection may induce bystander activation of T lymphocytes, including autoreactive T-cells directed against β-cell antigens. Inflammation in the pancreas may cause the damage and release of islet antigens (Horwitz et al., 1998, Horwitz et al., 2004, Roep & Atkinson, 2004). CV-B4 infection in the pancreatic islets of CD-1 mice led to the engulfment of β-cells by resident macrophages with minimal induction of cell death (Horwitz et al., 2004). Presentation of previously sequestered antigens would further lead to the activation of pre-existing populations of autoreactive T-cells which mediate islet destruction and the development of diabetes.

In NOD mice, CV-B4 can accelerate the spontaneous development of diabetes through bystander activation, although pre-existing autoreactive T-cells seem to be a requirement for the introduction (Serreze et al., 2000). A similar observation has been made with a BDC2.5 mice model (Horwitz et al., 2001).

2.9.3.4 Molecular mimicry

Molecular mimicry is based on partial sequence homology between an antigen and the pancreatic islet autoantigen. The best known example is the sequence similarity of six amino acids (PEVREK) between the non-structural protein 2C of coxsackieviruses and of glutamate decarboxylase (GAD65) (Kaufman et al., 1992, Rudy et al., 1995, Vreugdenhil et al., 1998a, Vreugdenhil et al., 1998b). This sequence homology has been shown to be a part of a diabetes-related T-cell epitope both in NOD mice and man (Atkinson et al., 1994, Tian et al., 1994, Schloot et al., 1997, Vreugdenhil et al., 1998b). It induced cellular and humoral cross-reactive immune responses with the 2C region (Atkinson et al., 1994, Lönnrot et al., 1996a, Lönnrot et al., 1996b).

There is also sequence homology between proinsulin residues 24-36 and GAD65 residues 506-518, which may induce cross-reactive immune responses in an early autoimmune event of T1D (Rudy et al., 1995, Chen et al., 2001). Additionally, the

C-terminal part of tyrosine phosphatase IA-2/IAR, a major target autoantigen in pathological indication of T1D, has sequence homology with the preserved motif in the enteroviruses VP1 protein (PALTAVETGA/HT) (Härkönen et al., 2002). Immunization of female NOD mice with CV-B4 E2 induces a humoral response recognizing the diabetogenic peptide IA-2/IAR (Härkönen et al., 2002). Likewise, it has been indicated that during serologically confirmed enterovirus infections, antibodies to this epitope are developed in a small number of humans (Härkönen et al., 2003).

2.10 Genetic determinants responsible for enterovirus induced pathology

Human enterovirus infections are a significant cause of morbidity and mortality throughout the world. HEVs have been associated with many human diseases, including myocarditis (Klingel et al., 1992), pancreatitis (Ramsingh, 2008), and chronic inflammatory myopathy (Tam and Messner, 1999). Poliomyelitis has been a well-known human disease throughout the history of mankind. However, other non-polio human EVs are also common causes of epidemic infections.

Susceptibility and resistance to virus infections are influenced by genetic factors of both the host and the virus. The increase or attenuation of viral pathogenesis is affected by the degree of antigenic variation resulting from genetic variation among virus types and even within a single isolate due to the high frequency of point mutations and recombination events (Rotbart et al., 1990, Szopa et al., 1990, Domingo et al., 1998, Domingo., 1998). In animal models, HEV strains of the same type show different virulence phenotypes. Yoon and co-workers have demonstrated the significance of adaptation in the mouse pancreas in the pathogenesis of T1D (Yoon et al., 1978). Consistently, CV-B4 JVB and CV-B4 P15 induce diabetes after an adaptation process (Vella et al., 1992, Szopa et al., 1993). This reveals that the key elements of pathogenesis can be found within the viral genome resulting from changes occurring through passaging in the host. It is expected that changes in the viral genome may enhance or attenuate virulence.

Several studies, including this thesis, indicate that very small changes in the viral capsid region may result in an altered viral phenotype. Alterations in the viral phenotype may concern cell type specificity, tissue tropism and the host species, finally affecting cytopathogenicity and also immune response in mice (Caggana et al., 1993, Jun et al., 1997, Halim & Ramsingh, 2000, Halim et al., 2000). It has been indicated that a single point mutation at nucleotide position 3155 or 3156 of the recombinant encephalomyocarditis virus (EMC) genome, located on the major capsid protein VP1, which causes an amino acid change, results in the gain or loss of viral diabetogenicity (Jun et al., 1997). In the case of pancreatitis, CV-B4 replication in mouse acinar pancreatic tissues was mapped to Thr-129 of VP1 (Caggana et al., 1993). In addition, Thr-129 of VP1 has an effect on the recruitment of B and T cells

to the pancreas (Ramsingh et al., 1997b) and on the antigenicity of the virulent virus (Halim & Ramsingh, 2000, Halim et al., 2000).

Other parts of the genome may also affect the virulence of an enterovirus. Lessons learned from myocarditic phenotypes indicate that the genetic determinants have a key role in CV-B3 induced myocarditis in mice. The genetic determinant was mapped to a short RNA sequence within 5'-UTR, domain II, or stem–loop II (SLII) (Dunn et al., 2000, Dunn et al., 2003). Most RNA viruses have multiple virulence determinants (Tracy et al., 2006). For example, by using a clinical isolate and tissue culture-derived strains of CV-B1, Tam and co-workers have mapped five different sites in the CV-B1 genome that contribute differentially to skeletal muscle inflammation and hind limb weakness in a mouse model of myositis or chronic inflammatory myopathy (Tam et al., 1994, Tam & Messner, 1997, Tam & Messner, 1999, Tam et al., 2003).

Changes in virulence may result from nutrient deficiency, such as selenium (Beck et al., 1994a, Beck et al., 1994b, Beck et al., 2003a, Beck et al., 2003b, Jun et al., 2011). In these studies, selenium-deficient mice infected with avirulent CV-B3/0 have subsequently developed myocarditis. The conversion from avirulent to virulent was mapped to 6 nucleotide changes that fully correlated with the virulence of CV-B3.

3 Aims of the Study

3.1 General aim of the thesis

The aim of the thesis was to understand the influence of sequence variation of HEV-B on diabetogenicity. To accomplish this, laboratory isolates of the CV-B5 strain DS and isolate D207 isolated from a diabetic individual were used in different experimental settings. Firstly, the adaptation process of virus variants was studied in detail in a mouse model and in mouse insulinoma cell lines and human primary islets cells. Secondly, full genomes of viruses selected for this thesis were sequenced to obtain new information on the genetic changes that may be involved in HEV-B pathogenesis in the onset of type 1 diabetes.

3.2 Specific aims of the thesis

Examination of enteroviral genetic determinants that are relevant to the diabetogenic phenotype by:

1. using a virus strain multiply passaged in mouse pancreas and analysing its cytopathology
2. studying virus and β-cell interaction using murine insulinoma cell lines
3. identifying virus determinants critical for islet cell tropism
4. analysing the full length genome of the D207 strain isolated from a diabetic individual from Slovakia
5. studying the molecular epidemiology of E-11 isolates

4 Materials and Methods

4.1 Viruses

All viruses used in this thesis belong to the *Human enterovirus B* species (HEV-B). The laboratory isolate of the coxsackievirus B5 strain DS (CV-B5-DS-1, CV-B5-DS or DS) kindly provided by Dr. Darryl See, was plaque purified and confirmation of the virus serotype was done by a neutralization assay using type-specific antisera. Strains MPP and MCA were obtained by passaging strain DS in mouse pancreas and MIN-6 cells, respectively. The known diabetogenic strain E2 of CV-B4 (Edward strain), kindly obtained from Prof. Jin-Woo Yoon, was used as a control in the initial mouse experiment *in vivo* (I). The prototype coxsackievirus B5 strain Faulkner (CV-B5) was obtained originally from the American type culture collection (ATCC) (II).

Another enterovirus strain, kindly provided by Dr. Shubhada Bobegamage and designated as D207, was isolated from a diabetic child in Slovakia and originally serotyped as coxsackievirus A9 (CV-A9). Two E-11 strains were causative agents of uveitis outbreaks in Siberia in 1986 and 1987, E-11/Kust/86 and E-11/Kar/87, respectively, and a third virus was an isolate in the Russian Far East in 1997, E-11/kh3/97 (Lukashev et al., 2002, Lukashev et al., 2003b). These viruses were kindly provided by Dr. Alexander N. Lukashev (III).

Fifty-eight E-11 strains were originated from environmental samples in Finland. The other 11 E-11 strains were clinical isolates sent to the national enterovirus reference laboratory from other Finnish laboratories and untypable nonpolio-enteroviruses from the National Polio Laboratories in Iceland, Slovakia and Egypt (Blomqvist et al., 2008). Viruses from the clinical specimens were isolated from stool and cerebrospinal fluid (CSF) (IV, Table 1).

4.2 Virus-specific antisera

The polyclonal rabbit antisera against E-11 were purchased from SBL (Swedish Institute for Infectious Diseases Control, Solna, Sweden); the polyclonal rabbit antisera against CV-A9 was made in-house (861, (Hovi & Roivainen, 1993)); and the polyclonal rabbit antisera against strains E-11/MorM/82, E-11/Pah/89, E-11/Vlad/89, E-11/Kust/86 and E-11/Kar/87 were kindly provided by Dr G.A. Koroleva (Lukashev et al., 2002, Lukashev et al., 2003b).

4.3 Cell Culture

4.3.1 GMK cells (I-IV)

African green monkey kidney cell lines (GMK) were cultured in Eagle's minimal essential medium (MEM), supplemented with 10% foetal bovine serum (FBS),

penicillin (100 units/ml), streptomycin (0.1 mg/ml), 20 mmol/l HEPES (N-22-hydroxyethylpiperazine-N0-2-ethanesulfonic acid) pH 7.4, and 20 mmol/l $MgCl_2$. GMK cells were used in several assays, for virus passaging, for assays of total infectivity using end-point dilutions in microwell cultures ($TCID_{50}$), for transfection to obtain infective clones and site-direct mutagenesis, for assay of immunofluorescence (IF) to visualize virus antigens, and for plaque neutralization. GMK cells were incubated at 36 ^{o}C in an atmosphere of humidified air (95%) and CO_2 (5%).

4.3.2 MIN-6 cells (II)

The mouse insulinoma cell line, MIN-6 (Miyazaki et al., 1990), was kindly provided by Prof. Miyazaki (Kumamoto University Medical School, Japan). MIN-6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS, 50 μM β-mercaptoethanol, penicillin (100 units/ml), streptomycin (0.1 mg/ml), 20 mmol/l HEPES pH 7.4, and 20 mmol/l $MgCl_2$. The MIN-6 cells grew adherently and were incubated routinely as described above.

4.3.3 Primary human islets (III)

Human pancreatic islets were isolated and purified at the Uppsala University Hospital (coordinator Prof. Olle Korsgren) as previously described (Johansson et al., 2003), with the consent of the ethics committee of Uppsala University. The islets were received in Helsinki as free-floating islets after 1–5 days of culture in Ham's F10 medium supplemented with 10 mmol/l HEPES and 2% calf serum. Before the experiments, the islets were maintained for 1–3 days in sterile, non-adherent culture plates in serum-free incubation medium (Ham's F10 containing 25 mmol/l HEPES, pH 7.4, 1% bovine serum albumin, penicillin [100 units/ml], streptomycin [0.1 mg/ml]). Incubation was performed as above. For the experiments, the islet cultures were divided into parallel aliquots and infected with an apparent high multiplicity of isolate D207 or CV-A9 Griggs viruses (Ylipaasto et al., 2005).

4.4 Experiment on animal model *in vivo* (I)

4.4.1 Animal model (I-II)

Male CD-1 mice were obtained from Harlan (Harlan, Boxmeer, the Netherlands) and housed in a Macrolon M3 cage in groups of four animals. CD-1 mice were used in several experiments in articles I and II. At the age of 5 weeks, parallel groups of mice were infected intraperitoneally with either the CV-B5 strain DS, its mice pancreas passaged MPP strain, MIN-6 cell adapted MCA strain, or CV-B4 strain E2. All mouse studies were approved by the Institutional Committee on Animal Care and Use, with final permission being granted by the Provincial Government of Southern Finland.

4.4.2 Passaging *in vivo*

CV-B5-DS-1-MPP (also CV-B5-MPP or MPP) was obtained after passaging the laboratory isolate of CV-B5 strain DS 15 times in mice (2-12 mice/passage), hence strain DS is the parental type of stain MPP. In the first experiment, two mice were infected intraperitoneally using an infection dose of 3 million infectious units per mouse. The mice were sacrificed at 3 days postinfection and pancreatic tissue was extracted, frozen and crushed by slicing 200 µl buffer. In order to obtain the next passage, a new set of mice were infected in a similar way with 200 µl of the previous passage of the virus strain. All virus passages were assayed for total infectivity using end-point dilutions in GMK cultures on microwell plates.

4.4.3 Studying effect of viral replication *in vivo*

To study the viral replication in mice, parallel groups of mice were infected via the intraperitoneal route with the parental virus strain CV-B5-DS (I) (0.3 million infectious units/mouse), or with a corresponding amount of mouse pancreatic-passaged virus strain, CV-B5-MPP (I and II), or MIN-6 cells adapted virus strain, CV-B5-MCA (II). The mice were sacrificed on selected days after infection and pancreatic tissues were collected for determination of viral total infectivity and fixed with 10% formaldehyde for histological haematoxylin and eosin staining (I and II). Other organs, such as heart and spleen as well as blood were collected to be used in determining viral infectivity in these tissues (I).

4.5 Virus replication *in vitro* (II)

4.5.1 Infection of MIN-6 cells

MIN-6 cells were cultured on tissue-culture-treated glass slides obtained from Becton Dickinson (Falcon culture slides, Becton Dickinson, USA). After virus adsorption for 1h at 36 °C (multiplicity of infections, M.O.I 1–10), the virus solution was removed and cells were washed twice with Hanks's balanced salt solution supplemented with 20 mmol/l HEPES, pH 7.4. An incubation medium containing 1% FBS was added to all cultures and the virus was allowed to replicate at 36 °C. Samples of suspended cells taken at different times were freeze–thawed three times to release the virus and clarified by low-speed centrifugation. These were assayed for total infectivity using end-point dilutions in cultures of GMK cells on microwell plates.

4.5.2 Visualizing viral antigens by immunofluorescence staining

To visualize virus antigens utilizing immunofluorescence staining, MIN-6 and GMK cells on Falcon slides were fixed at selected intervals using pre-cooled 100% methanol and then washed three times with PBS. Subsequently, the slides were incubated with enterovirus-specific polyclonal rabbit antiserum (1:400, KTL-510;

(Hovi & Roivainen, 1993)) for one hour at 36 °C, followed by a secondary incubation for 30 minutes with FITC labelled anti-rabbit antibody (Jackson ImmunoResearch Laboratory). A confocal microscope (Leica TCS NT) and Paint Shop Pro software were used to obtain and edit the images.

4.6 RNA extraction, RT-PCR, sequencing and molecular typing (I-IV)

Two separate procedures were used to extract the RNA. In publications I-III, the kits used were Trizol Reagent (Gibco BRL, Life Technologies, Gaithersburg, MD) for total RNA extraction; the SuperScript First-Strand Synthesis System for the RT-PCR Kit (Gibco BRL, Life Technologies, Gaithersburg, MD) for first-strand synthesis of cDNA production; SuperScript One-Step RT-PCR for the Long Templates Kit (Gibco BRL, Life Technologies) for virus genome amplification without the room temperature step, and the pGEM-T Easy Vector System I Kit (Promega, Madison, WI) for cloning. All steps were done according to manufacturer instructions.

The kits used in Publication IV for RNA extraction and RT-PCR were different from the methods mentioned above. For RNA isolation, two commercial kits; the Rneasy Total RNA kit (Qiagen Gmbh, Hilden, Germany) or the E.Z.N.A. Total RNA kit (Omega Bio-Tek Inc., Doraville, GA, USA) were used and RNA was extracted from 100 µl of the infected cell cultures according to the manufacturers' instructions. RT-PCR was performed with primers 292 and 222 (Oberste et al., 2000, Oberste et al., 2003a). Purification of the PCR products, sequencing and molecular typing were done as described in Blomqvist et al., 2008.

4.7 Molecular sequencing and construction of infectious cDNA clones (I-III)

The extracted viral RNA was used as a template to produce cDNA according to the manufacturer's instructions as described above. EP4A and A83A oligonucleotides were utilized to produce cDNA of the CV-B5 variant (I and II) and CV-A9/D207 (III), respectively. The genomes of the parental CV-B5-DS and CV-B5-MPP were produced in two partially overlapping halves, 3605- and 3922-nucleotide-long PCR products, using EP1 and EP2, and EP3 and EP4A primer pairs (Table I-I). The purified PCR products of both halves were cloned in a vector and the clones were transfected into competent bacterial cells (*Escherichia coli* DH5α). Plasmid DNA was extracted from bacteria using the QIAprep Spin Miniprep Kit Protocol (QIAGEN, Hilden, Germany). The full-length genome of CV-B5-MCA was produced using EP1 and EP4A oligonucleotides as primers. Subsequently, the genome was cloned and transfected as above. The full length genome of strain D207 was produced using the A82S and A83A oligonucleotides as primers (III) and cloned as above.

The purified plasmid clones (I and III) were sequenced using the primer walking strategy. Each nucleotide was sequenced at least twice (III) or mutated nucleotides were re-sequenced in the reverse direction (I). The cycle sequencing reactions were performed using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). The automated sequencing equipment ABI Prism 310 Genetic Analyzer (Applied Biosystems) was used. Primers for sequencing for the CV-B5 variant (Table II-I) and strain D207 (Table S1-III) were purchased from DNA Technology A/S (DNA Technology A/S, Risskov, Denmark). The pUC/M13 forward primer was purchased from Promega (III). Sequence data was analysed using the Vector NTI Suite 6 program (Invitrogen), assembled using ContigExpress and aligned using AlignX software (I and III). The CV-B5-MCA clone was sequenced (sequencing primers from Table II-I) using the primer walking strategy at the Molecular Medicine Sequencing Laboratory, NPHI (Biomedicum, Helsinki, Finland). The sequence data was analysed using the Vector NTI Suite 7 program (Invitrogen) (II).

The CV-B5-DS and CV-B5-MPP infectious clones were produced in two steps. First, viral genomes were produced in two halves of the 5'- and 3'ends as described above. The second step included combining the two overlapping halves. The whole genome of E-11 was amplified by PCR and transformed into *E. coli*. Plasmid DNA was then extracted from bacteria using the QIAprep Spin Miniprep Kit protocol (QIAGEN, Hilden, Germany).

4.7.1 Induction of Xba1 restriction site

The 3' cDNA half was re-amplified to introduce a novel Xba1 site at position 3496 of the DS and MPP strains (A→T). Mutation from A to T did not alter the amino acid sequence. During this procedure, 150 bp overlapping PCR amplicons were produced using four mutagenic oligonucleotides. P211s and P213a were used to amplify the first amplicon, and P212s and P214a for the second amplicon. The two amplicons were combined using P211s and P214a. P213a and P212s had an Xba1 site at the same position, the P211s had two restriction sites, AatII and Cla1 and P214a had a restriction site for EcoRV. According to this, the combined amplicon had four restriction sites AatII, Cla1, Xba1 and EcoRV, respectively. The pGEM-T-easy vector contains a restriction site AatII, and the 3'-half of the viral genomes contain an EcoRV site. To insert the combined amplicons in the 3'half, they were digested together with the 3'half using AatII and EcoRV. After purification, both products were ligated and transformed into E.coli cells. The 5'half of the virus cDNA, which contained 5'-nontranslated and capsid regions, was re-amplified using EP1s and P213a to insert the Xba1 site. After gel extraction, the re-amplified 5'half and the clone of the 3'half of the virus genome were digested using Cla1 and Xba1, ligated, and transformed into *E. coli*. The plasmid containing full-length insert was extracted from the bacteria and the viral clone was sequenced (II).

4.7.2 Transcription and transfection

The CV-B5-DS, CV-B5-MPP, CV-A9/D207 clones were either linearized with Mlu1 (II) or digested with the NotI (III) restriction enzymes (New England Biolabs, Ipswich, MA) and then transcribed into RNA using T7 polymerase (Roche, Germany) for 1h at 37 °C. Transcribed RNA (5 and 20 µl, respectively) was transfected into sub-confluent GMK cells to determine their infectivity using Lipofectamine and Plus reagent (Invitrogen). The infectivity of RNA was identified as the ability to induce a cytopathic effect in cells.

4.7.3 Constructing the virus mutants (II)

Two capsid mutations were introduced to the full-length infectious cDNA clone of CV-B5-MPP, in VP3 (position 2118 bp G→A) and VP1 (position 2726 bp G→A) (Table 1-II). The infectious cDNA clone was first linearised using the Mlu1 restriction enzyme. The VP3 mutant was constructed by preparing two overlapping amplicons using EP1s and HMP6a, HMP5s and P213a primer pairs. The overlapping amplicons were combined using EP1s and P213a primers. The infectious cDNA clone of CV-B5-MPP and the combined PCR product were both digested using ClaI and XbaI restriction enzymes and extracted from agarose gel (QIAEXII, QIAGEN, Hilden, Germany). Subsequently, the combined PCR product was ligated to the cDNA clone of the CV-B5-MPP, and then transformed into competent bacterial cells (*Escherichia coli* DH5α). Plasmid DNA was extracted from bacteria using the QIAprep Spin Miniprep Kit Protocol (QIAGEN, Hilden, Germany). A VP1 mutant was prepared using primer pairs HP9s and HMP8a, HMP7s and P213a to generate two overlapping amplicons which were combined utilizing HP9s and P213a oligonucleotides. The infectious cDNA clone of CV-B5-MPP and the combined amplicons were digested using BglII and XbaI restriction enzymes, and then gel extracted (QIAEXII). Ligation, transfection, and plasmid purification were performed as above. The viral clone was sequenced to ensure that only the expected mutations were inserted.

4.8 Plaque neutralization assay (III-IV)

A virus dilution 10^{-2} was incubated for 2 h at 36 ^{o}C with an equal volume of virus-specific polyclonal antiserum. After neutralization, virus samples were quantified for infectious virus by administration in 1:10 (10^{-3}-10^{-8}) serial dilutions onto GMK cell monolayers on six-well plates. After 30 min incubation at 36 ^{o}C, the inoculum was removed and the plates were subsequently overlaid by 2 ml of 0.5% carboxy methyl cellulose in Eagle MEM. Cells were incubated at 36 ^{o}C for two days and stained with crystal violet after which the number of plaques were counted.

4.9 Phylogenetic analysis (III-IV)

The nucleotide and amino acid sequences were aligned by CLUSTAL_X 1.83 (Thompson et al., 1997). Phylogenetic trees were produced by Bootstrap N-J Tree (CLUSTAL_X 1.83) using 1000 bootstrap replicates (bootstrap value 700 was considered significant) and were visualized with NJPlot (Perriere & Gouy, 1996). Similarity analysis of complete genomes was performed with SimPlot 2.5 (Lole et al., 1999) with a window of 200 nucleotides.

4.10 Peptide scanning (III)

To identify the epitopes in the capsid region of the D207 isolate responsible for dual neutralization, overlapping peptides of 12 amino acids with three-residue shift forming 384 peptides were synthesized. The synthesized peptides were coupled onto polyethylene glycol derivatized cellulose membranes (Hartman Analytic). The epitope mapping assay was performed based on the manufacturers' instructions (ABIMED and Genosys) and as described previously (Pulli et al., 1998, Härkönen et al., 2002, Härkönen et al., 2003). In brief, a blocking buffer (Tris-buffered saline (TBS), pH 8.0), containing 0.05% Tween-20 (Fluka), 1% casein (Genosys), and 5% w/v sucrose, was used to block unspecific binding. The peptide-coupled membranes were incubated at 4 °C overnight with the blocking buffer. All subsequent steps were performed at room temperature. Membranes were incubated for 2 h with virus-specific hyperimmune rabbit serum, diluted in blocking buffer, washed 5x5 min with washing buffer and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulins (Bio-Rad; 1:1500) for 30 min. The membranes were washed as above and incubated for 1 min with chemiluminescence reagent plus (NEN Life Science Products). Chemiluminescence was counted with a Victor scintillation counter (PerkinElmer) and verified by autoradiography. The membranes were regenerated with 8 M urea, 1 % SDS/0.1 % β-mercaptoethanol (3x30 min at 50 °C), 5x10 min (room temperature) in 50 % ethanol/40 % water/10 % acetic acid and 5x5 min with TBS 0.05 % Tween. The success of regeneration was tested with the conjugate.

5 Results and Discussion

5.1 Model for studying diabetogenicity

Enteroviruses induce or accelerate β-cell destruction by two major mechanisms according to whether the virus reaches the pancreas or other tissues (Chapter 2.9.3, Fig 5). Direct cytolysis occurs when the virus reaches the pancreatic islets and destroys the β-cells when multiplying in them. According to the current knowledge, several different enteroviruses are associated with T1D. Firm evidence is available for some coxsackie- and echoviruses. The overall aim of the study was to answer the question, what makes an enterovirus diabetogenic? In this thesis, diabetogenic enterovirus is described as virus strain, which is capable of inducing a diabetes-like syndrome in a mouse model or human islet cell death *in vitro.* The diabetes-like syndrome is characterised by chronic inflammation in the mouse pancreas together with mild dysregulation in glucose metabolism, loss of pancreatic acinar tissue and mild insulitis. The potential viral determinants responsible for the diabetogenic phenotype may be identified through analysis of full-length genomes of selected virus strains and site-direct mutagenesis.

Several studies have indicated that CV-B infection can induce murine type 1 diabetes (Yoon et al., 1979, See & Tilles, 1995, Ramsingh et al., 1997a). It has also been demonstrated that induction of type 1 diabetes in a mouse model is restricted to a specific CV-B strain (Yoon et al., 1979, Toniolo et al., 1982, Szopa et al., 1985, Szopa et al., 1989). However, to date the viral genetic determinants responsible for the variation of virulence remain undetermined. In order to study the adaptation process of the diabetogenic CV-B4 strain E2, as described by See & Tilles (1995), and to explore the genetic determinants responsible for the resulting phenotype, a virus stock obtained kindly from the laboratory of Dr. Darryl See was used. Using plaque isolation and virus neutralization assay, the virus stock was confirmed as containing CV-B5 and poliovirus 1, and not the CV-B4 strain E2. Consistent results were also described by our colleague in Sweden who received the same virus stock from the same laboratory. This emphasizes the significance of checking virus strains and other reagents before starting the experimentation. The plaque purified CV-B5, which may be part of the original clinical stock, was designated as strain DS and later used to study its pancreotropism in animals, and subsequently, to study virus genetic variation resulting from adaptation *in vivo*.

Studies were carried out to investigate the genetic diversity and molecular evolution of the D207 strain originally typed as CV-A9 isolated from a diabetic child in Slovakia. The D207 clinical isolate presented us with an opportunity to study the genetic variation of a clinical isolate circulating in nature.

5.2 Pancreotropism of HEV-B (I and II)

The ability of selected virus isolates to infect pancreatic tissue was analysed. CV-B5 DS was used to infect animal models *in vivo* (I), and D207 was utilised in human pancreatic islet infection *in vitro* (II). According to previous data, CV-B4 and CV-B5 have often been linked to the pathogenesis of type 1 diabetes (Yoon et al., 1979, Champsaur et al., 1982), and results from primary human islet infection showed impaired β-cell function or cell death or both (Frisk & Diderholm, 2000, Roivainen et al., 2001, Roivainen et al., 2002, Ylipaasto et al., 2004, Dotta et al., 2007, Klemola et al., 2008). To study the adaptation of strain DS and explore its genomic determinant, an animal model was used. This provided powerful tools for studying the development of the disease and the establishment of its complications in detail. A comparative experiment was conducted using the extensively studied diabetogenic CV-B4 strain E2 as a control. A set of parallel groups of 5-week-old CD-1 mice were infected intraperitoneally with either of the selected coxsackie B viruses. The mice were inoculated with equal amounts of viruses. Three days after infection, the mice were sacrificed and one half of the tissues were collected for infectivity measurements. In the case of the diabetogenic CV-B4 strain E2, the virus had similar distribution to all examined organs including the pancreas, whereas the CV-B5 strain DS was found to be more virulent in the pancreas giving yields 10- to 100-fold greater virus titres than the E2 strain (Fig 1 – I). It has been demonstrated in this thesis and others (Vuorinen et al., 1989, Gomes et al., 1991, Ramsingh et al., 1997a, Ramsingh., 1997, Tracy et al., 2000) that the titres resulting from CV-B replication in murine pancreas tend to be as high as or even higher than the titres in other tested tissues. Subsequently, the virus was rapidly cleared from the pancreas at three days after inoculation. These results indicated that the isolate CV-B5 strain DS has a natural tendency to infect the pancreatic exocrine tissue in mice (I).

According to the first Finnish DiMe-study, the infections by CV-A9, CV-B1, 3, and 5 were associated either with seroconversion of islet cell autoantibodies or the onset of T1D or both (Hyöty et al., 1995, Hiltunen et al., 1997, Roivainen et al., 1998). These data encouraged us to investigate the replication of strain D207, originally serotyped as CV-A9, in cultures of primary human islets. At this stage, strain D207 was still considered as CV-A9 and the results were compared to the prototype CV-A9. The virus was shown to cause rapid cytolysis coinciding with severe functional damage of the surviving islet cells (II). The results were surprising, since the prototype of coxsackievirus A9 (CV-A9) is known to cause only minimal damage in primary human islets. These results opened the door to investigate the genetic background of the D207 isolate and to define the determinants responsible for the observed phenotype (II).

5.3 Adaptation of coxsackievirus B5 strain DS to mouse pancreas (I)

The laboratory isolate of CV-B5 strain DS induced acute inflammation but failed to induce prolonged inflammation in infected mice. Adaptation of an enterovirus may enhance its replication in the secondary target tissues. To study the adaptation process in detail, the pancreotropic CV-B5 strain DS was selected for repeated passaging in mice *in vivo*. The produced virus strain from mouse pancreas passaging was called CV-B5-DS-1-MPP (CV-B5-MPP or MPP). As a result of fifteen successive passages, virus titres in the pancreatic tissues increased. The titres in other tested tissues also increased (Fig 2 – I). In order to gain better understanding of the effect of virus replication *in vivo*, parallel groups of mice were infected intraperitoneally with CV-B5-MPP or the parental CV-B5-DS virus, and the infection-induced consequences at various time points were compared between the groups. Three days after infection, five mice from each group were sacrificed and one half of the pancreas, heart and spleen were collected for infectivity measurements. The rest of animals were used for blood glucose measurements and histological analyses. Histological examination of tissues showed that 2-3 days post infection (p.i.) both virus strains, CV-B5 strain DS and MPP (mouse pancreas passaged virus strain), replicated in the pancreas causing acute pancreatic damage. However, inflammation induced by CV-B5 strain MPP following intraperitoneal injection in CD-1 male mice had three distinct immunovirological and pathological phases (the acute phase, subacute phase and chronic phase), while inflammation signs induced by the parental virus CV-B5 strain DS were restricted to the acute phase (Fig 6). The acute phase is characterized by prominent virus replication in blood and infected tissues. Viral antigen is detected in the islets of Langerhans. During the subacute phase, the presence of the progeny virus stimulates the infiltration of immune cells and probable proinflammatory cytokine released within infected pancreas. In this stage, the titre of the infectious virus began to decrease as a marker of tissue clearance of viral particles. The chronic phase is characterized by the absence of viral particles, atrophy/fibrosis, fat replacement, mild inflammation in the islets and dysregulation in glucose metabolism.

The histopathological changes in the mouse pancreas induced by infection were studied by infecting parallel groups of mice with the parental virus CV-B5-DS or the passaged virus CV-B5-DS-MPP. At various times after infection, subgroups of mice were sacrificed for histological examination of pancreatic tissue. Formaldehyde fixed paraffin blocks were cut into 5-µm sections and stained with hematoxylin and eosin. During the acute viremic phase, a high titre of virus was present in the blood, heart, spleen and pancreas. Aggressive virus replication within 2-3 p.i. led to early virus-induced injury in the acinar tissues with mild inflammation in the islet of Langerhans (Fig 3 and 4 – I and Fig 6). Signs of necrosis in the acinar tissue were more severe in mice infected with CV-B5-MPP than in those infected with the

parental strain, CV-B5-DS. Interestingly, the parental CV-B5-DS replicated to titres within the range of CV-B5-MPP, but both virus strains had different pathological changes in pancreatic tissue of the inoculated mice (Fig. 4-I). Thus, the analysis concerning virus titres in the pancreas, heart and spleen of inoculated mice indicated that virus replication in pancreatic tissue cannot be solely used as a marker for virulent viral phenotypes of the pancreas.

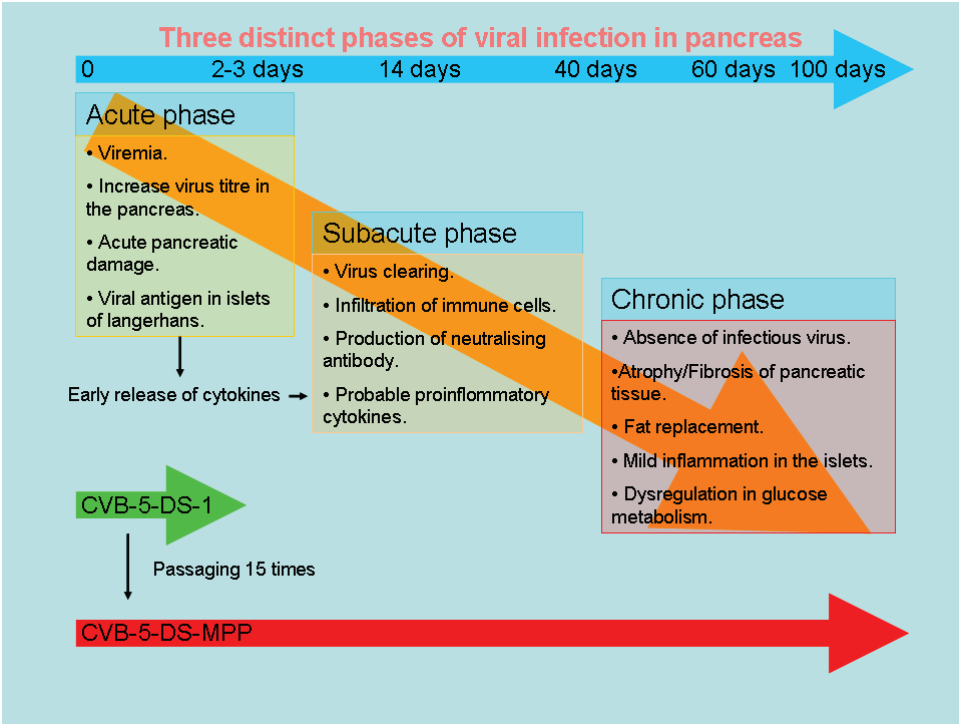


Figure 6. The three distinct phases of coxsackievirus B5 MPP infection in mouse pancreas *in vivo*.

Support for this observation came from previously published studies (Ramsingh et al., 1989). The pancreotropic variant CV-B4-V and the avirulent strain of CV-B4-P induced different pathologic changes in murine pancreas, but both viruses were detected at similar titres in murine pancreas. Tracy et al. (2000) showed that the avirulent CV-B3/GA replicated to the levels observed for the two pancreovirulent strains AS and CO at days 2 and 4 after infection. In spite of the high titre in the pancreas, the avirulent strain GA did not induce disease in the pancreas or in the heart. In this thesis, the parental CV-B5-DS strain induced only residual foci damages in the acinar tissue and the majority of exocrine pancreas remained

unaffected. Damage induced to the acinar tissue disappeared by 3 days p.i. (Fig. 3-I, Fig 6). In contrast, the destructive effect of the CV-B5-MPP virus was more prominent and evident for much longer. Hence, passaging of CV-B5-DS resulted in a virus with altered phenotype; this virus was called CV-B5-MPP.

The subacute phase of the disease can be induced only through infection with CV-B5-MPP (Fig 6). At this phase, infiltration of immune cells to the acinar tissue and islet cells of Langerhans were also observed, suggesting pancreatic expression of proinflammatory cytokines during the acute phase. It is likely that the inflammatory response and acinar cell division determine the progression from acute to subacute and chronic phases, as is the case for CV-B4-P/CV-B4-V (Ostrowski et al., 2004). In this stage, extensive CV-B5-MPP replication is associated with major inflammatory lesions. The virus titre began to decrease and was cleared by day 14 post infection probably because of the existence of high levels of neutralizing antibody and the establishment of B-cell mediating immunity. However, the MPP strain was capable of inducing damage for a much longer period of time, and signs of moderate atrophy/fibrosis and mild chronic inflammation were found in the acinar tissue at 6 weeks after infection (Fig. 4-I). Virus persistence was suggested as an explanation for CV-B-induced chronic immune disorders in pancreas (Bopegamage et al., 2005). Despite the fact that infectious virus could not be isolated from pancreatic tissue beyond three days after infection, signs of mild inflammation were seen for long periods of time in islets (Figs. 4 and 6-I). One could speculate that a recurrent infection at the subacute phase leads to the engulfment of infected β-cells by local antigen-presenting cells (APC) and to subsequent activation of the pre-existing autoreactive T-cell population which finally leads to T1D. A similar mechanism is likely to occur in humans (Elshebani et al., 2007, Schulte et al., 2010a, Schulte et al., 2010b). The continuous replication of HEV in human gut leads to genetic variation in the virus and may result in a more pathogenic phenotype. As such, it seems that enterovirus infection may be a causal agent of T1D in individuals with high risk of T1D but also in those with low risk of T1D. It is possible that in individuals with high T1D risk, entervirus may precipitate or accelerate an already ongoing autoimmune reaction leading to T1D. In individuals with low risk of T1D, enterovirus might initiate inflammation of the islet cells leading to T1D through several mechanisms (Fig 5).

In contrast to DS, the diabetogenic variant MPP was capable of spreading to the pancreas to cause rare islet cell infection (Fig 5 B and C – I), which may lead to the induction of an immune response against β-cells. As the immune system is activated against islet antigens, characterized by local lymphocyte infiltration (Fig. 6-I), this may lead to β-cell damage and a diabetes-like syndrome (See & Tilles, 1995). Consistent with this, an indication of MPP inducing β-cell alteration comes from glucose measurements. Mice infected with the parental virus strain had slightly but significantly increased blood glucose levels at 3 weeks after infection. On the other

hand, mice infected with MPP showed clearly higher median glucose levels at 3 weeks after infection (Fig. 8-I).

MPP-induced damage was monitored for longer period of time. The chronic phase, which was initiated after complete virus clearance from blood, was achieved between days 15–100. MPP-induced histopathological changes in the pancreas, and signs of both atrophy/fibrosis and chronic inflammation in the acinar tissue were still found at 9 and 15 weeks after infection (Fig. 7-I, Fig 6). Furthermore, mild islet inflammation was also evident (Fig. 7-I). HEV-B infections may be associated with chronic immune disorders by persisting in target organs. For example, genomic enteroviral sequences in endomyocardial tissues from adult patients with chronic dilated cardiomyopathy were detected by a rapid RT-PCR and hybridization assay (Rey et al., 2001). Isolation of an infectious virus from cell culture was, however, unsuccessful probably because defective viral genome viruses could not replicate. Kim and others were the first to isolate and successfully cultivate CV-B3 viruses from mouse heart (Kim et al., 2005) and primary cell culture (Kim et al., 2008), and CV-B2 from human heart (Chapman et al., 2008). These viruses were found to have terminal deletions (TDs) in the 5' cloverleaf. It should be emphasized here that these TDs viruses have not been isolated from other organs than the heart. Thus, one can hypothesize that the observed MPP-induced chronic inflammation may be a result of activation of the immune system by viral persistence in the pancreas. Supportive results for chronic inflammation induced by viral persistency were also reported in both human and experimental models (Frank et al., 1986, Vella et al., 1992, Berger et al., 1998, Ylipaasto et al., 2004, Dotta et al., 2007). Isolation of infectious MPP during the chronic stage was not within the research scope of this study.

5.4 Adaptation of MPP strain to murine insulinoma cell culture, MIN-6 (III)

Histological examinations of mouse pancreatic tissues infected with MPP and its parental strain show that both viruses infect and replicate more readily in exocrine tissue than in endocrine tissue, and that islet infection is only rarely detected in mice infected with MPP (Fig 3-I and Fig 5-I). The protection of islets against HEV infection has been proposed to be mediated by interferons. Flodström et al. (2002a) showed that the SOCS-1 transgenic mice were highly susceptible to CV-B4 infection, suggesting that IFN-α and IFN-γ are important in preventing viral infection of β-cells (Flodström et al., 2002a, Flodström et al., 2002b). However, the protection mechanism of islet cells is not fully understood. The interaction between MPP and islet cells was investigated using MIN-6 cells (Miyazaki et al., 1990). Despite the high infection titre used, the MPP virus strain was not capable of causing major infection of the MIN-6 cell monolayer and only few infected cells were visible at days 1 and 2 p.i (Fig 1A-III). However, after three days the virus was found to infect several cells, indicating adaptation to the cell line (Fig. 1A-III). After

an additional 24 hours of incubation, complete cell lysis of the MIN-6 cells was evident (data not shown) and the MIN-6 cell-adapted virus strain (CV-B5-MCA, MCA) was harvested on day 6 for further studies. When the progeny virus produced after adaptation was harvested and used for infection of new aliquots of MIN-6 cells, the infection started immediately and progressed efficiently, and the maximum titre was detected already at 72 hours p.i. (Fig. 1B-III).

Subsequently, MPP infection in other murine insulinoma cell line INS-1 was tested to see wether the phenotype induced by MPP is MIN-6 cells restricted. MPP infection in INS-1 cells gave the same phenotype as MIN-6 cells (data not shown). Then cultures of primary porcine foetal islets were used to test whether the phenotype of MPP is murine insulinoma cell-restricted. According to previously published data, CV-B5 is capable of infecting primary porcine foetal islets (Roivainen et al., 2001). MPP was confirmed in the experiments to readily infect primary porcine foetal islets (data not shown). Furthermore, other continuous cell lines that do not produce insulin are equally permissive to both virus strains (Table II-III and Fig. 3-III). These results suggest that MPP-induced phenotype in MIN-6 cells was murine insulinoma cell-restricted. The MPP variant infects a few islet cells *in vivo* (Fig 5–I) and MIN-6 cells *in vitro* (Fig 1A–III) within the first three days, suggesting that the MPP variant has a subpopulation, which is capable of infecting insulin-producing cells. This subpopulation is likely to be part of the initial quasispecies giving rise to the MCA variants.

The replicative ability of MCA clearly suggests that the virus is adapted to a new cell surface molecule. To determine whether adaptation of MPP has altered its receptor specifity, MIN-6 cells and GMK cells were pre-treated with monoclonal anti-receptor antibodies specific for HCAR (Bergelson et al., 1997a) and mDAF. After primary blocking of receptor, the attachments of viruses to cell surface were examined. The results showed that the mAb specific for HCAR blocked virus attachment of the three variants DS, MPP and MCA only to GMK cells but not to MIN-6 cells. The results of the protection experiment suggest that the virus variants have maintained ability to use HCAR only in primate cell cultures. Monoclonal DAF-specific anti-mouse antibody did not block the infection of the three variants either in MIN-6 cells or in GMK. To interpret the negative results of these experiments is hard; they may also not be completely reliable, since it is not known whether these monoclonal antibodies are protective against virus infections in mouse cells. On the other hand, the possibility that MPP has adapted to use an alternative receptor other than CAR and DAF should not be excluded. Several recent examples show that CV-Bs induce a cytolytic phenotype in cell cultures via a cellular receptor other than CAR or DAF, suggesting a new, yet unidentified, receptor (Schmidtke et al., 2000, Zautner et al., 2003, Polacek et al., 2005, Hodik et al., 2010). Another study suggests that even endogenous low-level receptor expression may support virus infection (Carson et al., 2007). It should be emphasized here that host cell permissiveness is a broad concept that is not only influenced by mere expression of

receptor molecules on the cell surface; also intracellular factors have an effect on host cell permissiveness. Species-specific and tissue-specific host factors are involved in the processes of virus transcription and translation. These host factors were not examined in the present thesis.

5.5 Genetic analysis of diabetogenic HEV-B variants (I-IV)

Identification of genetic determinants may provide an explanation for the observed virulence phenotype. Traditionally, a comparative genomic approach was utilized with virulent and avirulent strains to characterize mutations and their locations. Several clues prompted us to explore the genome of HEV-B strains presented in this thesis. These are the natural tropism to the pancreas presented by CV-B5 strain DS, the changed phenotypic feature of CV-B5 strain MPP, the infection phenotype of CV-B5 strain MCA, and the isolation of islet cell-destructive strain D207, originally typed by serological methods as CV-A9. The existence of strains with different properties within the same virus type further complicates the pathogenesis of HEV-B. Since the genetic determinants responsible for β-cell damage are still unknown, it is important to compare sequences of multiple strains in a single virus type and use the site-direct mutagenesis to identify virulence determinants.

Table 4. Summary of mutations induced during different adaptation processes.

Virla protein	Sequence nro.	CV-B5-DS		CV-B5-MPP		CV-B5-MCA	
	nt/aa	nt	aa	nt	aa	nt	aa
5'-NTR	49	A		A		U	
5'-NTR	512	C		C		U	
VP4	140/47	C	T	U	M	U	M
VP2	268/89	A	G	G	G	A	G
VP3	385/129	A	K	G	R	A	K
VP1	280/94	G	V	G	V	A	I
VP1	283/95	A	N	G	S	G	S
2A	73/24	T	H	C	H	T	H
2A	85/29	A	Q	A	Q	G	R
2C	780/261	A	N	G	D	A	N
3B	140/47	C	L	G	V	C	L
3C	415/139	T	T	C	T	C	T
3C	486/162	G	G	G	G	A	S
3C	490/163	U	V	U	V	G	G
3D	364/122	G	A	G	A	A	A
3D	1070/357	C	P	C	P	U	L

5.5.1 CV-B5 strains (I and III)

In order to determine the amino acids mediating the observed phenotypes in mouse pancreas *in vivo* and in insulinoma cell lines, MIN-6, *in vitro*, all three CV-B5 variants DS, MPP (I), and MCA (III) were cloned and sequenced. Comparison

between CV-B5-MPP and its parental virus strain revealed eight nucleotide changes resulting in five amino acid substitutions (Table III-I and Table 4). Three out of 5 substitutions were located in the capsid proteins VP1 (D→S, 95 aa), VP3 (K→R, 129 aa), and VP4 (T→M, 47 aa). The remaining two substitutions were located in the non-structural region 2C (D→N, 261 aa) and VPg (V→K, 74 aa). Adaptation to the murine insulinoma cell line MIN-6 indicated that MCA had acquired thirteen nucleotide changes resulting in eight amino acid substitutions (Table III-III and Table 4). Two of the amino acid substitutions were located in the capsid proteins VP1 (V→I, 94 aa) and VP3 (R→K, 129 aa). The remaining substitutions were located in non-structural proteins, 2A, 2C, 3B, two in 3C, and 3D. Two mutations were located in the 5'-NTR. No mutations were found in the 3'-NTR. The observed substitutions could have occurred during adaptation, or a possible pre-existing variant might have been selected during the growth in the MIN-6 cells.

5.5.2 Virus type identification and analysis of strain D207 (II and IV)

The interesting phenotype of D207 in primary human islets encouraged us to study this strain in detail. To identify the differences between the prototype CV-A9 and isolate D207 sequences, the full-length genome of D207 was cloned and sequenced. The genome comprised 7,432 nucleotides and coded for a 2,195 amino acid long polyprotein. Sequence analysis of the VP1 capsid protein revealed that the D207 was an isolate of E-11 and not CV-A9 as suggested by neutralization with antiserum pools and high titre antiserum. The following sequence comparisons of different capsid regions of the D207 genome with prototype strains E-11 Gregory and CV-A9 Griggs indicated that D207 was closer to E-11 (Gregory strain). In contrast, the comparisons of different non-structural regions were not as unambiguous as the capsid region. Non-structural proteins 3A and 3B of D207 were more similar to E-11 than CV-A9, while 2A, 2C, 3C and 3D and both the 3' and 5' non-coding regions were more similar to CV-A9 Griggs (Table 1-II). The phylogenetic analysis demonstrated that D207 was closely related to E-11 strains known to cause Uveitis (Lukashev et al., 2002, Lukashev et al., 2003b).

Recently, the molecular epidemiology and genetic features of E-11 isolates have been studied in various regions of the world (Lukashev et al., 2003b, Oberste et al., 2003b, Lukashev et al., 2004, Bouslama et al., 2006, Iwai et al., 2006, Bouslama et al., 2007). The Finnish genetic typing data of E-11 from the last 15 years (1993 – 2007) with available GenBank sequences were selected for more detailed phylogenetic analysis (Figure 1-IV). This analysis revealed that the Finnish strains clustered in the previously established genogroups (Oberste et al., 2003a) A (7 strains), C (2 strains), and D (53 strains). The genogroup D is further divided into five subclusters D1–D5. Within genogroup D, most strains (46) grouped in subcluster D5 while seven strains grouped in D4. E-11 strain D207 and the closely related uveitis causing strains were found to belong to subcluster D4. The Icelandic

(1) and Slovakian strains (2) clustered in D5. The Egyptian strains (4) clustered in genogroup C with one exception, Egy04-E290, which differed by 19.7% or more from all other strains and formed a possible new genotype.

At this point, the phenotype of D207 in primary human islets is similar to the phenotype of the prototype E-11 (Roivainen et al., 2002, Klemola et al., 2008). However, a new interesting feature of D207 concerning dual neutralization of the virus with CV-A9 high titre antiserum was found. The neutralization by two different antisera raised against different serotypes is a phenomenon, which is quite rare among serotypes. The neutralization of D207 by CV-A9 antiserum was confirmed by two independent laboratories, our laboratory and the laboratory that provided us with the D207 virus isolate.

The interesting genetic features of both HEV-B strains devided the subsequent studies into two directions. Firstly, the construction and analyses of recombinant and site-directed mutants of CV-B5-MPP were used to identify the critical amino acid(s) involved. Secondly, recombination between E-11 and CV-A9, with the joining point within the capsid coding region may explain the dual neutralizibility of D207. Subsequently, the genetic diversity and molecular evolution of E-11 strain D207 was studied using closely-related field strains. Moreover, subjecting these isolates to both monotypic antisera may reveal whether cross-reactivity is common within these closely related strains.

5.6 Dual neutralizibility with CV-A9 and E-11 specific antisera (II, IV)

The sequence comparison of the D207 VP1 capsid region was surprising, since the neutralization assay is largely used to distinguish between different virus serotypes after being isolated in cell culture. The assay is highly specific and based on neutralizing virus serotypes using hyperimmune sera raised against their corresponding prototypes. Therefore, dual neutralization of the virus is usually considered a technical error. The observed dual neutralizibility was tested to see whether it was a technical error or a real property shared by D207 and possibly strains belonging to genogroup D. A plaque-neutralization assay with polyclonal antisera against E-11 and CV-A9 (Hovi & Roivainen, 1993) was performed. In parallel with the original isolate (D207), the first passage of D207 and the virus strain produced from the infectious clone constructed in the study (Lipo-D207) were analyzed with an E-11 specific antiserum and CV-A9 specific antiserum. The results indicate a reduction in infectivity of about 3 logs by the E-11 specific antisera and about 2-3 logs by the CV-A9 specific antisera (Table 2A-II). The prototype strains were shown to neutralize only with their own virus-specific antiserum.

In order to further confirm that D207 is not a virus mixture, non-neutralized plaques were collected from wells containing E-11 antisera and CV-A9 antisera, respectively. The non-neutralized plaques were re-neutralized with the same antisera

as previously (E-11 specific antiserum and CV-A9 specific antiserum). As shown by Table 2B-II, the result of the non-neutralized plaques did not differ from those obtained with the original strain. The corresponding results were seen in plaques isolated in the presence of E-11 or CV-A9 specific antisera. Polyclonal antiserum contains antibodies to several different epitopes, some of which might be cross-reactive among related virus serotypes. This kind of cross-reactivity can easily be demonstrated in binding assays. However, neutralizing antibodies are serotype specific, so only virus strains belonging to the serotype used as an immunogen can be neutralised. In the case of virus isolate E-11 strain D207, dual neutralization with CV-A9 and E-11 specific antisera seems to be a genuine property of the virus. Exceptions to such strong reaction have also been reported between E-1 and E-8, E-9 and CV-A23, CV-A11 and CV-A15, and CV-A13 and CV-18. However, these viruses are now considered a single virus type.

Experiments were then carried out to see whether the phylogenetic relationship of the closest strains in the E-11/D subgroup and dual neutralizability with CV-A9 and E-11 specific antisera go together. Kust/86, Kar/87 and E-11/Kh3/97, belonging to the E-11/D4 subcluster (Table 2C-II), were subjected to neutralization testing. Kust/86 and Kar/87 were neutralized with both antisera (E-11 specific antiserum and CV-A9 specific antiserum) whereas E-11/Kh3/97 was neutralized only by E-11 antiserum. The results were interesting, since both dual neutralized virus strains were uveitis-causing strains.

Subsequently, ten strains of E-11 from genogroups A (3), B (1) and D (6) (IV) were analysed with antisera against E-11 Gregory and CV-A9 Griggs (Figure 2-IV). Neutralization with E-11 antiserum was shared by all tested strains. Variation was found in neutralization with CV-A9 antiserum. Five of the six tested D group strains were also neutralized with CV-A9 antisera, while only one out of four of genogroup A and B was neutralized with CV-A9 antisera with a reduction of less than one log. The anti-CV-A9 resistant strain belonging to the D-genogroup was Fin06-33A, and the anti-CV-A9 sensitive strain belonging to the genogroup A strains was Fin07-6A. Previously studies have demonstrated that the A, B and C genogroups are more efficiently neutralized by antiserum against Gregory, whereas the D genogroup is neutralized more readily by antisera against E-11 Silva (Oberste et al., 2003b). These results indicate that the dual serotype specificity and phylogenetic relationships of all tested E-11 strains are consistent.

5.7 Candidate determinants identified (II, III)

Candidate determinants explaining the behaviour of CV-B5-MCA and D207 E-11 viruses were mapped using site-directed mutagenesis and the peptide scanning technique. Sequence comparison of CV-B5 variants revealed that adaptation to murine insulinoma cell lines was associated with changes in viral genomes either at the same (VP3-129 aa) or neighbouring (VP1-94 aa and VP1-95 aa) capsid positions

as in MPP, which was passaged *in vivo* in mouse pancreas. The role of amino acid 94 of VP1 in virus cell interactions was further corroborated by the fact that the adaptation of coxsackievirus B5 strain DS to murine pancreas *in vivo* was associated with a change in the neighbouring amino acid 95 (comparison Table 4). According to the tertiary structure of the swine vesicular disease virus (SVDV) (Verdaguer et al., 2003), the VP1 substitution is located within the βC strand and is exposed to the surface. Moreover, the comparison of CV-B5 Faulkner and the DS strain revealed only a single amino acid substitution at position 94 in the VP1 capsid protein. This supports the suggested involvement of position 94 substitution in virus-cell surface interactions. On the other hand, during passaging in mouse the VP3 substitution changed from lysine to arginine and, after infection of MIN-6 cells, the same position changed back to lysine, suggesting that this position might have a role in adaptation to MIN-6 cells (cf. Table III-I, Table II-III and Table 4). The VP3 substitution forms the first amino acid of the βE strand around the two-fold axis. In order to find out which of the found substitutions is the most critical for the observed phenotype of CBV-5-MCA, single mutants were produced by site-directed mutagenesis.

In the CV-B5-MPP-VP1 mutant, the valine at position 94 was substituted by isoleucine, while in the CV-B5-MPP-VP3 mutant the arginine at position 129 was substituted by lysine. The infectivities of both mutants were determined in GMK cells. The capsid regions of rescued viruses were sequenced to ensure that the mutation was successfully inserted and no other substitutions had taken place. The determinant for MIN-6 cell infection was situated at VP1-94, since this mutant replicated immediately after infection (5h p.i. Fig 5A-III), while the replication of the VP3-129 mutant was initiated only after a delay of several days (Fig 5 – III). The replication phenotype of the CV-B5-MPP-VP3 mutant mimicked the replication of MPP, and the replication phenotype of the CV-B5-MPP-VP1 mutant was similar to the MCA. This result suggests that the substitution of amino acid 94 alone is enough for CV-B5-MPP replication in MIN-6 cells. However, it is impossible to state, on the basis of these results, whether other substitutions of MCA in both VP3 and non-structural proteins might enhance virus replication in a synergistic manner.

Phylogenetic analyses were conducted to analyse the genetic relationship of D207 to other E-11 strains (Fig 1-II). According to the capsid region, the closest relatives of the D207 isolate were E-11 strains that were isolated from infants with severe enterovirus infections in 1986-1990, strains E-11/Kust/86, E-11/Kar/87 and E-11/Hun/90. These strains were found to be part of the group designated as E-11/D4, which is part of the genogroup D containing subcluster D1–D5 described previously, due to their marked serological differences in comparison to the prototype E-11 strain Gregory (Lukashev et al., 2002). The close relationship between D207 and uveitis-causing strains indicated that these viruses have a common evolutionary history and that they have diverged from the other E-11 strains a long time ago. In the non-structural region, not all E-11 strains clustered

together (Fig. 1-II), but the relationship of D207 to the uveitis causing strains E-11/Kust/86, E-11/Kar/87 and multisystem disease-causing (MSD) strain E-11/Hun/90 was still seen. It is possible that the ancestor of the isolate acquired the non-structural region through recombination with some other HEV-B (Chevaliez et al., 2004, Lukashev et al., 2004), or the result may be explained by the overall elevated similarity of most recent HEV-B strains in this part of the genome (Lukashev et al., 2003a). Likewise, in the 5' non-coding region, these strains showed a similarity of 90% or more to D207. When the sequence of the D207 capsid region was compared to other HEV-B sequences, D207 showed remarkable similarity to CV-A9 (Figure 1a-II). One could speculate that recombination between E-11 and CV-A9 may explain the dual neutralizibility of D207. Computer-assisted analysis of the capsid region using Simplot software was carried out to find out major transferred motifs. The result of Simplot analyses revealed only a short motif (VFCGSAMATGKFLLAY) in the VP3 region of D207 and subcluster D4, concerning two uveitis-causing strains and one MSD-causing strain, to be identical with CV-A9 Griggs and different from that of E-11/Gregory.

The peptide scanning technique was conducted to identify possible regions responsible for the cross-reactivity. Peptides of the D207 capsid region recognised during the peptide scanning technique were arranged in three categories: peptides that reacted only with D207 antiserum (Fig 3-II and Fig4A-II), with both D207 and CV-A9 antisera (Fig 3-II and Fig4B-II), or only with CV-A9 antiserum (Fig 3-II and Fig4C-II). The results indicated that the CV-A9 antiserum recognized several E-11 capsid protein-derived peptide sequences, including the 15 sporadic amino acid peptides included in EILNYYAHWSGSVKLTFVFCGSAMATGKFLLAY from the capsid protein VP3 that was previously recognized by Simplot. Amino acid sequence analysis could not explain the dual neutralization, since some of the regions reacting with CV-A9 induced antiserum were not recognized by the D207 induced antiserum (Fig 3-II, Fig 4 panel C-II), even though they share great similarity in their amino acid sequences. Furthermore, some regions reacting with the D207 antiserum only, did not show more sequence divergence than the two other categories (Fig 4 panel A-II). The results support the suggestion that the epitopes responsible for the observed phenotype of dual neutralization are located within the peptides reacting with antisera. However, further studies are needed to identify responsible epitopes.

5.8 Determinants of the studied HEV-B strains

Reverse mutagenesis and peptide scanning techniques were utilized to identify the genetic determinants responsible for the observed phenotypes. Both approaches indicated that these genetic determinants of both studied HEV-B strains (CV-B5-DS and E-11 D207) are located in the capsid region. The capsid interacts primarily with the cell surface receptors. The presence or absence of a receptor is considered a

major determinant of tissue and species tropism for enteroviruses. The enteroviral capsid has been shown to contain determinants contributing to the pathogenic phenotype of CV-B4, CV-B3, and CV-B2 (Bae & Yoon, 1993, Caggana et al., 1993, Knowlton et al., 1996, Jun et al., 1997, Schmidtke et al., 2000, Polacek et al., 2005), and in certain cases adaptation may result in extensive receptor usage (Schmidtke et al., 2000, Zautner et al., 2003, Polacek et al., 2005, Hodik et al., 2010). Mutation and recombination provide a strategy for viruses to enhance their chances of survival in new environments. Hypothetically, intertypic recombination could result in a phenotypic characteristic for strain D207; however, neither visual nor coputer-assisted Simplot analysis gave the support for this view. Phylogenetic analysis revealed that D207 belongs to the large genogroup D, and has probably long time ago diverged from E-11 strain Gregory. Most viruses of this genogroup have a similar dual neutralization phenotype to D207. One could hypothesize that transition toward CV-A9 is beneficial for E-11 viruses. There is the possibility that the viruses begin to adapt to new cell surface receptor(s).

However, adaptation in cell culture may limit the quasispecies of the adapted virus. To answer the question of whether MCA strain has lost its ability to cause chronic inflammation in CD-1 mice, the mice strain which was previously used in the characterization of CV-B5-MPP. Parallel group of CD-1 mice were infected intraperitoneally with equal amounts of CV-B5-MPP and CV-B5-MCA. At various times after infection, subgroups of mice were sacrificed for histological examination of pancreatic tissue. Formaldehyde fixed paraffin blocks were cut into 5-µm sections and stained with hematoxylin and eosin. Virus-induced chronic inflammations were found in pancreatic acinar tissue but also in the islets (Fig 6-III). The signs of infection resembled that of the MPP virus strain (I), suggesting that, in spite of MIN-6 cell adaptation, the MCA virus strain still has the capacity obtained from the CV-B5-MPP strain. These results reflect the possibility of multiple viral determinants mediating chronic pancreatic inflammation in mice. The sites determining the virulence phenotypes of these viruses do not necessarily co-localize to a single capsid protein or even a single capsid region, and they may also involve the non-structural region. If so, this may also explain the previous failure to identify the precise genetic determinants of the CV-B5-MPP phenotype in mice by constructing site-direct mutagenesis and chimeras from MPP and DS strains by joining the 5’ part of MPP with the 3’ part of DS and vice versa (the 5’ part of DS with the 3’ part of MPP). In addition to the viral determinant, the host genetic background also influences the capacity of CV-Bs to induce diabetes.

6 Conclusions

Hypothetically, the diabetogenic phenotypes of enteroviruses are restricted to certain genetic determinants. Identification of the diabetogenic determinant was sought from the genome of laboratory isolate CV-B5 strain DS and another HEV-B member strain D207, which was isolated from a diabetic individual, by using different cell models and methods.

The pathogenesis of coxsackie B viruses can be enhanced by repeated passaging either in mice *in vivo* or in cultured mice β-cells. Fifteen successive passages of the virus through a mouse pancreas resulted in the capability of causing diabetes-like syndrome in mice. The diabetes-like syndrome was characterized by chronic pancreatic inflammation together with mild glucose dysregulation, loss of pancreatic acinar tissue, and mild insulitis. However, the *in vivo* mouse pancreas passaged virus strain was not capable of infecting the murine insulinoma cell lines, MIN-6, *in vitro*.

Constructing infectious cDNA clones of the adapted virus strain (MPP) and from their parental prototype strain (DS) showed that the chronic pancreatic inflammation *in vivo* is mediated by multiple viral determinants in the capsid and in the non-capsid region as tested by producing site-direct mutagenesis and chimeras from both virus strains. However, the adaptation to murine insulinoma cell lines MIN-6 was mediated by a single amino acid within the VP1 (94 aa).

Since other human enterovirus B (HEV-B) types, such as echoviruses, are known to have the ability to infect human islet cells, a HEV-B virus strain isolated from a diabetic child and originally typed as CV-A9 was included in this thesis. However, D207 was found to belong to genogroup D of the most recent E-11 isolates, but was also neutralized with monotypic antiserum to coxsackievirus A9. Even though relatively large amino acid sequence variability existed within the genogroup, dual neutralizability was shared by a large spectrum of E-11 isolates that belong to genogroup D. Despite of several attempts to determine the epitopes responsible for the phenotype, they remain unknown. However, numerous antigenic peptides were found to cross-react with both antisera.

In summary, viruses can acquire new features after only few changes in their genome, as in the cases of MPP and MCA. In addition, dual neutralizibility was maintained by a wide range of the D subcluster of E-11 despite their genetic divergence. These studies emphasize that the position of mutations has a greater affect on the viral phenotype than the amount of mutations. Furthermore, MCA virus strain, which most probably has small quasispecies size, seems to survive in different environments, in cell culture and *in vivo*. The dual-phenotype specificity of this virus strain emphasizes the importance of the synergistic interaction between substitutions.

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