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2015-12-02

Smura , T , Natri , O , Ylipaasto , P , Hellman , M , Al-Hello , H , Piemonti , L & Roivainen , M
2015 , ' Enterovirus strain and type-specific differences in growth kinetics and virus-induced
cell destruction in human pancreatic duct epithelial HPDE cells ' , Virus Research , vol. 210 ,
pp. 188-197 . <https://doi.org/10.1016/j.virusres.2015.08.003>

<http://hdl.handle.net/10138/223831>

<https://doi.org/10.1016/j.virusres.2015.08.003>

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Enterovirus strain and type-specific differences in growth kinetics and virus-induced cell destruction in human pancreatic duct epithelial HPDE cells

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ARTICLE INFO

Article history:

Received 29 April 2015

Received in revised form 31 July 2015

Accepted 5 August 2015

Available online 7 August 2015

Keywords:

Enterovirus

Type 1 diabetes

Pancreatic duct

Coxsackievirus

Cell tropism

ABSTRACT

Enterovirus infections have been suspected to be involved in the development of type 1 diabetes. However, the pathogenetic mechanism of enterovirus-induced type 1 diabetes is not known. Pancreatic ductal cells are closely associated with pancreatic islets. Therefore, enterovirus infections in ductal cells may also affect beta-cells and be involved in the induction of type 1 diabetes. The aim of this study was to assess the ability of different enterovirus strains to infect, replicate and produce cytopathic effect in human pancreatic ductal cells. Furthermore, the viral factors that affect these capabilities were studied.

The pancreatic ductal cells were highly susceptible to enterovirus infections. Both viral growth and cytolysis were detected for several enterovirus serotypes. However, the viral growth and capability to induce cytopathic effect (cpe) did not correlate completely. Some of the virus strains replicated in ductal cells without apparent cpe. Furthermore, there were strain-specific differences in the growth kinetics and the ability to cause cpe within some serotypes. Viral adaptation experiments were carried out to study the potential genetic determinants behind these phenotypic differences. The blind-passage of non-lytic CV-B6-Schmitt strain in HPDE-cells resulted in lytic phenotype and increased progeny production. This was associated with the substitution of a single amino acid (K257E) in the virus capsid protein VP1 and the viral ability to use decay accelerating factor (DAF) as a receptor.

This study demonstrates considerable plasticity in the cell tropism, receptor usage and cytolytic properties of enteroviruses and underlines the strong effect of single or few amino acid substitutions in cell tropism and lytic capabilities of a given enterovirus. Since ductal cells are anatomically close to pancreatic islets, the capability of enteroviruses to infect and destroy pancreatic ductal cells may also implicate in respect to enterovirus induced type 1 diabetes. In addition, the capability for rapid adaptation to different cell types suggests that, on occasion, enterovirus strains with different pathogenetic properties may arise from less pathogenic ancestors.

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1. Introduction

Enteroviruses (family Picornaviridae, genus *Enterovirus*) are small non-enveloped positive strand RNA viruses. Enterovirus infections have been suspected to be involved in the development of type 1 diabetes, mainly on the basis of seroepidemiological studies (reviewed in (Yeung et al., 2011)) Human pancreatic

islets are highly susceptible for enterovirus infections in vitro (Roivainen et al., 2000; Roivainen et al., 2002) and enterovirus specific RNA (Ylipaasto et al., 2004) and proteins (Richardson et al., 2009) have been detected in the pancreatic islets of deceased type 1 diabetes patients. However, the pathogenetic mechanism of enterovirus-induced type 1 diabetes is not known. The potential mechanisms, which are not mutually exclusive, include direct beta-cell damage after cytolytic virus infection, indirect beta-cell damage due to virus-induced cytokine expression and virus-induced auto-immune reactions against beta-cells. Direct or indirect virus-induced beta-cell damage may lead to exposure of

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sequestered beta-cell autoantigens and therefore predispose for subsequent auto-immune reactions against beta-cells.

Enteroviruses are a large and genetically diverse group of RNA viruses. Currently, the genus *Enterovirus* consists of 12 species, *Enterovirus* (EV) A–H, EV-J and *rhinovirus* (RV) A–C (Knowles, 2015). Of these, the species EV-A to -D and RV-A to -C contain serotypes that cause infections in humans. In this article, the term ‘enterovirus’ refers to the members of EV species A–D (i.e., acid stable primate enteroviruses that are transmitted mostly via fecal–oral route). Over 100 EV-A to -D serotypes have been characterized so far (Knowles, 2015). Although enteroviruses are often implicated as triggers of type 1 diabetes, it is not known precisely which of the enterovirus serotypes or strains are involved in type 1 diabetes development. *In-vitro* studies suggest that enterovirus serotypes and strains within a given serotype differ in their capability to induce destruction and cytokine response in human pancreatic islets (Anagandula et al., 2014; Paananen et al., 2003, 2013; Roivainen et al., 2002; Sarmiento et al., 2013; Smura et al., 2010; Ylipaasto et al., 2012). In addition, virus strain-dependent differences have been detected in plasmacytoid dendritic cell mediated immunogenicity (Hamalainen et al., 2014).

Strain-specific differences in cell tropism, cell destruction and immunogenicity have important implications in respect to enterovirus induced type 1 diabetes. The primary site of enterovirus infection is the mucosal tissue of the respiratory or gastrointestinal tract. The primary infection can occasionally be followed by a viremic phase, during which the virus spreads through the lymphatic system and circulation, and may gain access to the secondary target tissues, including pancreas. Most enterovirus-related diseases are consequences stemming from the viral infection of secondary target tissues. Pancreatic ductal cells are closely associated with pancreatic islets (Bertelli and Bendayan, 2005; Zhao et al., 2008). Therefore, enterovirus infections in ductal cells may affect also beta-cells and be involved in the induction of type 1 diabetes. Notably, enterovirus genomes/capsid proteins have been detected in ductal cells of type 1 diabetes patients (Richardson et al., 2009; Ylipaasto et al., 2004).

Hypothetically, enterovirus infection in pancreatic ductal cells may (1) amplify the infection in pancreas leading to higher local viral loads and more efficient spread of the virus to the beta-cells, (2) induce local pro-inflammatory environment that may be directly harmful to pancreatic beta-cells or attract immune cells, such as autoreactive T-cells, close to pancreatic islets. Lytic infection (with necrotic cell death) is likely to induce a strong inflammatory response that, together with presentation of beta cell autoantigens, could induce autoimmunity. Non-lytic infection, on the other hand, may induce persistent infection, and thus, lead to sustained stimulation of autoimmunity. In addition, persistent enterovirus infection in the duct cells has been shown to reduce ductal cell differentiation (Sane et al., 2013). This may impede trans-differentiation of duct cells to beta cells, thus impeding beta cell replacement (Lysy et al., 2013).

In this study we assessed the tropism of different enterovirus types and strains for pancreatic ductal cells and the factors affecting ductal cell tropism.

2. Material and methods

2.1. Viruses

The prototype strains of enterovirus serotypes coxsackievirus (CV) B1-Conn-1, CV-B2-Ohio-1, CV-B3-Nancy, CV-B4-JVB, CV-B5-Faulkner, CV-B6-Schmitt, CV-A9-Griggs, echovirus (E-) 3-Morrissey, E-6-D'Amori, E-9-Hill, E-9-Barty, E-11-Gregory, E-30-Bastianni, poliovirus (PV-) 1-Sabin, enterovirus (EV-) D68-Fermon and EV-

D70-J670/71 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and passaged twice in green monkey kidney cells (GMK). EV-D94-E210 was originally isolated from waste water in Egypt (Smura et al., 2007) and passaged twice in human rhabdomyosarcoma cells (RD). CV-B4-E2 strain was kindly provided by Prof. J.W. Yoon. The production of CV-B5-DS strain, mouse pancreas (in vivo) adapted CV-B5-MPA strain and murine insulinoma cell adapted CV-B5-MCA strain is described elsewhere (Al-Hello et al., 2005, 2009). The strains E-9-DM (Vreugdenhil et al., 2000) and E-11-D207 (Al-Hello et al., 2008) were originally isolated from patients at the clinical onset of type 1 diabetes and passaged twice in GMK cells. The E-30 strain 14916net87 was originally isolated from an aseptic meningitis patient in the Netherlands (Savolainen et al., 2001). CV-B2 strain 94.ST256 was isolated from a child participating in the babydiet dietary intervention study (Schmid et al., 2004) before the onset of beta cell autoantibodies (Simonen-Tikka et al., 2011). CV-B2 strain 1864 was isolated from waste water in Finland using a two-phase concentration method (World Health Organization (World Health Organization (WHO), 2003) and plaque purified and passaged in GMK cells.

2.2. Cell lines

The green monkey kidney cell line (GMK) has been maintained in the laboratory since the 1960s. Human rhabdomyosarcoma (RD) cell line was provided by the WHO Polio Labnet. The GMK and RD cells were maintained in Eagle MEM growth medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). Pancreatic duct epithelial cell line (HPDE) (Furukawa et al., 1996) was maintained in RPMI1640 growth medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% FBS.

2.3. Infection and viral growth curve assessment

The cells were infected with virus strains diluted in Hanks' balanced salt solution supplemented with 20 mM HEPES, pH 7.4. Multiplicity of infection (m.o.i.) of 1 was used. After incubation for 1 h at +36 °C in a 5% CO₂ atmosphere, the cells were washed twice with Hanks balanced salt solution and growth medium supplemented with 20 mM HEPES, pH 7.4, 20 mM MgCl₂, 1% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin was added to the cultures. The cultures were incubated at +36 °C in 5% CO₂ atmosphere.

For virus titration, the cell cultures were harvested at different intervals and freeze–thawed three times. The total infectivity of each sample was determined by end-point titration in microwell cultures of GMK or RD cells. RD cells were used for EV-D strains and GMK cells for the other enterovirus strains. Cytopathic effect (cpe) was read on day 6 after infection by light microscope, and 50% tissue culture infectious dose (TCID₅₀) titers were calculated using the Kärber formula (Lennette, 1969).

2.4. Antibodies

Polyclonal antibodies against poliovirus receptor (PVR) (NAETZ8) and integrin $\alpha\beta 3$ were produced by immunizing rabbits with three to four doses of purified PVR or $\alpha\beta 3$ proteins together with Freund's complete (the first dose) or incomplete adjuvant (with boosters) (Ylipaasto et al., 2010). Monoclonal antibodies against decay accelerating factor (DAF) (IH4 and IF7) (Kinoshita et al., 1985) were provided by T. Kinoshita, Department of Immunoregulation, Research Institute of Microbial Diseases, Osaka University, Japan. The monoclonal antibody against human coxsackievirus and adenovirus receptor (HCAR) (RmcB) (Hsu et al.,

Fig. 1. Infectious progeny production of selected enterovirus prototype strains in HPDE cells. The cells were infected and samples were collected 0 h, 5 h, 8 h, 24 h and 48 h post infection. The total infectivity of each sample was determined by end-point titration in microwell cultures of GMK or RD cells. Cytopathic effects (cpe) were read on day 6 after infection by microscope, and 50% tissue culture infectious dose (TCID₅₀) titers were calculated using the Kärber formula.

1988) was kindly provided by L. Philipson, Karolinska Institute, Stockholm, Sweden.

2.5. Receptor antibody-mediated cpe-protection assay

The inhibitory effect of anti-HCAR (RmcB) and anti-DAF (IH4, IF7) antibodies on virus infections was assessed using cell protection assay. HPDE cells were cultured in 96-well plates for 2 days, incubated for 1 hour at room temperature with RmcB, IH4 or IF7 (1:50 dilution of ascites fluids) or growth medium without antibodies, followed by inoculation with the virus for 1 h in Hanks balanced salt solution. After infection the cells were washed twice with Hanks balanced salt solution and maintained in growth medium supplemented with 20 mM HEPES, pH 7.4, 20 mM MgCl₂, 1% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The presence of antibodies in the culture medium was maintained throughout the experiment.

The inhibitory effect of heparin on virus infection was assessed by incubating virus (m.o.i. of 1) with porcine intestinal mucosa heparin (Sigma-Aldrich) diluted in RPMI1640 (concentration 0, 10 and 100 µg/ml) at +36 °C for 60 min. After pre-incubation, the virus-heparin mixture was inoculated on confluent HPDE-cells cultured in 96-well plates. The virus induced cpe was evaluated using light microscope 24 h post infection and the surviving cells were stained with crystal violet.

2.6. Immunocytochemistry of cell surface expression of enterovirus receptors

Expression of enterovirus receptors on cells was carried out by staining cells with receptor-specific antibodies for 2 h at +22 °C. After staining with receptor antibodies, the cells were incubated with FITC labeled conjugate (Jackson ImmunoResearch), then fixed with paraformaldehyde (PFA) and made permeable with Triton X100 followed by the staining of the cell nuclei with DRAQ5. The samples were analyzed using a confocal microscope (Leica TCS SPE, Wetzlar, Germany).

Fig. 2. The viability of HPDE cells after CVB infection was assessed using Sytox-green assay (Life Technologies) and spectrophotometer (Wallac 1420 Victor2™, PerkinElmer) 24 h post infection. The serotypes that induced visible cpe by 24 h post infection are indicated with solid black bars and the serotypes that did not induce visible cpe by 24 h are indicated with grey bars. Note that CV-B3 induced visible cpe in the later time point (by 48 h post infection).

2.7. Cell viability assay

The viability of cells was assessed using Sytox green nucleic acid stain (Life Technologies, Carlsbad, CA, USA). Sytox green penetrates cells with compromised plasma membranes and binds to the nucleic acids of the dead cells. This binding leads to 500-fold fluorescence enhancement. The cells (five parallel wells per virus) were incubated for 10 min in 1 µM Sytox green concentration followed by fluorescence quantification using a spectrophotometer (Wallac 1420 Victor2™, PerkinElmer, Waltham, MA).

2.8. Sequencing

The complete genomes of pancreatic ductal cell adapted CV-B6-DUCT strain and the parental strain CV-B6-Schmitt were sequenced. Total RNA was extracted from infected cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions. The primers for cDNA synthesis and PCRs were designed on the basis of CV-B6 sequences (AF039205, AF105342 and AF114384) available in the GenBank. The cDNA synthesis was conducted using SuperScript First-Strand Synthesis System

for RT-PCR kit (Life technologies). SuperScript One-Step RT-PCR for Long Templates kit (Life Technologies) was used for the PCRs. PCR products were purified using a QIAEXII agarose gel extraction kit (Qiagen). Sequencing reactions with BigDye Terminator cycle sequencing ready reaction kit v3.1 (Life Technologies) and sequencing with ABI3730 Automatic DNA Sequencer (Life Technologies) were performed by Institute for Molecular Medicine Finland (FIMM) Sequencing Laboratory. The electropherograms were analysed using Geneious Pro 5.6 software (Biomatters, Ltd., Auckland, New Zealand, <http://www.geneious.com>).

3. RESULTS

3.1. Susceptibility of ductal cells to enterovirus infections

To assess the capability of enteroviruses to infect and form infectious progeny in pancreatic ductal cells, HPDE cells were infected with different enterovirus strains. Both prototype strains and field isolates of enterovirus serotypes that have previously been associated with type 1 diabetes were used.

Several enterovirus prototype strains were able to grow in HPDE cells (Fig 1). However, infectious progeny production and appearance of classical cytopathic effect (cpe), as detected using light microscope, did not correlate completely. Both infectious progeny production and cytopathic effect were detected for CV-B1-Conn-5, CV-B2-Ohio-1, CV-B3-Nancy, CV-B5-Faulkner, CV-A9-Griggs, E-3-Morrissey, E-6-D'Amori, E-9-Hill, PV-1-Sabin, EV-D70-J670/71 and EV-D94-E210. Progeny production without apparent cpe was detected for CV-B4-JVB and CV-B6-Schmitt. CV-B3-Nancy induced delayed cytolysis that appeared, at earliest, by 48 h post infection. No definitive progeny production was detected for EV-D68-Fermon. The cell viabilities after infection with the six Coxsackie B virus serotypes (CV-B1 to CV-B6) were further assessed using Sytox Green assay (Fig 2). The Sytox Green assay indicated congruent results with cpe assessment using light microscope.

In addition to the prototype strains, also virus isolates that have been linked previously to virus-induced pathogenesis of type 1 diabetes (i.e., were isolated from type 1 diabetes patients or pancreatic islet autoantibody positive donors or have previous experimental in vitro/in vivo evidence for pathogenetic processes relevant for type 1 diabetes) were studied for their capability to infect and form infectious progeny viruses in HPDE cells. For some serotypes (CV-B4, CV-B5, E-9, E-11, E-30), there were no clear differences in the growth and cytolytic capabilities between different strains (Fig 3). In contrast, the three CV-B2 strains had different growth kinetics and capabilities to induce cpe. CV-B2 strain 94.ST256 (isolated from pancreatic islet autoantibody positive individual (Simonen-Tikka et al., 2011)) and the strain 1864 (isolated from sewage) had slower progeny production (Fig 4a) and delayed appearance of cpe (Fig 4b) compared to the prototype strain CV-B2-Ohio in HPDE cells. In GMK cells, all three CV-B2 strains showed strong progeny production (Fig 4c) and cell lysis, although the strain CV-B2-1864 had slower growth kinetics than the other two strains. This difference was not statistically significant, however. Immunofluorescence staining indicated that by 8 h post infection (i.e., during the first infection cycle) fewer cells stained positive for viral capsid protein after CV-B2-1864 or CV-B2-94.ST256 infection than after the infection with the prototype strain CV-B2-Ohio (Fig 4d). The positively stained cells showed morphological characteristics typical of enterovirus induced cpe.

3.2. EV receptor expression

Correspondingly with virus growth curves, the receptors for CVA-9 and E-9 (α_{VP3}) and poliovirus (PVR) were detected in the

Fig. 3. Infectious progeny production of selected prototype vs. other enterovirus strains in HPDE cells. The infection and viral growth assays were conducted as indicated in Fig. 1. Both CV-B4-JVB and the strain CV-B4-E2 (originally isolated from a patient after onset of type 1 diabetes (Yoon et al., 1979)) strains formed productive infection but did not induce cpe. Both virus growth and cytolysis were detected for CV-B5 prototype strain Faulkner and the strains DS (that is highly cytolytic in human pancreatic islets in vitro (Ylipaasto et al., 2012)), MPP (in vivo mouse pancreas adapted strain that induces chronic pancreatic inflammation, dysregulation in glucose metabolism, loss of pancreatic acinar tissue, and mild insulinitis in mouse (Al-Hello et al., 2005)) and MCA (in vitro adapted to mouse insulinoma cell line, MIN6 (Al-Hello et al., 2009)), E-9 prototype Hill, laboratory strains Barty and strain DM isolated from a patient at the onset of type 1 diabetes (Vreugdenhil et al., 2000), E-11 prototype Gregory and strain D207 isolated from a stool sample of a patient at the onset of type 1 diabetes (Al-Hello et al., 2008) as well as for E-30 prototype strain Bastianni and strain 14916net87 that is highly lytic in human pancreatic islets in vitro (Roivainen et al., 2002).

surface of most of the cells using immunofluorescence technique (Fig 5). The receptor for Coxsackie B viruses (Coxsackie-adenovirus receptor, CAR) was detected in the surface of a subset of cells. However, decay accelerating factor (DAF), a co-receptor for CV-B1 and -5, could not be detected on the surface of HPDE cells.

3.3. Adaptation of non-lytic CV-B6 strain to HPDE cells

The growth curve and viability analyses together with immunofluorescence staining suggested that there are strain-specific differences in the ability of enteroviruses to infect pancreatic ductal cells. Since genetic diversity among circulating enteroviruses is very high and even the strains of the same serotype may have differences of hundreds of amino acids, viral adaptation experiments were carried out to study the potential genetic determinants behind the phenotypic differences.

The strains CV-B4-E2 and CV-B6-Schmitt (both of which had non-lytic phenotype in HPDE cells) were blind-passaged five times in HPDE cells. The passage of CV-B6-Schmitt in HPDE cells led to increased cytolysis (Fig 6a and b) and infectious progeny production (Fig 6c). The replication advantage was specific for ductal cells, since in GMK-cells both the parental strain Schmitt and the adapted strain (referred here as CV-B6-DUCT) had similar growth and lytic capabilities (Fig. 6d and e). The immunofluorescence staining of EV-capsid suggested that fewer cells were productively infected by CV-B6-Schmitt than by CV-B6-DUCT (Fig 6f). No changes in the growth curve or the ability to induce cpe were observed for CV-B4-E2 during passage (not shown).

3.4. Viral genetic changes during the adaptation to HPDE cells

To study the genetic determinants of the adaptation process, the complete genomes of both the parental strain CV-B6-Schmitt and the ductal cell adapted strain CV-B6-DUCT were sequenced.

Fig. 4. Infectious progeny production of CVB-2 prototype Ohio and strains CVB-2-1864 and CVB-2-94.ST256 in HPDE cells (a) and GMK cells (c). The cells were infected with m.o.i. of 1 and samples were collected 0 h, 5 h, 8 h, 24 h and 48 h post infection. The total infectivity of each sample was determined by end-point titration in microwell cultures of GMK cells. Cytopathic effects (cpe) were read on day 6 after infection by microscope, and 50% tissue culture infectious dose (TCID₅₀) titers were calculated using the Kärber formula. Mean TCID₅₀ ($\pm$ standard deviation) from three independent experiments is plotted. The statistical significance of differences between TCID₅₀ values was calculated with ANOVA-test (*0.05 > P > 0.01; ** 0.01 > P > 0.001; *** P > 0.001). (b) The viability of HPDE cells after infection with different CVB-2 strains was assessed using Sytox-green assay (Invitrogen) and spectrophotometer (Wallac 1420 Victor2™, PerkinElmer) 24 h post infection. The strain that induced visible cpe by 24 h post infection is indicated with solid black bar and the strains that did not induce visible cpe by 24 h are indicated with grey bars. Means of three experiments ($\pm$ SD) is shown. The statistical significance of the absorbance differences between mock infected control cells and virus infected cells was calculated with ANOVA-test (* 0.05 > P > 0.01; ** 0.01 > P > 0.001; *** P > 0.001). Note that CVB-2-1864 and CVB-2-94.ST256 strains induced visible cpe in the later time point (by 48 h post infection). (d) Immunofluorescence staining of CV-B2 infected HPDE cells. The cells were stained with enterovirus-specific polyclonal rabbit antiserum and made visible with FITC labeled anti-rabbit conjugate (green) 8 h after infection. The nuclei of the cells were stained with DRAQ5 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Immunofluorescence staining of selected human enterovirus receptors (green). The HPDE cells were stained with anti-PVR (Naez8), anti-hCAR (polyclonal), anti-DAF (IA10 (shown) and VIIIA7 (not shown) monoclonal) or anti- $\alpha_v\beta_3$ Ab (NAKG4) antibodies. Anti-rabbit FITC was used as a conjugate. The nuclei of the cells were stained with DRAQ5 (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

There was only one nucleotide difference (A3216G) between the HPDE adapted CV-B6-DUCT (accession no. KT380952) and the parental CV-B6-Schmitt (accession no. KT380953). This mutation resulted in lysine to glutamic acid substitution in the viral capsid protein VP1 amino acid site 257 (K257E). The three dimensional structure of CV-B6 is not known, but if superimposed over the structures of CV-B3 or swine vesicular disease virus SVDV (which is a

swine adapted strain of CV-B5), the amino acid site K257E is most likely located at the surface of the virus in the so-called canyon region (a depression around the five-fold axis of viral capsid) (Supplementary Fig. 1).

There are currently three different sequences for the prototype strain CV-B6-Schmitt available in the GenBank (AF039205, AF105342 and AF114384). None of these sequences were identical

Fig. 6. (a) Virus induced cpe in HPDE cells. The cells were visualized with Olympus IX81 light microscope using 200 fold magnification. (b) The viability of HPDE cells after infection with CVB-6 prototype Schmitt and ductal cell adapted strain CVB-6-DUCT in HPDE cells using Sytox-green assay (Invitrogen) and spectrophotometer (Wallac 1420 Victor2™, PerkinElmer) 24 h post infection. The statistical significance of the absorbance differences between mock infected control cells and virus infected cells was calculated with ANOVA-test (* 0.05 > P > 0.01; ** 0.01 > P > 0.001; *** P < 0.001). (c and d) Infectious progeny production of CVB-6 prototype Schmitt and ductal cell adapted strain CVB-6-DUCT in HPDE (c) and GMK (d) cells. The cells were infected with equal m.o.i. and samples were collected 0 h, 5 h, 8 h, 24 h and 48 h post infection. The total infectivity of each sample was determined by end-point titration in microwell cultures of GMK cells. Cytopathic effects (cpe) were read on day 6 after infection by microscope, and 50% tissue culture infectious dose (TCID₅₀) titers were calculated using the Kärber formula (Lenette, 1969). Mean TCID₅₀ (±standard deviation) from three independent experiments is plotted. The statistical significance of differences between TCID₅₀ values was calculated with ANOVA-test (* 0.05 > P > 0.01; ** 0.01 > P > 0.001; *** P < 0.001). (e) Immunofluorescence staining of HPDE cells infected with CVB-6-Schmitt or CVB-6-DUCT. The cells were infected with equal m.o.i. The cells were stained with enterovirus-specific polyclonal rabbit antiserum and made visible with FITC-labeled anti-rabbit conjugate (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to our CVB-6-Schmitt strain (accession no. KT380953). There were 7–12 nucleotide (corresponding to 2–5 amino acid) differences between the strain sequenced in this study and the strains retrieved from the GenBank (Supplementary Table 1). Three of these differences T4124C, T4751C and A5923T (corresponding amino acid site Y1974F) were specific for our CVB-6-Schmitt strain (i.e., were found only in the sequence).

3.5. Changes in the receptor-usage of CV-B6 during the adaptation to HPDE cells

Since the receptor binding site for several enteroviruses (including CV-B) is located in the viral capsid canyon, it was hypothesized that VP1 K257E mutation might alter the receptor binding of CV-B6. Furthermore, a homologous amino acid in SVDV VP1 (Q258K/E) has

been suggested to be among the putative heparin sulfate binding sites (Verdaguer et al., 2003).

HPDE cells were infected with CVB-6-Schmitt or CVB-6-DUCT in presence of heparin or monoclonal antibodies against CAR or DAF. CAR antibody treatment delayed the growth of CVB-6-Schmitt but had no effect on the growth of CVB-6-DUCT (Fig 7). DAF antibody treatment induced hundred-fold reduction in the growth of CVB-6-DUCT but had no effect on the growth of CVB-6-Schmitt. Furthermore, DAF antibody treatment prevented CVB-6-DUCT induced cell lysis in CPE protection assay. Heparin treatment had no effect on the growth or cpe production of either one of the virus strains.

Fig. 7. Receptor antibody-mediated protection assay. Infectious progeny production of CVB-6 prototype Schmitt and ductal cell adapted strain CVB-6-DUCT in HPDE cells in presence of monoclonal antibodies against HCAR or DAF. The cells were incubated for 1 h at room temperature in growth medium with or without antibodies, followed by inoculation with the virus for 1 h. After infection the cells were washed twice and maintained in growth medium. The total infectivity of each sample was determined by end-point titration in microwell cultures of GMK cells.

4. Discussion

The aim of this study was to assess the ability of different enterovirus strains to infect, replicate and produce cytopathic effect in human pancreatic ductal cells. Furthermore, the viral factors that affect these capabilities were studied. The capability of enteroviruses to infect pancreatic ductal cells has important implications in respect to enterovirus induced type 1 diabetes. Since ductal epithelial cells are anatomically close to pancreatic islets (Bertelli and Bendayan, 2005; Zhao et al., 2008), the productive infection of ductal cells may amplify the infection and provide a route for viruses to gain access to pancreatic islets (and beta-cells), which have also been shown to be susceptible to enterovirus infections (Chehadeh et al., 2000; Roivainen et al., 2000, 2002; Sarmiento et al., 2013; Smura et al., 2010). Furthermore, virus replication in ductal cells may induce proinflammatory cytokine expression that may be harmful for beta-cells either directly or due to recruitment of immune cells to the site of infection (Eizirik and Mandrup-Poulsen, 2001; Eizirik et al., 2009).

The pancreatic ductal cells were highly susceptible to enterovirus infections. Both viral growth and cytolysis were detected for several enterovirus serotypes. However, the viral growth and appearance of the classical cytopathic effect (cpe) in HPDE cells did not correlate completely. Some virus strains, such as CV-B4-JVB, CV-B4-E2 and CV-B6-Schmitt, replicated in the HPDE-cells but no cpe was detected in the light microscope or using Sytox green assay. In accordance with our results, it has been shown previously that in another pancreatic ductal cell line PANC-1 and primary human ductal cells CV-B4-E2 induces persistent infection with low cytopathic effect (Sane et al., 2013; Triantafilou and Triantafilou, 2004). The capability of inducing cpe in HPDE cells may have implications on type 1 diabetes pathogenesis. The virus-induced cytopathic effect is likely to be highly pro-inflammatory, since the contents of the affected cells are leaked into the extracellular space leading to harmful effects in the neighbouring cells. Therefore, the cytolytic infection of pancreatic ductal cells might lead to local inflammatory milieu, where also beta cells are (either directly or indirectly) affected. Strong inflammatory response, together with presentation of beta cell autoantigens from damaged beta-cells could induce autoimmunity and result in type 1 diabetes. Non-lytic infection, on the other hand, may induce persistent infection and lead to sustained stimulation of autoimmunity.

The amplification of infection in pancreas due to viral replication in ductal cells may also have implications on the development of type 1 diabetes. The experiments using primary cultures of human pancreatic islets suggest that the extent to which inflam-

matory mediators are produced may correlate to the severity of the infection (virus yield) (Schulte et al., 2012; Ylipaasto et al., 2012). Likewise, in mouse models it has been shown that the viral replication efficiency in pancreas contributes to the development of type 1 diabetes (Kanno et al., 2006) or chronic pancreatic inflammation (Al-Hello et al., 2005) after CVB infection. Therefore, virus strains with high productivity in pancreas are more likely to induce stronger and potentially harmful inflammatory response.

Intriguingly, there were strain-specific differences within some serotypes (CV-B2) in the growth kinetics and the capability to induce cpe in HPDE cells. The immunofluorescent staining of viral capsid proteins suggested that despite high multiplicity of infection, for CV-B4-E2, CV-B6-Schmitt, CV-B2-1864 and CV-B2-94.ST256 only few cells were initially infected. This observation was in line with enterovirus cell surface receptor staining, in which CAR (a receptor for CVB viruses) was detected only in a subset of cells. Since thousands of progeny viruses can be produced by a single enterovirus infected cell, it is possible that the viral growth detected with single-step growth curves were due to productive infection of few individual cells (possibly with strong CAR expression). In comparison, the immunofluorescence staining after infection with the highly lytic CV-B2-Ohio strain suggested that a large number of cells were infected during the first virus replication cycle when infected with m.o.i. equal to that used with CV-B2-1864 and CV-B2-94.ST256. A similar observation was made with CV-B6 strains. Fewer cells were productively infected by non-lytic CV-B6-Schmitt than by lytic CV-B6-DUCT. The appearance of strong cpe may therefore be due to enhanced infection rate of certain virus strains.

In order to study the potential determinants of the strain-dependent differences in the growth and lytic capabilities of enteroviruses in HPDE cells, adaptation experiments were carried out. The adaptation of CV-B6-Schmitt to HPDE cells resulted in pronounced cpe and more efficient viral multiplication in single-step growth curve experiments. These effects were specific to HPDE cells since in GMK cells both virus strains grew efficiently and induced cpe readily. Treatment of HPDE cells with DAF antibody reduced the growth of HPDE adapted CV-B6-DUCT strain equivalent to the growth rate of CV-B6-Schmitt and abolished the virus induced cpe. This suggests that the adaptation was associated with the viral capability of binding to DAF.

All six CVB serotypes use CAR as a receptor (Bergelson et al., 1997a,b; Martino et al., 2000; Tomko et al., 1997). CAR is a cell adhesion molecule that is expressed in the cell junctions (Cohen et al., 2001). In addition to CAR, several CV-B1, CV-B3 and CV-B5 strains attach to DAF (Bergelson et al., 1997b; Riabi et al., 2014; Shafren et al., 1995). However, DAF-binding alone does not lead to A-particle formation (i.e., conformational changes in the viral capsid that lead to release of viral RNA into cytoplasm) (Milstone et al., 2005) or cytolytic infection (Bergelson et al., 1995, 1997b; Shafren et al., 1997). DAF is present on the apical surface of polarized cells (Shieh and Bergelson, 2002). The binding of CVB to DAF on the apical surface leads to the transportation of the virus to the tight junction, where it interacts with CAR leading to virus entry (Coyne and Bergelson, 2006). To our knowledge, no CV-B2, -4 or -6 strains with DAF binding capacity have been characterized previously. There are, however, several similarities between CV-B6-DUCT strain described in this study and the two well-studied DAF-binding CV-B3 strains; CV-B3-RD-strain (Bergelson et al., 1995; Reagan et al., 1984) and CV-B3-PD-strain (Schmidtke et al., 2000). Specifically, DAF-binding appears to be required for lytic infection for all three virus strains (in pancreatic duct epithelial cells, colon epithelial cells and fibroblast, respectively) (Schmidtke et al., 2000; Shieh and Bergelson, 2002). CAR blockage (with RmcB antibody) had little or no effect on the growth of DAF-binding CV-B3-RD (Shieh and Bergelson, 2002) or CV-B6-DUCT in epithelial cells, although the CV-B3-RD strain had retained its capability to

use CAR as a receptor in CHO-CAR cells (Shieh and Bergelson, 2002). Since CV-B strains with strong DAF-binding properties require the CAR protein to mediate lytic infection (Bergelson et al., 1995, 1997b; Shafren et al., 1997), it is possible that CV-B6-DUCT strain has not lost its capability to use CAR as a receptor. Unfortunately, we could not verify this due to lack of suitable cell model. However, the possibility that DAF alone is sufficient for infection of pancreatic ductal cells by CV-B6-DUCT strain can not be excluded. Inconsistently, we were not able to detect DAF in the membrane of HPDE cells using immunofluorescence microscopy. This may be due to failure to detect the molecule rather than the absence of molecule.

The VP1 K257E was the only substitution that was identified during the adaptation of CV-B6-Schmidt to HPDE cells, suggesting that this single amino acid mutation may account for the more efficient infection and viral progeny production in HPDE cells. Further, the lack of synonymous mutations in the consensus sequence of CV-B6-DUCT suggests a strong (positive) selection for the non-synonymous K257E substitution. However, further reverse-genetic studies using site-directed mutagenesis should be conducted to verify the role of this substitution in the DAF-binding capacity and cytolytic infection in pancreatic ductal cells. On the other hand, the differences between the CV-B6-Schmitt sequenced in this study (accession no. KT380953) and the three CV-B6-Schmitt sequences (AF039205, AF105342 and AF114384) retrieved from the GenBank are most likely due to different passage histories of the strains in different laboratories.

Intriguingly, despite the phenotypic similarities, the amino acids that mediate DAF-binding of CV-B3-RD strain (Pan et al., 2011; Yoder et al., 2012) or CV-B3-PD strain (Schmidtke et al., 2000) are not homologous to the CV-B6 VP1 K257E substitution identified as a putative DAF-binding determinant of CV-B6-DUCT strain in this study. The identification of a single CV-B6 amino acid that is most likely responsible for the lytic phenotype, more efficient progeny production and DAF-usage is in line with previous reports that have shown strong effects for single or few amino acid substitutions on the receptor usage, cell and tissue tropism and pathogenesis (Al-Hello et al., 2005, 2009; Caggana et al., 1993; Chua et al., 2008; Cifuentes et al., 2011; Gullberg et al., 2010; Halim and Ramsingh, 2000; Kim and Racaniello, 2007; Knowlton et al., 1996; Novoselov et al., 2012; Paananen et al., 2013; Pan et al., 2011; Pelletier et al., 1998; Polacek et al., 2005; Ramsingh and Collins, 1995; Ramsingh et al., 1997; Schmidtke et al., 2000). A phenomenon similar to our CV-B6 model has been described previously using CV-B2-Ohio strain that induces persistent non-cytolytic infection in CAR-deficient RD-cells. A single amino acid substitution in VP1 protein transforms the virus phenotype from non-cytolytic to cytolytic (Gullberg et al., 2010; Polacek et al., 2005). However, this adapted CV-B2 strain does not bind to DAF (i.e., uses yet un-identified receptor but retains CAR-binding) and the amino acid substitution responsible for the cytolytic phenotype of CV-B2 (Q164K at VP1) is not homologous to the amino acid responsible for CV-B6-DUCT adaptation (although both map to the adjacent regions in capsid surface). Intriguingly, CV-B2-Ohio strain induces lytic infection in the HPDE-cells although it does not have DAF-binding capacity (Bergelson et al., 1997b; Gullberg et al., 2010; Shafren et al., 1995). Therefore, the ability to use DAF as a receptor is, most likely, not the sole determinant of lytic infection in pancreatic ductal cells. Further studies should be conducted to reveal the determinants of lytic phenotype of CV-B2 in ductal cells.

5. Conclusions

The present and previous studies demonstrate considerable plasticity in the receptor usage and cytolytic/pathogenetic properties of enteroviruses. The opportunistic nature of RNA-viruses has

resulted in a significant convergence in the evolution of receptor usage and the precise mutations required for the adaptation of the virus to a given cell type seems to be dependent on the genetic background of a virus (as exemplified by the independent adaptation to DAF usage by CV-B3 and CV-B6).

In respect to type 1 diabetes pathogenesis, this and the previous studies underline the strong effect of single or few amino acid substitutions in the pathogenetic properties (e.g., cell and tissue tropism, lytic capabilities, immune response) of different enteroviruses. The capability of enteroviruses for rapid adaptation to different cell types suggests that, on occasion, strains with different pathogenetic properties (e.g., cell tropism) may arise from less pathogenic ancestors. This hampers the attempts to detect “diabetogenic” enterovirus strains by genetic or antigenic (serotypic) properties and the attempts to develop vaccines against “diabetogenic” enterovirus strains since the antigenic and pathogenetic properties of the virus are likely to be at least partially distinct features.

Acknowledgments

The authors thank M. Eskelinen (National Institute for Health and Welfare [THL], Helsinki, Finland) for expert technical assistance.

This study was supported by The European Union (EP7-HEALTH-2007, DIAPREPP N202013) and The Diabetes Research Foundation (Finland).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.virusres.2015.08.003>

References

- Al-Hello, H., 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, 566–574.
- Al-Hello, H., Paananen, A., Eskelinen, M., Ylipaasto, P., Hovi, T., Salmela, K., Lukashov, A.N., Bobegamag, S., Roivainen, M., 2008. An enterovirus strain isolated from diabetic child belongs to a genetic subcluster of echovirus 11, but is also neutralised with monotypic antisera to coxsackievirus A9. *J. Gen. Virol.* 89, 1949–1959.
- Al-Hello, H., Ylipaasto, P., Smura, T., Rieder, E., Hovi, T., Roivainen, M., 2009. Amino acids of Coxsackie B5 virus are critical for infection of the murine insulinoma cell line, MIN-6. *J. Med. Virol.* 81, 296–304.
- Anagandula, M., Richardson, S.J., Oberste, M.S., Sioofy-Khojine, A.B., Hyoty, H., Morgan, N.G., Korsgren, O., Frisk, G., 2014. Infection of human islets of langerhans with two strains of Coxsackie B virus serotype 1: assessment of virus replication, degree of cell death and induction of genes involved in the innate immunity pathway. *J. Med. Virol.* 86, 1402–1411.
- Bergelson, J.M., Mohanty, J.G., Crowell, R.L., St John, N.F., Lublin, D.M., Finberg, R.W., 1995. Coxsackievirus B3 adapted to growth in RD cells binds to decay-accelerating factor (CD55). *J. Virol.* 69, 1903–1906.
- Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L., Finberg, R.W., 1997a. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* 275, 1320–1323.
- Bergelson, J.M., Modlin, J.F., Wieland-Alter, W., Cunningham, J.A., Crowell, R.L., Finberg, R.W., 1997b. Clinical coxsackievirus B isolates differ from laboratory strains in their interaction with two cell surface receptors. *J. Infect. Dis.* 175, 697–700.
- Bertelli, E., Bendayan, M., 2005. Association between endocrine pancreas and ductal system. More than an epiphenomenon of endocrine differentiation and development? *J. Histochem. Cytochem.* 53, 1071–1086.
- Caggana, M., Chan, P., Ramsingh, A., 1993. Identification of a single amino acid residue in the capsid protein VP1 of coxsackievirus B4 that determines the virulent phenotype. *J. Virol.* 67, 4797–4803.
- Chehadeh, W., Kerr-Conte, J., Pattou, F., Alm, G., Lefebvre, J., Wattre, P., Hober, D., 2000. Persistent infection of human pancreatic islets by coxsackievirus B is associated with alpha interferon synthesis in beta cells. *J. Virol.* 74, 10153–10164.

- Chua, B.H., Phuektes, P., Sanders, S.A., Nicholls, P.K., McMinn, P.C., 2008. The molecular basis of mouse adaptation by human enterovirus 71. *J. Gen. Virol.* 89, 1622–1632.
- Cifuentes, J.O., Ferrer, M.F., Jaquenod de Giusti, C., Song, W.C., Romanowski, V., Hafenstein, S.L., Gomez, R.M., 2011. Molecular determinants of disease in coxsackievirus B1 murine infection. *J. Med. Virol.* 83, 1571–1581.
- Cohen, C.J., Shieh, J.T., Pickles, R.J., Okegawa, T., Hsieh, J.T., Bergelson, J.M., 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc. Natl. Acad. Sci. U. S. A.* 98, 15191–15196.
- Coyne, C.B., Bergelson, J.M., 2006. Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions. *Cell* 124, 119–131.
- Eizirik, D.L., Mandrup-Poulsen, T., 2001. A choice of death – the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* 44, 2115–2133.
- Eizirik, D.L., Colli, M.L., Ortis, F., 2009. The role of inflammation in insulinitis and beta-cell loss in type 1 diabetes. *Nat. Rev. Endocrinol.* 5, 219–226.
- Furukawa, T., Duguid, W.P., Rosenberg, L., Viallet, J., Galloway, D.A., Tsao, M.S., 1996. Long-term culture and immortalization of epithelial cells from normal adult human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16. *Am. J. Pathol.* 148, 1763–1770.
- Gullberg, M., Tolf, C., Jonsson, N., Polacek, C., Precechtelova, J., Badurova, M., Sojka, M., Mohlin, C., Israelsson, S., Johansson, C., Bopegamage, S., Hafenstein, S., Lindberg, A.M., 2010. A single coxsackievirus B2 capsid residue controls cytolysis and apoptosis in rhabdomyosarcoma cells. *J. Virol.* 84, 5868–5879.
- Halim, S., Ramsingh, A.L., 2000. A point mutation in VP1 of coxsackievirus B4 alters antigenicity. *Virology* 269, 86–94.
- Hamalainen, S., Nurminen, N., Ahlfors, H., Oikarinen, S., Siiofy-Khojine, A.B., Frisk, G., Oberste, M.S., Laheesmaa, R., Pesu, M., Hyoty, H., 2014. Coxsackievirus B1 reveals strain specific differences in plasmacytoid dendritic cell mediated immunogenicity. *J. Med. Virol.* 86, 1412–1420.
- Hsu, K.H., Lonberg-Holm, K., Alstein, B., Crowell, R.L., 1988. A monoclonal antibody specific for the cellular receptor for the group B coxsackieviruses. *J. Virol.* 62, 1647–1652.
- Kanno, T., Kim, K., Kono, K., Drescher, K.M., Chapman, N.M., Tracy, S., 2006. Group B coxsackievirus diabetogenic phenotype correlates with replication efficiency. *J. Virol.* 80, 5637–5643.
- Kim, M.S., Racaniello, V.R., 2007. Enterovirus 70 receptor utilization is controlled by capsid residues that also regulate host range and cytopathogenicity. *J. Virol.* 81, 8648–8655.
- Kinoshita, T., Medof, M.E., Silber, R., Nussenzweig, V., 1985. Distribution of decay-accelerating factor in the peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. *J. Exp. Med.* 162, 75–92.
- Knowles, N.J., 2015. Picornavirus home page. <http://www.picornaviridae.com/> (accessed 08.09.15).
- Knowlton, K.U., Jeon, E.S., Berkley, N., Wessely, R., Huber, S., 1996. A mutation in the puff region of VP2 attenuates the myocarditic phenotype of an infectious cDNA of the Woodruff variant of coxsackievirus B3. *J. Virol.* 70, 7811–7818.
- Lennette, E.H., 1969. General principles underlying laboratory diagnosis of viral and rickettsial infections. In: Lennette, E.H., Schmidt, N.J. (Eds.), *Diagnostic Procedures for Viral and Rickettsial Infections*. American Public Health Association, New York, pp. 1–2–63.
- Lysy, P.A., Weir, G.C., Bonner-Weir, S., 2013. Making beta cells from adult cells within the pancreas. *Curr. Diab. Rep.* 13, 695–703.
- Martino, T.A., Petric, M., Weingartl, H., Bergelson, J.M., Opavsky, M.A., Richardson, C.D., Modlin, J.F., Finberg, R.W., Kain, K.C., Willis, N., Gauntt, C.J., Liu, P.P., 2000. The coxsackie-adenovirus receptor (CAR) is used by reference strains and clinical isolates representing all six serotypes of coxsackievirus group B and by swine vesicular disease virus. *Virology* 271, 99–108.
- Milstone, A.M., Petrella, J., Sanchez, M.D., Mahmud, M., Whitbeck, J.C., Bergelson, J.M., 2005. Interaction with coxsackievirus and adenovirus receptor, but not with decay-accelerating factor (DAF), induces A-particle formation in a DAF-binding coxsackievirus B3 isolate. *J. Virol.* 79, 655–660.
- Novoselov, A.V., Rezaykin, A.V., Sergeev, A.G., Fadeyev, F.A., Grigoryeva, J.V., Sokolova, Z.I., 2012. A single amino acid substitution controls DAF-dependent phenotype of echovirus 11 in rhabdomyosarcoma cells. *Virus Res.* 166, 87–96.
- Paananen, A., Ylipaasto, P., Rieder, E., Hovi, T., Galama, J., Roivainen, M., 2003. Molecular and biological analysis of echovirus 9 strain isolated from a diabetic child. *J. Med. Virol.* 69, 529–537.
- Paananen, A., Ylipaasto, P., Smura, T., Lempinen, M., Galama, J., Roivainen, M., 2013. A single amino acid substitution in viral VP1 protein alters the lytic potential of clone-derived variants of echovirus 9 DM strain in human pancreatic islets. *J. Med. Virol.* 85, 1267–1273.
- Pan, J., Narayanan, B., Shah, S., Yoder, J.D., Cifuentes, J.O., Hafenstein, S., Bergelson, J.M., 2011. Single amino acid changes in the virus capsid permit coxsackievirus B3 to bind decay-accelerating factor. *J. Virol.* 85, 7436–7443.
- Pelletier, I., Duncan, G., Colbere-Garapin, F., 1998. One amino acid change on the capsid surface of poliovirus sabin allows the establishment of persistent infections in HEP-2c cell cultures. *Virology* 241 (1), 1–13.
- Polacek, C., Ekstrom, J.O., Lundgren, A., Lindberg, A.M., 2005. Cytolytic replication of coxsackievirus B2 in CAR-deficient rhabdomyosarcoma cells. *Virus Res.* 113, 107–115.
- Ramsingh, A.L., Collins, D.N., 1995. A point mutation in the VP4 coding sequence of coxsackievirus B4 influences virulence. *J. Virol.* 69, 7278–7281.
- Ramsingh, A.L., Lee, W.T., Collins, D.N., Armstrong, L.E., 1997. Differential recruitment of B and T cells in coxsackievirus B4-induced pancreatitis is influenced by a capsid protein. *J. Virol.* 71, 8690–8697.
- Reagan, K.J., Goldberg, B., Crowell, R.L., 1984. Altered receptor specificity of coxsackievirus B3 after growth in rhabdomyosarcoma cells. *J. Virol.* 49, 635–640.
- Riabi, S., Harrath, R., Gaaloul, I., Bouslama, L., Nasri, D., Aouni, M., Pillet, S., Pozzetto, B., 2014. Study of Coxsackie B viruses interactions with Coxsackie adenovirus receptor and decay-accelerating factor using human CaCo-2 cell line. *J. Biomed. Sci.* 21, 50.
- Richardson, S.J., Willcox, A., Bone, A.J., Foulis, A.K., Morgan, N.G., 2009. The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. *Diabetologia* 52, 1143–1151.
- Roivainen, M., Rasilainen, S., Ylipaasto, P., Nissinen, R., Ustinov, J., Bouwens, L., Eizirik, D.L., Hovi, T., Otonkoski, T., 2000. Mechanisms of coxsackievirus-induced damage to human pancreatic beta-cells. *J. Clin. Endocrinol. Metab.* 85, 432–440.
- Roivainen, M., Ylipaasto, P., Savolainen, C., Galama, J., Hovi, T., Otonkoski, T., 2002. Functional impairment and killing of human beta cells by enteroviruses: the capacity is shared by a wide range of serotypes, but the extent is a characteristic of individual virus strains. *Diabetologia* 45, 693–702.
- Sane, F., Caloone, D., Gmyr, V., Engelmann, I., Belaich, S., Kerr-Conte, J., Pattou, F., Desailoud, R., Hober, D., 2013. Coxsackievirus B4 can infect human pancreas ductal cells and persist in ductal-like cell cultures which results in inhibition of Pdx1 expression and disturbed formation of islet-like cell aggregates. *Cell. Mol. Life Sci.* 70, 4169–4180.
- Sarmiento, L., Frisk, G., Anagandula, M., Cabrera-Rode, E., Roivainen, M., Cilio, C.M., 2013. Expression of innate immunity genes and damage of primary human pancreatic islets by epidemic strains of Echovirus: implication for post-virus islet autoimmunity. *PLoS One* 8, e77850.
- Savolainen, C., Hovi, T., Mulders, M.N., 2001. Molecular epidemiology of echovirus 30 in Europe: succession of dominant sublineages within a single major genotype. *Arch. Virol.* 146, 521–537.
- Schmid, S., Buuck, D., Knopff, A., Bonifacio, E., Ziegler, A.G., 2004. BABYDIET, a feasibility study to prevent the appearance of islet autoantibodies in relatives of patients with Type 1 diabetes by delaying exposure to gluten. *Diabetologia* 47, 1130–1131.
- Schmidtke, M., Selinka, H.C., Heim, A., Jahn, B., Tonew, M., Kandolf, R., Stelzner, A., Zell, R., 2000. Attachment of coxsackievirus B3 variants to various cell lines: mapping of phenotypic differences to capsid protein VP1. *Virology* 275, 77–88.
- Schulte, B.M., Lanke, K.H., Piganelli, J.D., Kers-Rebel, E.D., Bottino, R., Trucco, M., Huijbens, R.J., Radstake, T.R., Engelse, M.A., de Koning, E.J., Galama, J.M., Adema, G.J., van Kuppeveld, F.J., 2012. Cytokine and chemokine production by human pancreatic islets upon enterovirus infection. *Diabetes* 61, 2030–2036.
- Shafren, D.R., Bates, R.C., Agrez, M.V., Herd, R.L., Burns, G.F., Barry, R.D., 1995. Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment. *J. Virol.* 69, 3873–3877.
- Shafren, D.R., Williams, D.T., Barry, R.D., 1997. A decay-accelerating factor-binding strain of coxsackievirus B3 requires the coxsackievirus-adenovirus receptor protein to mediate lytic infection of rhabdomyosarcoma cells. *J. Virol.* 71, 9844–9848.
- Shieh, J.T., Bergelson, J.M., 2002. Interaction with decay-accelerating factor facilitates coxsackievirus B infection of polarized epithelial cells. *J. Virol.* 76, 9474–9480.
- Simonen-Tikka, M.L., Pflueger, M., Klemola, P., Savolainen-Kopra, C., Smura, T., Hummel, S., Kaijalainen, S., Nuutila, K., Natri, O., Roivainen, M., Ziegler, A.G., 2011. Human enterovirus infections in children at increased risk for type 1 diabetes: the babydiet study. *Diabetologia* 54, 2995–3002.
- Smura, T.P., Junttila, N., Blomqvist, S., Norder, H., Kaijalainen, S., Paananen, A., Magnus, L.O., Hovi, T., Roivainen, M., 2007. Enterovirus 94 a proposed new serotype in human enterovirus species D. *J. Gen. Virol.* 88, 849–858.
- Smura, T., Ylipaasto, P., Klemola, P., Kaijalainen, S., Kyllonen, L., Sordi, V., Piemonti, L., Roivainen, M., 2010. Cellular tropism of human enterovirus D species serotypes EV-94, EV-70, and EV-68 in vitro: implications for pathogenesis. *J. Med. Virol.* 82, 1940–1949.
- Tomko, R.P., Xu, R., Philipson, L., 1997. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc. Natl. Acad. Sci. U. S. A.* 94, 3352–3356.
- Triantafyllou, K., Triantafyllou, M., 2004. Coxsackievirus B4-induced cytokine production in pancreatic cells is mediated through toll-like receptor 4. *J. Virol.* 78, 11313–11320.
- Verdaguer, N., Jimenez-Clavero, M.A., Fita, I., Ley, V., 2003. Structure of swine vesicular disease virus: mapping of changes occurring during adaptation of human coxsackie B5 virus to infect swine. *J. Virol.* 77, 9780–9789.
- Vreugdenhil, G.R., Schloot, N.C., Hoorens, A., Rongen, C., Pipeleers, D.G., Melchers, W.J., Roep, B.O., Galama, J.M., 2000. Acute onset of type 1 diabetes mellitus after severe echovirus 9 infection: putative pathogenic pathways. *Clin. Infect. Dis.* 31, 1025–1031.
- World Health Organization (WHO), 2003. Guidelines for Environmental Surveillance of Poliovirus Circulation. World Health Organization, Geneva.
- Yeung, W.C., Rawlinson, W.D., Craig, M.E., 2011. Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. *BMJ* 342, d35.
- Ylipaasto, P., Klingel, K., Lindberg, A.M., Otonkoski, T., Kandolf, R., Hovi, T., Roivainen, M., 2004. Enterovirus infection in human pancreatic islet cells, islet

- tropism in vivo and receptor involvement in cultured islet beta cells. *Diabetologia* 47, 225–239.
- Ylipaasto, P., Eskelinen, M., Salmela, K., Hovi, T., Roivainen, M., 2010. [Vitronectin receptors, alpha v integrins, are recognized by several non-RGD-containing echoviruses in a continuous laboratory cell line and also in primary human Langerhans' islets and endothelial cells.](#) *J. Gen. Virol.* 91, 155–165.
- Ylipaasto, P., Smura, T., Gopalacharyulu, P., Paananen, A., Seppanen-Laakso, T., Kaijalainen, S., Ahlfors, H., Korsgren, O., Lakey, J.R., Lahesmaa, R., Piemonti, L., Oresic, M., Galama, J., Roivainen, M., 2012. [Enterovirus-induced gene expression profile is critical for human pancreatic islet destruction.](#) *Diabetologia* 55, 3273–3283.
- Yoder, J.D., Cifuentes, J.O., Pan, J., Bergelson, J.M., Hafenstein, S., 2012. [The crystal structure of a coxsackievirus B3-RD variant and a refined 9-angstrom cryo-electron microscopy reconstruction of the virus complexed with decay-accelerating factor \(DAF\) provide a new footprint of DAF on the virus surface.](#) *J. Virol.* 86, 12571–12581.
- Yoon, J.W., Austin, M., Onodera, T., Notkins, A.L., 1979. [Isolation of a virus from the pancreas of a child with diabetic ketoacidosis.](#) *N. Engl. J. Med.* 300, 1173–1179.
- Zhao, H.L., Sui, Y., Guan, J., Lai, F.M., Gu, X.M., He, L., Zhu, X., Rowlands, D.K., Xu, G., Tong, P.C., Chan, J.C., 2008. [Topographical associations between islet endocrine cells and duct epithelial cells in the adult human pancreas.](#) *Clin. Endocrinol. (Oxford)* 69, 400–406.