CELLULAR TROPISM OF HUMAN ENTEROVIRUS D SPECIES SEROTYPES EV-94, EV-70 AND EV-68 IN VITRO – IMPLICATIONS FOR PATHOGENESIS

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**CELLULAR TROPISM OF HUMAN ENTEROVIRUS D SPECIES
SEROTYPES EV-94, EV-70 AND EV-68 IN VITRO –
IMPLICATIONS FOR PATHOGENESIS**

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Keywords:	enterovirus, tissue tropism, leukocyte, endothelial cell, pancreatic islet

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Table 1. The geometric means of the end-point titres for neutralizing antibodies against EV-68, EV-70 and EV-94 in the sera of Finnish women at the end of the first trimester of pregnancy.

	1983	1993	2002
EV-68	178.8	88.1	44.5
EV-70	1.3	1.4	1.2
EV-94	7.8	9.6	7.1

Table 2. Cytokines showing more than five fold increase in the endothelial cell culture supernatants after infection with EV-94.

	Time post infection	Peak concentration pg/ml		
		EV-94 high m.o.i.	EV-94 low m.o.i.	Mock infected
IL-1a	48 h	45.3	12.9	4.6
SCGF-b	48 h	87.8	20.7	0
SDF-1a	48 h	191.1	170.0	0
IL-5	48 h	11.2	11.2	0
Eotaxin	6 h	105.8	0	0
TRAIL	48 h	42.5	0	0
HGF	48 h	5.7	0	0
IFN-g	48 h	153.9	380.4	36.2
MIG	48 h	3.0	0	0
VEGF	6h	73.5	0	11.9

Fig 1
128x77mm (300 x 300 DPI)

Fig 2a
123x71mm (300 x 300 DPI)

Fig. 2b
125x77mm (300 x 300 DPI)

Fig 2c
125x76mm (300 x 300 DPI)

Fig.2d
158x109mm (300 x 300 DPI)

Fig 3a
163x111mm (300 x 300 DPI)

Fig 3b
155x96mm (300 x 300 DPI)

Fig 4a
88x77mm (300 x 300 DPI)

Fig 4b
265x102mm (300 x 300 DPI)

Fig 4c
70x30mm (300 x 300 DPI)

Fig 4d
85x94mm (300 x 300 DPI)

**CELLULAR TROPISM OF HUMAN ENTEROVIRUS D SPECIES SEROTYPES EV-94,
EV-70 AND EV-68 *IN VITRO*
– IMPLICATIONS FOR PATHOGENESIS**

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Running head: Cellular tropism of HEV-D viruses

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ABSTRACT

Enterovirus 94 (EV-94) is an enterovirus serotype described recently which, together with EV-68 and EV-70, forms human enterovirus D species. This study investigated the seroprevalences of these three serotypes and their abilities to infect, replicate and damage cell types considered to be essential for enterovirus-induced diseases. The cell types studied included human leukocyte cell lines, primary endothelial cells and pancreatic islets.

High prevalence of neutralizing antibodies against EV-68 and EV-94 was found in the Finnish population. The virus strains studied had wide leukocyte tropism. EV-94 and EV-68 were able to produce infectious progeny in leukocyte cell lines with monocytic, granulacytic, T-cell or B-cell characteristics. EV-94 and EV-70 were capable of infecting primary human umbilical vein endothelial cells, whereas EV-68 had only marginal progeny production and did not induce cytopathic effects in these cells. Intriguingly, EV-94 was able to damage pancreatic islet β -cells, to infect, replicate and cause necrosis in human pancreatic islets, and to induce proinflammatory and chemoattractive cytokine expression in endothelial cells. These results suggest that HEV-D viruses may be more prevalent than has been thought previously, and they provide *in vitro* evidence that EV-94 may be a potent pathogen and should be considered a potentially diabetogenic enterovirus type.

KEY WORDS

enterovirus, tissue tropism, leukocyte, endothelial cell, human pancreatic islet, type 1 diabetes

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INTRODUCTION

Enterovirus genus (family *Picornaviridae*) contains six species causing infections in humans, *Human enterovirus A* (HEV-A) to HEV-D, *Human rhinovirus A* (HRV-A) and HRV-B, classified into over 200 currently known serotypes. Although the majority of enterovirus infections are subclinical, they can lead to a variety of acute and chronic diseases including mild upper respiratory illness, febrile rash, aseptic meningitis, encephalitis, acute hemorrhagic conjunctivitis, pleurodynia, acute flaccid paralysis (AFP), myocarditis, type 1 diabetes (T1D) and neonatal sepsis-like disease (Pallansch & Roos, 2001).

The primary site of enterovirus infection is the mucosal tissue of the respiratory or gastrointestinal tract. Virus replication can continue in the intestinal mucosa for several weeks. During this period, the progeny virus is excreted in the faeces. 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. Most enterovirus-related diseases are consequences stemming from the viral infection of secondary target tissues. Thus the tissue tropism of a virus is a major factor influencing the incidence and severity of enterovirus-induced diseases.

Molecular typing methods have led to characterization of several new enterovirus types. Enterovirus 94 (EV-94) was recently detected during poliovirus surveillance of waste water in Egypt and in acute flaccid paralysis patients in the Democratic Republic of Congo (Smura *et al.*, 2007, Junttila *et al.*, 2007). EV-94 is a member of the HEV-D species. This species is known to have only two other serotypes: enterovirus 68 (EV-68), which has been associated with respiratory infections (Schieble *et al.*, 1967, Oberste *et al.*, 2004); and enterovirus 70 (EV-70), a causative agent of acute haemorrhagic conjunctivitis (Mirkovic *et al.*, 1973). Both EV-70 and EV-68 can

occasionally infect the central nervous system (Pallansch & Roos, 2001, Khetsuriani *et al.*, 2006). Seroprevalence studies have revealed a high prevalence of EV-94 in the Finnish population over the past two decades (Smura *et al.*, 2007). As yet, however, the pathogenesis and possible clinical manifestations of this virus have not been studied in detail.

EV-94, unlike EV-68 and some strains of EV-70 (Blomqvist *et al.*, 2002, Oberste *et al.*, 2004), is acid stable and thus probably able to use the faecal-oral route of transmission to gain access to intestinal tissues (Smura *et al.*, 2007). If the primary infection in mucosal tissue is followed by viremia, the leukocytes may provide an additional reservoir for viral replication and/or persistence. This could increase the viral load in the circulation, thereby lengthening the viremic period. Endothelial cells, on the other hand, act as a barrier between vascular space and tissue parenchyma. In order to infect secondary target tissues, the virus has to pass through endothelial cells either by infecting them or by transcytosis. Alternatively, the viruses can be transported through the endothelial barrier by infected leukocytes migrating to the target tissues.

The pancreas is one of the secondary target tissues for enteroviruses (Pallansch & Roos, 2001, Huber & Ramsingh, 2004), and enteroviruses are considered to be a major environmental factor causing predisposition to type 1 diabetes (Varela-Calvino & Peakman 2003, Knip *et al.*, 2005, Roivainen 2006). The enterovirus genome or enterovirus-specific antibodies can be detected more often from the blood samples of patients with type 1 diabetes than from blood samples of control subjects (reviewed in Jaidane & Hober, 2008). Many enterovirus strains are capable of infecting pancreatic islets *in vitro* (Roivainen *et al.*, 2000, 2002), and enterovirus genome/proteins have been detected in the pancreatic islets of patients with type 1 diabetes (Ylipaasto *et al.*, 2004, Dotta *et al.*, 2007, Richardson *et al.*, 2009). However, the pathogenetic processes leading to virally-induced type 1 diabetes have not been characterized in detail.

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This study investigated the seroprevalences of HEV-D serotypes and their abilities to infect and damage cell types that are thought to play an essential role in enterovirus-induced diseases. A high seroprevalence and wide leukocyte tropism were detected for EV-68 and EV-94. EV-94 was able to damage pancreatic islet β -cells, to infect, replicate and cause necrosis in human pancreatic islets and to induce proinflammatory and chemoattractive cytokine expression in endothelial cells. The results suggest that HEV-D viruses may be more prevalent than has previously been thought. They also provide *in vitro* evidence that EV-94 may be a potent pathogen and should be considered a potentially diabetogenic enterovirus type.

MATERIAL & METHODS

Viruses

The isolation and plaque purification of EV-94 strain E210 have been described in a previous study (Smura *et al.*, 2007). The prototype strains EV-68-Fermon and EV-70-J670/71 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Cell lines

The human rhabdomyosarcoma cell line (RD) was provided by the World Health Organization (WHO) Polio Labnet. The green monkey kidney cell line (GMK) has been maintained in the laboratory since the 1960s. The leukocyte cell lines KG-1, HL-60, THP-1, U-937, Jurkat and Raji were purchased from ATCC (Manassas, VA, USA). MOLT and RC-2A leukocyte cell lines were kindly provided by Leif Andersson (University of Helsinki, Finland). The leukocyte cell lines were maintained in RPMI 1640 medium (Cat. No. R-6504, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS), except for the KG-1 cell line, which was cultured in Iscove's modified Dulbecco's Medium (IMDM) (Invitrogen, Gibco, Carlsbad, CA, USA) supplemented with 10% FBS.

Human pancreatic islets

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. After 1–5 days of culture in Ham's F10 (Cat. No. N6013, Sigma-

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Aldrich) medium supplemented with 10 mmol/l HEPES and 2% FBS, the islets were sent to Helsinki. Before the experiments, the islets were maintained for 1–6 days in sterile, non-adherent culture plates in incubation medium (Ham’s F10 containing 25 mmol/l HEPES, pH 7.4, 2% FBS, penicillin and streptomycin). The islet cell studies were approved by ethics committees both in Finland (The Institutional Review Board) and in Sweden (the ethical committee of Uppsala University).

Endothelial cells

Human primary endothelial cells were isolated from umbilical cords obtained from the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, Finland, with parents’ permission. The umbilical cords were collected in phosphate buffered saline (PBS). The cells were isolated as described by Saijets *et al.*, (2003). Briefly, the tissue was digested with 0.03% collagenase (Cat. No. C-5138, Sigma-Aldrich, NY, USA) in Hanks balanced salt solution supplemented with 20 mM Hepes pH 7.4, at +37° C for 10 minutes. Isolated cells were washed with growth medium (DMEM, Cat. No. D-7777, Sigma-Aldrich, NY, USA) supplemented with 50 µg/ml gentamycin, 150 µg/ml endothelial cell growth supplement (ECGS, Cat. No. E0760, Sigma-Aldrich, NY, USA), 2.5 U/ml heparin (Cat. No. H3149, Sigma-Aldrich, NY, USA) and 15% FBS, and subsequently suspended in the same medium. The cells were cultured in gelatine (Cat. No 4070, Merck, Darmstadt, Germany) coated flasks (0.2% sterile filtered gelatine in PBS) for 7 days at +36° C in a 5% CO₂ atmosphere. The medium was changed three times a week. Before the experiments, the cells were harvested and seeded (approximately 0.3 × 10⁶ cells/ml) into 8 chamber cell culture slides (Cat. No 354108, Falcon, Becton Dickinson, Lincoln Park, NJ, USA) for immunofluorescence assays, or into 96-well plates for infectivity assays. The purity of endothelial

cell culture was determined by staining von Willebrand's factor (vWF) with the immunofluorescence technique as described below.

Infection and viral progeny production

The cells were infected in Hanks' balanced salt solution supplemented with 20 mM Hepes pH 7.4. For the leukocyte cell lines, 10^6 cells/ml and a multiplicity of infection (m.o.i.) of 0.2–0.5 were used. The endothelial cells were infected at 80–95% confluency with various multiplicities (m.o.i. 0.01–10). The human pancreatic islets were infected as described previously (Ylipaasto *et al.*, 2005). Approximately 2000 islets/ml and a high m.o.i. were used. After incubation of 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 MgCl, 1% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin) was added to the cultures. For pancreatic islets, a serum-free medium was used (Ham's F10 supplemented with 25mM Hepes pH 7.4, 1% BSA, 100 U/ml penicillin and 0.1 mg/ml streptomycin). The cultures were incubated at +36° C in a 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 RD cells. Cytopathic effects (cpe) were read on day 6 after infection by microscope, and 50% tissue culture infectious dose (TCID₅₀) titres were calculated using the Kärber formula (Lenette, 1969).

Immunofluorescence assays

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The infected cells were fixed with cold methanol for 15 min at +4° C, washed three times with PBS and incubated with antiserum for 1 h at +36° C. A polyclonal rabbit antiserum produced against EAIPALTAVETGHTSQVC-peptide designed according to the VP1 region of the enterovirus genome (Harkonen *et al.*, 2002), or polyclonal rabbit antiserum against EV-94 (K-HAX), was used. The endothelial cells were identified with Factor VIII related antigen (vWF) specific antibody (Cat No. MS-722-S, Neomarkers). Insulin-producing islet β -cells were identified with insulin-specific polyclonal sheep antiserum (30 μ g/ml; PC059, The Binding Site, Birmingham, UK). Unbound antibody was removed by washing three times with PBS. The conjugates for rabbit (anti-rabbit FITC, Cat No. 711-095-152, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and mouse antibodies (antimouse REDX, Cat No. 715-295-150 Jackson ImmunoResearch Laboratories, Inc.) were incubated for 30 min at +36° C. After staining, the slides were washed five times with PBS. The slides were analysed under a confocal microscope (Leica TCS SPE, Wetzlar, Germany).

Activation of endothelial cells

The endothelial cells were either infected with viruses at m.o.i. of 1–10, incubated for 24 h with recombinant human interleukin-1 β (10 ng/ml, 1500 U/ml, Becton Dickinson) or mock infected. The samples were fixed with cold methanol and stained with mouse anti-human intercellular adhesion molecule 1 (ICAM-1) (Cat. No. HM4004, HyCult Biotechnology bv., Uden, The Netherlands) or mouse anti-human E-selectin (Cat. No. HM4001 HyCult Biotechnology bv., Uden, The Netherlands) antibodies as described above.

Cell viability assays

Cell proliferation reagent WST-1 (Cat. No. 1644807, Roche Diagnostics, Mannheim, Germany) and Sytox Green nucleic acid stain (Cat. No. S7020, Invitrogen) were used for quantification of leukocyte and endothelial cell viabilities, respectively. In WST-1 assay, a tetrazolium salt WST-1 is added to the cell culture medium. The tetrazolium salts are cleaved to formazan by cellular enzymes of metabolically active cells. The number of viable cells is proportional to the amount of formazan dye, which was quantified by using a spectrophotometer (Wallac 1420 Victor²™, PerkinElmer, MA, USA) to measure the absorbance of the culture media.. The viability of human pancreatic islets was assessed using Live/Dead assay (Cat No L3224, Molecular Probes, Leiden, The Netherlands) and a confocal microscope.

DNA and insulin content of pancreatic β -cells.

For the measurement of DNA and insulin content, the islet cells were homogenized ultrasonically in distilled water. DNA was measured using Fluoreportex Blue Fluorometric dsDNA quantitation kit (Cat. no. F2962, Molecular probes). Insulin was measured with a solid-phase insulin RIA kit (DPC, Los Angeles, CA, USA) after overnight extraction with acid ethanol as described previously (Otonkoski et al., 1993).

Serum neutralization assay

The study group for the serum neutralization assay consisted of 281 women at the end of the first trimester of pregnancy. The sera were derived from the Finnish Maternity Cohort and had been collected in 1983 (n=86), 1993 (n=99) and 2002 (n=96).

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Aliquots of the sera were inactivated at +56° C for 20 minutes and stored at +4° C. Serial fourfold dilutions of sera were mixed with an equal volume of virus (100 TCID₅₀). The mixtures were neutralized first at +36° C for 1 hour and then overnight at +4° C. The mixtures were transferred into 96–well cell culture plates with RD cells for EV-70 and EV-94, or with GMK cells for EV-68 (~0.3 million cells ml⁻¹), and incubated at +36° C in a 5% CO₂ atmosphere for 6 days. The highest dilution completely inhibiting the viral cytopathic effect was regarded as the end point titre of the serum.

Cytokine assay

The capability of EV-94 to induce cytokine expression in endothelial cells was measured using multiplex bead-based assays based on xMAP technology (Bio-Plex; Biorad Laboratories, Hercules, CA, USA). The parallel wells of endothelial cells (~75 000 cells/well) were infected with EV-94 at the m.o.i. of 4 or 0.01, or they were mock infected. The culture media supernatant was collected 0, 6, 24 and 48 hours post infection and assayed for 48 cytokines. The limits of detection and the coefficients of variability (intra Assay % CV and inter Assay % CV) of the cytokine/chemokine assay were: IL-1α: 0.5 pg/ml (4; 4) ; IL-1β: 0.6 pg/ml (6; 8); IL-1ra: 5.5 pg/ml (9;8); IL-2: 1.6 pg/ml (7; 9); IL-2Rα: 2.1 (6;4); IL-3: 4.8 pg/ml (7;4); IL-4: 0.7 pg/ml (9;8); IL-5: 0.6 pg/ml (8;10); IL-6: 2.6 pg/ml (7;11); IL-7: 1.1 pg/ml (6;8); IL-9: 2.5 pg/ml (8;9); IL-10: 0.3 pg/ml (5;6); IL-12p40: 23.3 pg/ml (5;8); IL-12p70: 3.5 pg/ml (6;6); IL-13: 0.7 pg/ml (8;7); IL-15: 2.4 pg/ml (5;6); IL-16: 0.4 pg/ml (6; 4); IL-17: 3.3 pg/ml (8;6); IL-18: 0.2 pg/ml (4;5); INFγ: 6.4 pg/ml (15;9); TNFα: 6 pg/ml (8;6); TNFβ: 0.3 pg/ml (4;4); GM-CSF: 2.2 pg/ml (12;6); G-CSF: 1.7 pg/ml (10;5); M-CSF: 0.9 pg/ml ((4,5); CCL2/MCP-1: 1.1 pg/ml (9;7); CCL3/MIP-1α : 1.6 pg/ml (7;8); CCL4/MIP-1β: 2.4 pg/ml (8;8); CCL5/Rantes: 1.8 pg/ml (9;6);CCL7/MCP-3: 1 pg/ml (7;8); CCL11/Eotaxin: 2.5 pg/ml (8;11); CCL27/CTAK: 3.4 pg/ml (5;6); CXCL1/GRO-α: 6.3 pg/ml (5;

8); CXCL9/MIG: 1.2 pg/ml (6;6); CXCL10/IP-10: 6.1 pg/ml (11; 9); CXCL12/SDF1 α : 8.7 pg/ml (6;6); VEGF: 3.1 pg/ml (9;7); CXCL8/IL-8: 1.0 pg/ml (9;4); basic FGF: 1.9 pg/ml (8;8); PDGF-BB: 2.9 pg/ml (9; 8); HGF: 4.9 pg/ml (5; 6); IFN- α 2: 4.3 pg/ml (7;3); LIF: 5.5 pg/ml (4;3); MIF: 1.5 pg/ml (5;8); β -NGF: 0.2 pg/ml (4;7); SCF: 1 pg/ml (5;4); SCGF- β : 45.4 pg/ml (6;8); TRAIL 2.1pg/ml (4;8).

Statistical analysis

One-way analysis of variance (ANOVA) and Kruskal-Wallis one-way analysis of variance were used for statistical evaluation of cell viabilities and serum antibody levels, respectively.

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RESULTS

Seroprevalence of HEV-D serotypes in Finland

A previous study detected a high seroprevalence of EV-94 in the Finnish population (Smura *et al.*, 2007). In this study, the prevalence of antibodies against the two other HEV-D serotypes, EV-68 and EV-70, in Finland was assessed using the serum neutralization assay. Serum samples obtained from pregnant women and collected in 1983 (n=86), 1993 (n=99) and 2002 (n=96) were studied. Neutralizing antibodies against EV-68 were found in 100%, against EV-70 in 15.1%, 21.2 % and 13.5 %, and against EV-94 in 79.1%, 79.8% and 79.2% of the subjects in the years 1983, 1993 and 2002, respectively (Fig. 1).

The geometric means for the serum end point titres are shown in Table 1. The serum samples studied had significantly higher antibody levels against EV-68 than against EV-70 or EV-94 in all of the years for which samples were studied (Kruskal-Wallis $p < 0.01$). The mean antibody levels against EV-68 showed a decreasing temporal trend whereas those against EV-70 and EV-94 remained constant over the study period (Kruskal-Wallis $p < 0.01$).

Susceptibility of human leukocyte cell lines to EV-94 and EV-68

Replication in blood cells may provide enteroviruses an additional replication site during viremia, thus lengthening the viremia and/or increasing the viral load during viremia. The viruses may also spread to secondary target tissues within infected circulating leukocytes. Therefore, the susceptibilities of monocytic (THP-1, RC-2A and U937), granulocytic (HL-60 and KG-1), T-cell (MOLT and Jurkat) and B-cell (Raji) lineages to EV-94 and EV-68 infections were studied.

For EV-94, infectious progeny production was detected in all studied cell lines 24 hours after infection (Fig.2 a). The viabilities of the cells were reduced significantly three days after EV-94 infection (ANOVA $p < 0.005$) (Fig. 2 b). For EV-68, an increase in viral titre was detected in T-cell lines MOLT and Jurkat, in the B-cell line Raji, the granulocytic line KG-1 and in the monocytic line U-937, whereas no definitive infective virus production was detected in the monocytic RC-2A line or in the granulocytic cell line HL-60 (Fig. 2 c). EV-68 infection did not induce statistically significant changes in the viabilities of the leukocytes (Fig. 2 d).

Susceptibility of endothelial cells to viral infections

Replication in the endothelial cell lining of blood vessels may provide a virus with an access route to secondary target tissues. To assess the susceptibility of endothelial cells to EV-68, EV-70 and EV-94 infections, single-step growth curve experiments were carried out in primary human umbilical vein derived endothelial cells.

EV-94 and EV-70 showed a definite cytopathic effect and progeny production in endothelial cells (Fig. 3a). However, there was substantial donor-specific variation in the susceptibility of endothelial cells to HEV-D infections. Infective progeny formation was detected in the cells isolated from twelve out of fourteen donors after EV-94 infection and in seven out of eleven donors after EV-70 infection. A low level of progeny formation but no definitive cytopathic effect was detected after EV-68 infection in the cells derived from two out of six donors.

To verify that the viruses infected endothelial cells specifically, the endothelial cell specific Factor VIII related antigen (von Willebrand factor) and enterovirus capsid protein were co-stained. EV-94

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capsid protein positive cells were detected in a subset of endothelial cells (Fig. 3b). Owing to a lack of suitable antibody against EV-68 and EV-70, such staining could not be performed for these viruses.

EV-94 induced cytokine expression in endothelial cells

The capability of EV-94 to induce cytokine expression in endothelial cells was measured using multiplex bead-based assays based on xMAP technology. The parallel wells of endothelial cells (~75 000 cells/well) were infected with EV-94 at the m.o.i. of 4 or 0.01, or they were mock infected. The culture media supernatant was collected 6, 24 and 48 hours post infection and assayed for 48 cytokines.

Ten cytokines showed over fivefold increase in EV-94 infected cell supernatants compared to mock-infected controls (Table 2). Seven of these peaked 48 h post infection and showed a stronger response to high m.o.i. infection than to low m.o.i. infection. The interferon γ (IFN- γ) levels showed a stronger response to low m.o.i. infection. CCL11/Eotaxin and VEGF peaked 6 h after high m.o.i. infection and 24 h after low m.o.i infection, respectively.

Fifteen cytokines showed detectable expression, but no differences were detected between EV-94 and mock-infected cell cultures. The highest concentrations were detected for CXCL1/GRO- α (max 1117 pg/ml), MIF (max 2685 pg/ml), PDGF-BB (max 601 pg/ml), IL-6 (max 1497 pg/ml) and CXCL8/IL-8 (max 7722 pg/ml). MCP-1 showed strong expression with a slight down regulation after high m.o.i. EV-94 infection (concentrations at 48 h post infection: 5526 pg/ml, 10,791 pg/ml and 10,649 pg/ml for high m.o.i. EV-94, low m.o.i. EV-94 and mock-infected control, respectively). Basic FGF showed strong expression with slightly higher concentrations after high m.o.i. EV-94

infection (concentrations 48 h post infection: 661 pg/ml, 485 pg/ml and 440 pg/ml for high m.o.i. EV-94, low m.o.i. EV-94 and mock-infected controls, respectively). CXCL10/IP-10, CCL3/MIP-1 α , CCR5/RANTES, G-CSF, CCL4/MIP-1 β , IL-1Ra, IL-7, IL-9, IL-15 and CCL7/MCP-3 showed low but consistent expression. No expression was detected for IL-2Ra, IL-3, IL-16, IFN- α 2 and IL-17. The concentrations of CCL27/CTAK, IL-18, β -NGF, SCF, TNF- β , IL-10, LIF, M-CSF and IL-1 β fell under the detection limit. TNF- α showed low but detectable expression 24 h after high m.o.i. infection and 48 h after both high and low m.o.i. infections, whereas the concentrations of mock-infected controls fell under the detection limit.

Activation of endothelial cells by viruses

The capability of HEV-D viruses to induce the activation of endothelial cells was studied by immunohistochemical staining of E-selectin and ICAM-1, which are surface molecules associated with the activation of endothelial cells (Collins *et al.*, 1995). None of the viruses (EV-68, EV-70 or EV-94) was able to induce E-selectin or ICAM-1 expression in the endothelial cell surface 6–24 h after infection, whereas cells treated with IL-1 showed definite E-selectin and ICAM-1 expression (the number of donors studied: : n=5 for EV-94; and n= 2 for EV-68 and EV-70).

Human pancreatic islet infection with EV-94 and EV-68

The pancreas is one of the most important secondary target tissues for human enteroviruses, and enteroviruses have been considered to play a significant role in the aetiology of type 1 diabetes. The pancreatic islets of three donors were infected with both EV-94 and EV-68. Viral infection led to the detection of an infectious progeny formation with both viruses (Fig. 4a). The viability of the islets was drastically reduced 4–7 days after infection with EV-94 (n=4), whereas the mock-infected

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control islets and the islets infected with EV-68 (n=3) remained viable during the same period of time (Fig 4b). The co-staining of viral protein and insulin indicated that both viruses were able to infect insulin-producing pancreatic β -cells (Fig. 4c). To assess the β -cell specific destruction, the insulin to cellular DNA ratios were measured at several time points after infection. The insulin to DNA ratio decreased significantly 8–14 days after infection with EV-94 (n=3). There was no change in the insulin to DNA ratio after EV-68 infection (n=2) (Fig. 4d).

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DISCUSSION

In this study the *in vitro* pathogenetic properties of the enterovirus serotypes of the HEV-D species (EV-68 and EV-70) were compared in cell types that are considered to be essential for enterovirus-induced diseases. The cell types studied included human leukocyte cell lines, primary endothelial cells and pancreatic islets.

The results for EV-68 and EV-94 obtained in this study and in earlier studies on EV-70 (Haddad *et al.*, 2004) suggest that HEV-D viruses have an unusually wide leukocyte tropism. However, the role of leukocytes in enterovirus pathogenesis is somewhat obscure. After primary infection of the gastrointestinal tract, the viremia could be started via leukocytes if the virus were able to infect the dendritic cells and/or macrophages of Payer's patches. Leukocytes may also serve as an additional reservoir for virus replication, thereby lengthening the viremic phase and increasing the amount of virus in circulation. Accordingly, a correlation between poliovirus neurovirulence and replication efficiency in monocytes has been observed (Freistadt & Eberle 1996). Tissue migrating leukocytes may also provide a virus with an access to secondary target tissues. Viral replication in leukocytes is also likely to affect the host immune system (Kramer *et al.*, 2007, 2008). At least some enteroviruses (e.g. CBV-3, CBV-4 and PV-1) are able to induce pro-inflammatory cytokine expression in human leukocytes (Henke *et al.*, 1992, Vreugdenhil *et al.*, 2000). This can cause and/or maintain local inflammation in the secondary target tissues, thus contributing to immune-mediated chronic diseases such as type 1 diabetes.

It seems there are considerable differences between the abilities of different enterovirus serotypes to infect and replicate in leukocytes. Poliovirus is able to infect the monocytic fraction of peripheral blood mononuclear cells (Eberle *et al.*, 1995), monocytic and granulocytic cell lines (Kitamura *et*

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al., 1985, Okada *et al.*, 1987, Roivainen & Hovi 1989, Freistadt & Eberle 1996, Vuorinen *et al.*, 1999) and monocyte-derived dendritic cells and macrophages (Wahid *et al.*, 2005). However, both T-cells and B-cells seem to be resistant to poliovirus infection (Okada *et al.*, 1987, Vuorinen *et al.*, 1999). Coxsackie B viruses, in contrast, are able to replicate and persist in T-cell and B-cell lines (Matteucci *et al.*, 1985, Vuorinen *et al.*, 1994, 1996), but not in the granulocytic or monocytic lineages or monocyte-derived dendritic cells (Vuorinen *et al.*, 1996, Kramer *et al.*, 2007). The other members of HEV-B species – echoviruses – seem to have a very different leukocyte tropism. Echo 1 is able to replicate in monocytic (U937) cells but not in B-cells or T-cells (Raji or Molt-4) (Vuorinen *et al.*, 1999). Several echovirus strains are able to infect monocyte-derived dendritic cells and to form productive progeny in them, but are not able to infect PBMC-isolated monocytes (Kramer *et al.*, 2007).

Virus receptors are considered to be one of the key determinants of viral tissue tropism. Receptor usage may thus be one of the causative factors of the wide leukocyte tropism of HEV-D viruses. The receptor for EV-94 is not known, but both EV-70 and EV-68 use DAF as a receptor in Hela cells and sialic acid-containing receptors in leukocytes (Uncapher *et al.*, 1991, Karnaukhov *et al.*, 1996, Alexander *et al.*, 2002, Blomqvist *et al.*, 2002).

Another important site in enterovirus pathogenesis is the endothelial cell lining of the circulatory system. Infection of endothelial cells may affect viral pathogenesis through several mechanisms: 1) viral progeny production in endothelial cells can provide the virus a route for infecting the parenchymal tissue; 2) viral replication in endothelial cells can increase the local amount of virus, thereby increasing the probability, and possibly altering the outcome, of secondary target tissue infection; 3) the virus-induced activation of endothelial cells can promote chemoattraction and the

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3 homing of inflammatory leukocytes; 4) virally-induced proinflammatory cytokine production can
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5 induce inflammation or can be directly harmful for parenchymal cells.
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10 In this study, EV-94 and EV-70 were capable of infecting primary human umbilical vein
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12 endothelial cells, whereas EV-68 had only marginal progeny production and did not induce
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14 cytopathic effect in these cells. It has previously been shown that many other enterovirus strains,
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16 including poliovirus 1 (MacGregor *et al.*, 1980, Friedman *et al.*, 1981), coxsackie B viruses
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18 (Conaldi *et al.*, 1997, Zanone *et al.*, 2003) and some of the coxsackie A and echoviruses (CVA-13,
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20 E-6, E-7, E-9, E-11, E-30) (Friedman *et al.*, 1981, Saijets *et al.*, 2003) are able to infect and
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22 replicate in primary human endothelial cells. Most of the serotypes (e.g. echoviruses, PV-1 and
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24 CVA-13) induce cell lysis (Saijets *et al.*, 2003), whereas persistent infection is a more prominent
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26 outcome of infection with coxsackie B viruses (Conaldi *et al.*, 1997, Zanone *et al.*, 2003).
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34 An enterovirus infection can induce tissue damage either by direct cytolysis or by
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36 immunopathogenetic mechanisms (autoimmunity or virus-targeted immunity). With regard to virus-
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38 induced type 1 diabetes, the possible pathogenetic mechanisms (which are not mutually exclusive)
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40 include direct cytolysis of pancreatic β -cells after viral infection, virally-induced bystander
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42 activation of autoreactive T-cells (Horwitz *et al.*, 1998) and molecular mimicry (Atkinson *et al.*,
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44 1994).
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50 While direct cytolysis and/or virus-targeted immunity can be a possible cause for some cases of
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52 fulminant type 1 diabetes (Imagawa & Hanafusa, 2006, Akatsuka *et al.*, 2009), it is likely that
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54 autoimmune reactions triggered by viral infection are also involved in enterovirus-induced type 1
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56 diabetes. In this scenario, a virus infection in the pancreas and a (limited) β -cell damage (by a virus
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58 and/or virally-induced cytokines) would result in the exposure of autoantigens in a local
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inflammatory milieu, leading to upregulation of MHC molecules and up-take and presentation of autoantigens by activated antigen-presenting cells, which in turn would promote further β -cell damage by activating autoreactive T-cells (Filippi & Von Herrath, 2005).

With regard to the virus-induced development of autoimmune diabetes, it is intriguing that EV-94 is capable both of damaging human pancreatic islet β -cells and of inducing IFN- γ (along with other proinflammatory cytokines and chemokines like IL-1 α and CXCL12/SDF-1 α) expression in endothelial cells. In addition to antiviral effects, IFN- γ is considered to play a significant role in autoimmune diseases. Among other effects, IFN- γ enhances the expression of MHC-I proteins and increases the repertoire and quantity of peptides displayed to CD8 $^{+}$ T-cells. It also promotes Th1 differentiation and suppresses Th2 differentiation (Goodbourn *et al.*, 2000). Moreover, IFN- γ (in combination with TNF- α , IL-1 or synthetic dsRNA analogue polyinosinic-polycytidylic acid) is sufficient to induce apoptosis and to upregulate chemokine expression in pancreatic β -cells (Eizirik & Mandrup-Poulsen 2001, Liu *et al.*, 2001, Ylipaasto *et al.*, 2005). Thus the endothelial cells can both provide an accession route for a virus to infect pancreatic β -cells and contribute to the necessary proinflammatory milieu for the induction of beta cell autoimmunity.

Endothelial cell activation could not be detected after EV-94 infection (data not shown), even though activation promoting cytokines – e.g. IL-1A, which has been shown to be the predominant endothelial cell activating factor after EV-70 infection (Chang *et al.*, 2004) – were detected. This may be due to the rapid lysis of endothelial cells following high m.o.i. EV-94 infection. Some other enterovirus serotypes (e.g. CBV-2, CBV-4, CBV-5, CVA-9, CVA-13, E-1, E-11, E-30, PV-1, EV-70 and EV-71) have been shown to induce endothelial cell activation (Saijets *et al.*, 2003, Chang *et al.*, 2004, Liang *et al.*, 2004, Zanone *et al.*, 2007).

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3 The endothelial cells derived from different organs show distinct susceptibility to enterovirus
4 infections, and the cellular response of macrovascular and microvascular endothelial cells to viral
5 challenge may differ (Huber *et al.*, 1990, Conaldi *et al.*, 1997, Zanone *et al.*, 2003, 2007, 2008).
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7 Further studies are needed to assess the capability of different enterovirus types to replicate in
8 microvascular endothelial cells and to determine the host cell responses after enterovirus infection.
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17 EV-68 and EV-70 differ from most enteroviruses by infecting their primary target tissues
18 (respiratory tract for EV-68 and conjunctiva for EV-70) directly. The primary target tissue of EV-94
19 is not known, but the acid stability of this virus suggests that it may use the faecal-oral route of
20 transmission (Smura *et al.*, 2007). Leukocyte and endothelial cell tropism of HEV-D viruses
21 suggests that these viruses also have the potential to cause secondary target tissue infections,
22 although the low endothelial cell tropism of EV-68 suggests that secondary target tissue infections
23 may be less common after EV-68 than after EV-70 or EV-94 infection. Indeed, both EV-70 and, to
24 an lesser extent, EV-68 can infect occasionally the central nervous system (Pallansch & Roos, 2001,
25 Khetsuriani *et al.*, 2006), and both *in vitro* results in neuroblastoma cells (Smura *et al.*, 2007) and
26 the association with acute flaccid paralysis cases (Junttila *et al.*, 2007) suggest that EV-94 may also
27 be neurovirulent. According to the *in vitro* results of this study, EV-94 may also have another
28 secondary target: the pancreatic islets.
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48 Since an unexpectedly high seroprevalence of EV-94 was detected previously in the Finnish
49 population (Smura *et al.*, 2007), the seroprevalences of the other two HEV-D serotypes (EV-68 and
50 EV-70) were assessed. EV-68 seems to have an extremely high prevalence in Finland. This
51 observation is in line with the findings of a previous study on a limited number of individuals
52 (Blomqvist *et al.*, 2002). The high seroprevalence of EV-68 is in an apparent contrast with the
53 findings of enterovirus surveillance studies from clinical or environmental samples (Khetsuriani *et*
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al., 2006, Blomqvist *et al.*, 2008). However EV-68 may be underrepresented in clinical samples as a result of mild or subclinical symptoms. In most studies, enteroviruses are sought from stool samples or from waste water; EV-68 may remain undetected in these studies owing to respiratory tract tropism, temperature sensitivity and acid lability. Several EV-68 strains have been detected recently from respiratory tract specimens (Savolainen-Kopra *et al.*, 2009, She *et al.*, 2010). The mean antibody levels against EV-68 have decreased during the study period, suggesting antigenic drift among circulating EV-68 strains.

In conclusion, the results of this study suggest that HEV-D viruses may be more prevalent than has been thought previously, and that they have wide tropism for leukocytes and endothelial cells. It was also shown that EV-94 is able to damage human pancreatic islet β -cells *in vitro* and to induce proinflammatory and chemoattractive cytokine expression in endothelial cells, and should therefore be considered a potentially diabetogenic enterovirus serotype.

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FIGURES

Fig. 1. Prevalence and end-point titres of neutralizing antibodies against EV-68, EV-70 and EV-94 in the sera of Finnish women collected in 1983 (n=86), 1993 (n=99) and 2002 (n=96) at the end of the first trimester of pregnancy. The highest serum dilution (1:1024, 1:256, 1:64, 1:16 or 1:4) completely inhibiting the viral cytopathic effect was regarded as the end point titre of the serum.

Fig. 2. Infectious progeny production of EV-94 (a) and EV-68 (c) in leukocyte cell lines. The cells were infected with m.o.i. of 0.2–0.5 and harvested 0 hours (0 h), 24 hours (24 h) or 48 hours (48 h) post infection. The infectivity of each sample was determined by end-point titration (mean + standard deviation) in RD cells. The viabilities of leukocyte cells (% of control) 3 days after infection with EV-94 (b) and EV-68 (d). The cells were infected with m.o.i. of 0.2–0.5 and the viabilities were measured with a colorimetric WST-1 (Roche Diagnostics) assay.

Fig 3. (a) Infectious progeny production of EV-68, EV-70 and EV-94 in primary human umbilical cord derived endothelial cells. The cells were infected with m.o.i. of 0.01-1 and harvested at several time points after infection. The total infectivity of each sample was determined by end point titration in microwell cultures of RD cells. Mean titres from several donors (n=6 for EV-68, n=5 for EV-70 and for EV-94) are shown. (b) Immunofluorescence staining of EV-94 infected (right-hand panel) and mock-infected (left-hand panel) endothelial cells. The cells were stained with enterovirus-specific polyclonal rabbit antiserum (green) and endothelial cell-specific Factor VIII related antigen (vWF) specific antibody (red) 6 h after infection. Scale bar, 25 µm.

Fig 4. (a) Viral infectious progeny production of EV-68 and EV-94 in human pancreatic islets. The islets were infected with an apparently high m.o.i and the samples were collected immediately (0 h),

24 hours (24 h) and 48 hours (48 h) after infection. The total infectivity of each sample was determined by end point titration in microwell cultures of RD cells.

(b) The viabilities of pancreatic islets 7 days after infection with EV-68 or EV-94. The viabilities were assessed using Live/Dead assay (Molecular Probes). Because of their esterase activity, live cells are stained green by calcein, while nuclei of dead cells are stained red by ethidium homodimer-1.

(c) A co-staining of enterovirus-specific polyclonal rabbit antiserum (green) and insulin-specific polyclonal sheep antiserum (red) (Binding Site) 24 hours after infection with EV-94. The yellow colour indicates infected β -cells. Scale bar, 25 μ m.

(d) Insulin to DNA ratios of islet preparations 8–14 days after infection with EV-68 and EV-94. DNA was measured using a Fluoreportex Blue Fluorometric dsDNA quantitation kit (Molecular probes), and insulin was measured with a solid-phase insulin RIA kit (DPC).

TABLES

Table 1. The geometric means of the end-point titres for neutralizing antibodies against EV-68, EV-70 and EV-94 in the sera of Finnish women at the end of the first trimester of pregnancy.

Table 2. Cytokines showing more than a fivefold increase in the endothelial cell culture supernatants after infection with EV-94.