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

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Abstract

Enterovirus (EV) infections have been associated with the pathogenesis of type 1 diabetes (T1D). They may cause β-cell destruction either by cytolytic infection of the cells or indirectly by triggering the autoimmune response. Evidence for EV involvement have been presented in several studies, EV-IgM antibodies have been reported in T1D patients, EV-RNA has been found in the blood from T1D patients at onset, and EV have been isolated from newly diagnosed T1D. Our aim was to study infections with EV isolates from newly diagnosed T1D patients in human pancreatic islets in vitro. Two of them (T1 and T2) originated from a mother and her son diagnosed with T1D on the same day, the other two (A and E) were isolated from a pair of twins at the time of diagnosis of T1D in one of them. Isolated human pancreatic islets were infected and viral replication, viability and degree of cytolysis as well as insulin release in response to high glucose were measured. All four EV isolates replicated in the islet cells and virus particles and virus-induced vesicles were seen in the cytoplasm of the β-cells. The isolates varied in their ability to induce cytolysis and to cause destruction of the islets and infection with two of the isolates (T1 and A) caused more pronounced destruction of the islets. Infection with the isolate from the healthy twin boy (E) was the least cytolytic. The ability to secrete insulin in response to high glucose was reduced in all infected islets as early as 3 days post infection, before any difference in viability was observed. To conclude, strains of EV isolated from T1D patients at clinical presentation of T1D revealed β-cell tropism, and clearly affected the function of the β-cell. In addition, the infection caused a clear increase in the number of dead cells.

Keywords: Enterovirus; β-cells; Type 1 diabetes

1. Introduction

Type 1 diabetes (T1D) is a chronic multi-factorial disease in which the insulin producing β-cells in the pancreas are selectively destroyed. The prevailing view has long been that T1D is an autoimmune disease with genetic susceptibility linked to HLA DR3, DR4 and to DQβ alleles (Gale et al., 2001). Due to lack of concordance in identical twins, an environmental trigger is also thought to be involved, which has been suggested to be a virus. Several lines of epidemiological evidence suggest that enterovirus (EV) infections might cause or trigger T1D (Yoon et al., 1979; King et al., 1983; Banatvala et al., 1985; Yoon, 1990; Frisk et al., 1992; Dahlquist et al., 1995; Chehadeh et al., 2000; Helfand et al., 1995; Hyoty et al., 1995; Hiltunen et al., 1997; Frisk and Diderholm, 1997; Yin et al., 2002a; Nairn et al., 1999; Lonnrot et al., 2000a; Roivainen et al., 1998; Salminen et al., 2003; Lonnrot et al., 2000b; Champsaur et al., 1982). The basic evidence for the involvement of EV in the aetiology of T1D has been presented in a number of studies (King et al., 1983; Hyoty et al., 1995; Frisk and Diderholm, 1997; Yin et al., 2002a; Nairn et al., 1999; Lonnrot et al., 2000a,b). EV have also been isolated from newly diagnosed T1D patients in a few cases (Yoon et al., 1979; Vreugdenhil et al., 2000; Hindersson et al., 2005) and some of the isolated virus strains have been shown to cause diabetes in animal models (Yoon, 1995). There are instances where the diagnosis of T1D in one member of a family has been

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rapidly followed by its appearance in other members of the same family (Nelson et al., 1977; Phillips and Pauli, 1981). We have previously published a case report where EV was isolated from a mother and from her son diagnosed with T1D the same day (Hindersson et al., 2005). Such clustering of T1D cases in time within a family is a strong indication of an infectious agent as a trigger or cause of the disease.

EV are prime candidates for constituting an environmental risk factor because they induce strong immune responses and they can infect the β-cells leading to local inflammation (Ylipaasto et al., 2004). The genus EV, a member of the picornavirus family are divided into five major groups: Human enterovirus-A (HEV-A), HEV-B, HEV-C, HEV-D and Poliovirus (King, 2000), as many as 97 different EV serotypes are recognised and this number is still increasing. EV is transmitted by faecal, oral or by the respiratory route from one person to another. EV infections have been associated with a wide range of clinical manifestations including meningitis, encephalitis, paralysis, skin disease, common cold-like symptoms but mostly the infection is asymptomatic. EV infection usually starts from the respiratory tract or from the gastrointestinal mucosa, from where it might be spread to specific tissues and organs.

Upon infection, EV induces shut off of almost all host cell translation and induces a severe cytopathic effect (CPE) in infected cells. The shut off of host cell translation has been thought to result from the cleavage of the eukaryotic translation factor eIF4G (Ehrenfeld, 1982; Lamphear et al., 1995). The cleavage of eIF4G blocks the initiation of cap-dependent translation (Etchison et al., 1982). EV-infected cells reveal typical signs of CPE such as rounding up, accumulation of membranous vesicles (Dales et al., 1965) condensation of chromatin (Tolskaya et al., 1995; Romanova et al., 2005) and detachment from the basal surface of the culture dish or from other cells. Broadly speaking, there are two types of cell death; necrosis and apoptosis: It has been suggested that EV trigger the apoptotic pathway and that they before cell death has ensued terminated the process and initiate the alternative pathway (Agol et al., 2000).

It has been known for several years that the secretory pathway is disrupted in cells infected with picornaviruses (Barco and Carrasco, 1998), and this is characterized by the appearance of large number of membrane vesicles in the cytoplasm (Bienz et al., 1987; Rust et al., 2001; Suhy et al., 2000).

Different strains of EV have earlier been shown to infect isolated human pancreatic islets in vitro and such infections could either result in cell lysis or in a persistent infection depending on the viral strain (Chehadeh et al., 2000; Paananen et al., 2003; Frisk and Diderholm, 2000; Roivainen et al., 2000; Roivainen et al., 2002). However, the pathophysiological mechanism of virus induce T1D is still a matter of debate. Direct apoptosis/necrosis induced by the EV and indirect autoimmune responses induced by cytokines in the infected pancreatic islet cells remain attractive but unconfirmed hypothesis to explain the induction of T1D. Generally, viral proteins in infected cells interact with cellular proteins and interfere with cellular protein production to enable viral replication and propagation. Therefore, understanding the virus–host relationship is important if we are to understand the cellular responses against viral infections and the pathological mechanisms behind virus-induced T1D.

EV infections have for decades been associated with T1D, our successful isolation of four strains of EV from members of T1D families at clinical presentation of T1D together with the knowledge that such infections in non-β-cells interferes with the cell protein processing, made us design the following tasks (i) to study the ability of the isolated EV strains to induce cytolysis in isolated human islets; (ii) to study the ability of such an infection to affect insulin release in response to high glucose; (iii) to what extent these isolates would cause morphological changes of the islet and/or of the islet cells.

2. Material and methods

2.1. Patients

In an otherwise healthy family comprising mother, father and two sons, the mother and a 10-year-old son developed symptoms of increasing thirst and frequent urination. At a visit to the local physician, both were diagnosed with diabetes. Both were referred to the university hospital, T1D was immediately diagnosed in the son and he was put on insulin the same day. In the case of the mother it took some time before the type of diabetes was clarified, but it was clearly also diagnosed as T1D. The son had a short history of about a week with obvious symptoms of diabetes, and he had lost 5 kg in weight. He was in good general condition; his first HbA1c was 8.4% (ref. 3.6–5.0), and the first blood glucose 18.2 mmol/l and pH 7.40. The mother had always been susceptible to infections and she was diagnosed with an acute myocarditis some years prior to the onset of T1D. Her first blood glucose was 23 mmol/l, HbA1c 9.2% and ICA 1/160.

A twin boy, Adrian, was referred to the university hospital at the age of 5 years and 2 months with 3 weeks of increased drinking and micturation. He also had secondary enuresis and he had lost about 1 kg weight. His HbA1c was 7.2%, the first blood glucose 23 mmol/l, and there was no ketoacidosis present. His identical twin brother, Eric, is still healthy 3 years later.

2.2. GAD65 antibodies

Antibodies against glutamic acid decarboxylase 65 (GAD65) were measured with Diamyd's Anti-GAD65 RIA (Mercodia AB, Uppsala, Sweden). Using a cut-off of 9.5 U/l the specificity was 99% and the sensitivity was 74%. Antibodies against GAD65 were also measured with an Anti-GAD65-ELISA (developed by Mercodia AB, Uppsala, Sweden) and this assay allowed detection of IgM antibodies as well as IgG antibodies against this autoantigen.

2.3. Viral isolation

Stool samples were obtained from the mother and the son 10 days after the diagnosis of T1D. Virus was also isolated from stool samples from the identical twins Erik (E) and Adrian (A), at the time of diagnosis of T1D in one of the twins (A). The faeces were re-suspended with glass beads in a phosphate buffer,

after removal of the beads the clarified suspensions were inoculated on Green Monkey Kidney (GMK), Rhabdo myosarcoma (RD) and Henrietta Lacks (HeLa) cells. The inoculated cells were examined every day for the appearance of CPE. The isolation and characterization of the T1 and T2 strains has been described earlier (Hindersson et al., 2005). Both isolates from the twin boys revealed typical EV CPE on GMK and HeLa cells five (A) and 10 (E) days post inoculation. None of the isolates caused CPE on RD cells. In addition, the isolates from the twin boys were further serotyped by the standard method of virus neutralization with antisera pools. Wells containing polyclone antibodies against Echovirus 21did not reveal CPE suggesting that these two isolates were Echovirus 21.

2.4. Cell culture

The pancreata were obtained from normoglycemic donors after appropriate consent for multiorgan donation. The isolation of the islets has been described earlier (Goto et al., 2004). Islets used in this study originated from 14 donors, 8 males with a mean age of 56.8 years (range 39–66 year) and the mean ischemia time was 10 h (range 5–21 h). Islets were cultured in CMRL-1066 (Mediatech, Cellgro, Herndon, US), supplemented with 10 mM nicotinamide (Sigma–Aldrich, St. Louis, US), 10 mM Hepes buffer (Gibco-BRL, Invitrogen, Paisley, Scotland, UK), 0.25 μg/ml fungizone (Gibco-BRL, Invitrogen), 50 μg/ml gentamicin (Gibco-BRL, Invitrogen), 2 mM L-glutamine (Gibco-BRL, Invitrogen), 10 μg/ml ciprofloxacin (Bayer Healthcare AG, Leverkusen, Germany), 10% heat-inactivated human serum and kept at 37 °C, for 5–8 days before infection with virus. The medium was changes every other day during this period. GMK cells: were used for $TCID_{50}$ titration's and they were cultured in 96-well plates in EMEM (SVA, Uppsala, Sweden) supplemented with 2% newborn bovine serum (Seromed, Biochrom, Berlin, Germany). RD cells, HeLa and GMK cells for isolation of virus were culture in 12-well plates in EMEM supplemented with 2% newborn bovine serum.

2.5. Quality test

The purity of the islets was determined by microscope characterization after staining with diphenylthiocarbazone. Evaluation of the islet viability was carried out by using a dynamic perfusion system. Islets were challenged with two glucose concentrations (1.67 mmol/l and 16.7 mmol/l and then 1.67 mmol/l again). Fractions were collected at 6-min interval for 120 min and the insulin content was determined by ELISA (Mercodia, Uppsala, Sweden).

2.6. RNA isolation and EV-RT-PCR

RNA was isolated from the four isolates by the use of RNeasy Mini Kit (Qiagen Gmbh, Germany). The extracted RNA was stored at −70 °C. EV-RT-PCR was then performed as described earlier (Yin et al., 2002a). Briefly, the RNA was reverse transcribed using an anti sense primer ECBV5 5′-GATGGCCAATCCAATAGCT-3′ A semi-nested Polymerase Chain Reaction (PCR) was then performed (Verheyden et al., 2003). EV-RT-PCR was also performed with the Enterovision kit (DNA Technology A/S Science Park Aarhus, Aarhus C, Denmark).

2.7. Immunostaining for EV

HeLa cells cultured on culture slides (BectonDickinson labware, France) uninfected and infected with the four isolates and with the CBV-4 strain E2 (positive control), were fixed in acetone at 4 °C. Monoclonal antibodies (MAbs), raised in mice, directed against a broad reacting epitope on the EV capsid (DakoA/S, Glostrup, Denmark) were added to the slides. The antibody reacts with the structural protein VP1. After washing of the slides, binding of primary antibodies was visualized by the addition of PicTure-Plus Kit (Zymed Laboratories Inc., San Francisco, CA, US) containing a polymer conjugate of horseradish peroxidase and Fab fragments. Potential background due to endogenous biotin activator Fc receptors is by such means completely avoided. Human islets were infected with the isolates and on day 3 and day 6 post infection they were handpicked, washed in phosphate-buffered saline (PBS) and fixed in 4% buffered PFA, dehydrated in graded ethanol and embedded in paraffin. Sections of infected and uninfected islets were deparaffinized in xylene, rehydrated in graded ethanol, followed by washing with PBS for 10 min. The EV specific monoclonal antibody was then added to the slides. After washing, binding of the primary antibody was visualized by the addition of polymer conjugate of horseradish peroxidase and Fab fragments, PicTure-Plus Kit (Zymed Laboratories Inc., San Francisco, CA, US).

2.8. Infection of isolated islets

Handpicked human islets were cultured (300–325 islets/well) in 8 well plates, in 3 ml RPMI 5.5 mM glucose (SVA, Uppsala, Sweden) supplemented with 10% heat inactivated human serum tested for presence of neutralizing antibodies against the isolates. Two islet containing wells/isolate were infected with 10^3 $TCID_{50}$ and two wells containing islets from the same donor were not infected and used as a control.

2.9. Virus replication and islet degeneration

The replication of the four isolated virus strains in isolated human pancreatic islets was studied for a period of 7 days. After allowing the virus to attach for 30–60 min at 37 °C, aliquots of the culture media were taken from each well and this was repeated every day for 7 days. The collected samples were frozen for subsequent $TCID_{50}$ titration on GMK cells. The culture medium was changed day 3 and day 4 (Table 1). Degree of degeneration and degree of virus-induced CPE were studied every day as described before (Frisk and Diderholm, 2000). Briefly, the degree of CPE/islet degenerations were ranked from 0 to 4 and 0 were no CPE/islet destruction and 4 were almost total destruction of the islets. Islet degeneration is characterized by the loss of islet integrity, disintegration, and partial dispersion of islets.

Table 1
Glucose stimulation in the human pancreatic islets infected with the four isolated viruses strains as well as with the uninfected controls

Well	Culture conditions (mM glucose)		
	Day 0–3	Day 3–4[a]	Day 4–7
A-1[b], E-1, T1-1 and T2-1	5.5	5.5	5.5
A-2[b], E-2, T1-2 and T2-2	5.5	16.5	5.5
Control-1	5.5	5.5	5.5
Control-2	5.5	16.5	5.5

[a] Culture medium was changed in all wells day 3 to RPMI containing either 5.5 mM or 16.5 mM glucose. Day 4 post infection all wells were changed back to RPMI containing 5.5 mM.
[b] Well number.

2.10. Islet viability

Viability of the islet cells was determined in a double-blind manner by the use of a light microscope. All cells (islets and detached cells) from all wells were centrifuged to remove the culture medium, the islet cells were trypsinized and analyzed by trypan blue staining for dead cells day 3 and day 7 post infection.

2.11. Apoptosis

Day 3 and day 6 post infection infected and un-infected islets were handpicked, washed in PBS and fixed in 4% buffered para formaldehyde (PFA), dehydrated in graded ethanol and embedded in paraffin. The 5 μm sections were deparafinized in xylene, rehydrated in graded ethanol, followed by 5 min wash in PBS with 0.05% Tween. The ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon® International, Temecula, CA, US) based on the TUNEL assay was used according to the manufacturer's instruction. Light counterstaining was carried out with hematoxylin.

2.12. Glucose stimulation

Glucose stimulation tests were performed on infected islets and uninfected controls as clarified in Table 1. Aliquots of the culture medium from all wells were withdrawn after the change of culture medium day 3 and before the change of culture medium day 4 for subsequent measurements of insulin. The samples were stored at −20 °C until analysed. A high range rat insulin ELISA (Mercodia AB) was used for the measurements of insulin to avoid dilution of the samples. The insulin release in response to high glucose was calculated per islet.

2.13. DNA content

Islets were handpicked day 6 post infection, washed in PBS and transferred into PBS containing 5 mM EDTA, 10 mM benzamide, 0.1 mg/ml of soybean trypsin inhibitor, and 1 mM PMSF and stored in −70 °C. All samples were homogenized ultrasonically and analyzed for DNA with the Pico Green® dsDNA Quantitation kit (P-7589, Molecular Probes, Europe BV, The Netherlands).

2.14. Electron microscopy

Virus-induced morphological changes were studied day 3 and day 7 post infection. Infected and uninfected islets were fixed in 2% glutaraldehyde and 1% formaldehyde, followed by 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in TAAB-812-resin. Ultrathin sections (500 Å) were counterstained with uranyl acetate and lead citrate before examination under the electron microscope.

2.15. Statistical analyses

Data presented are means ± S.E.M. All results were based on observations from at least three donors. A p-value of <0.05 was considered significant. Rates of insulin release (mU/islet/24 h) from infected and control islets, including calculated differences, have been computed using Wilcoxon signed ranks test and Mann–Whitney test. Differences in the degree of cytolysis/islet destruction were tested with the independent samples t-test for comparing paired samples. The comparisons were between islets infected with one isolate and islets infected with another or left uninfected for each time point.

3. Results

Two different GAD65 antibody assays were used and the Radioimmunoassay (RIA) results (detecting only IgG antibodies) obtained with the acute and the convalescent serum samples from the mother and the son revealed that the former had high levels of such antibodies (>198 IU) whereas no such antibodies was detected in the son's serum samples. When the same samples were analysed from the twin boys, the acute sample from both were totally negative while the antibody levels in the convalescent sample was somewhat increased in the diabetic twin (11 IU). When the ELISA (detecting both IgM and IgG antibodies) was used the GAD65 antibody levels in the acute serum from the T1D boy (T2) was 199 and in the convalescent serum

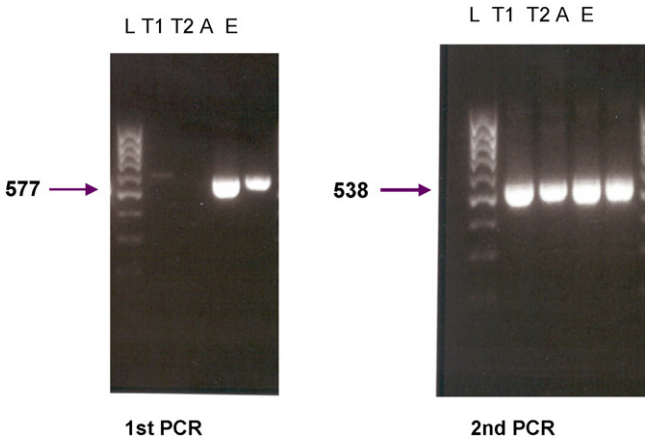

Fig. 1. Electrophoresis analysis of the PCR products from human pancreatic islets infected with the isolates. First PCR; lane 1 show bands between 100 and 1500 bp. Lanes 2–5 show the PCR product from human pancreatic islets infected with T1, T2, A, and E. Second PCR; line 1 shows the DNA ladder. Lanes 2–5 show the T1, T2, A, and E amplicons.

31.5. Analyses of the acute serum from the twin boys revealed 400 U/ml (A) and 43 U/l (E).

All four isolates were positive when the Enterovision-RT-PCR-kit was used indicating that they all belonged to the genera EV. To confirm these results a semi-nested EV-RT-PCR was performed using EV specific primers binding to the 5′NCR. Amplicons of the expected size (538 bp) could be seen on the agarose gel (Fig. 1) after the second PCR.

Immunostaining of isolated human islets infected with the four isolates (A, E, T1 and T2) with an EV specific antibody confirmed the results obtained above, i.e. that all four isolates belonged to the genus EV since a positive staining was only obtained in infected islet cells (Fig. 2). In addition, it indicates that these isolates could replicate in the islet cells. Islets infected with the A isolate can be seen in Fig. 2B. In addition, all four isolates (A, E, T1 and T2) revealed positive immunostaining of infected HeLa cells when using an EV specific antibody (Fig. 2C and D) compared to uninfected control HeLa cells (Fig. 2E).

Virus replication measurements were assessed every day during culture. The $TCID_{50}$ titers increased day 3 to day 7 post infection. Day 3 post infection changes of culture medium were performed, because of the glucose stimulation tests, causing a reduction of the virus titre. The increases in titre did not differ between the isolates day 7 post infection. The mean titre increases was $10^{0.6}$ (range $10^{0.2}$–$10^{1.25}$).

All four isolated EV strains were able to induce CPE and/or degradation of the islets. However, the degree of islet degradation varied with the isolate (Table 2 and Fig. 3). All infected islets were significantly more degraded than the uninfected controls day 7 post infection ($p < 0.001$). Islets infected with the A isolate (TID twin) differed from islets infected with the E (healthy twin) isolate day 3 post infection, $p < 0.087$ and day 7 post infection

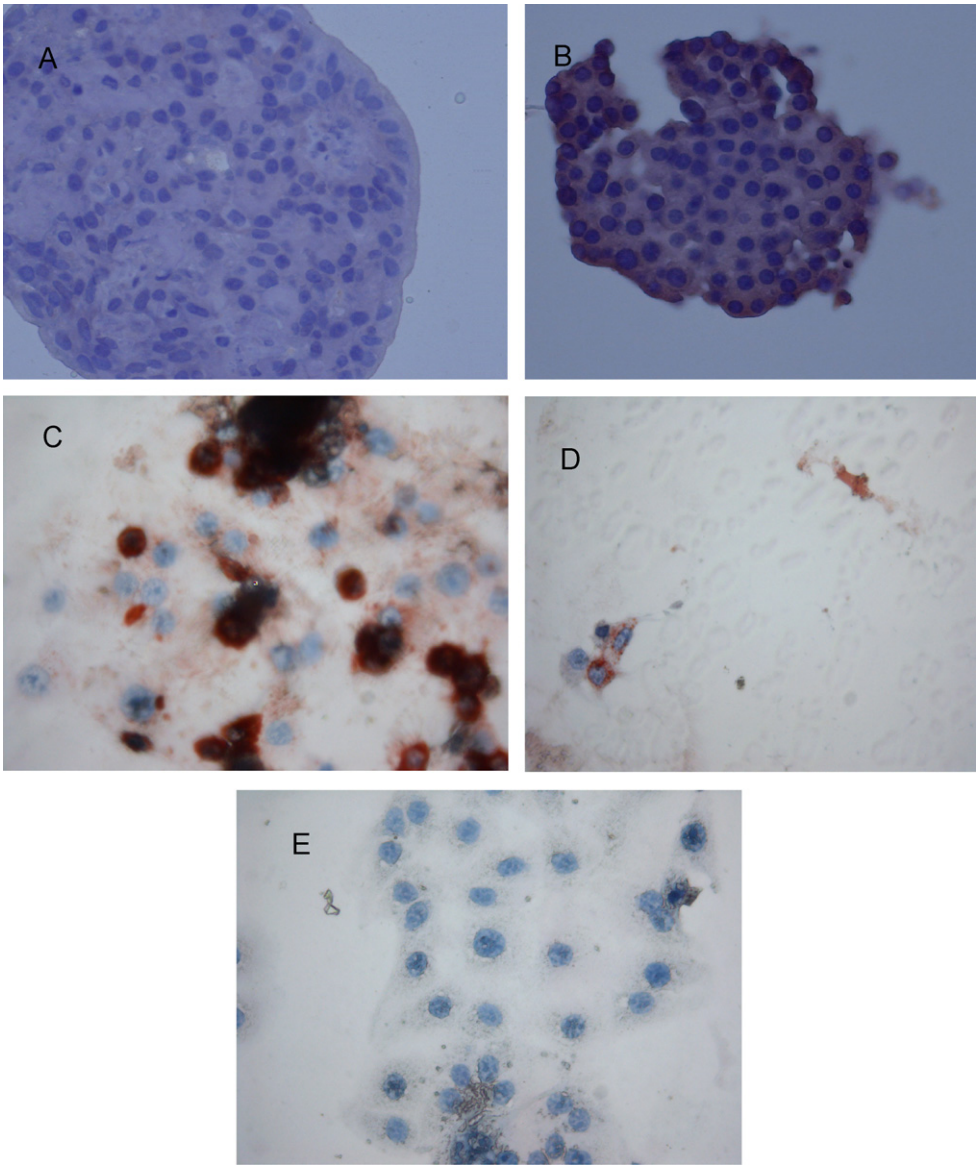

Fig. 2. Immunostaining for EV in isolated human islets and HeLa cells. (A) Uninfected control islets; (B) islets infected with the Adrian (A) isolate 3 days post infection (magnifications 60×). (C) HeLa cells infected with the Adrian (A) isolate 7 days post infection. (D) HeLa cells infected with the Erik (E) isolate 7 days post infection. (E) Uninfected HeLa cells (magnifications 40×).

Table 2
Degree of CPE/islet destruction in isolated human islets infected with Adrian (A), Erik (E), Tuvemo 1 (T1) and Tuvemo 2 (T2) compared with the degree of CPE/islet destruction in control islets

Virus	*n*	Day 3 (mean ± S.D.)	Day 7 (mean ± S.D.)
A-1[a]	10	1.7 ± 1.25	2.6 ± 1.71
A-2[b]	10	1.7 ± 1.25	2.7 ± 1.70
E-1	10	1.1 ± 0.92	1.4 ± 0.97
E-2	10	1.1 ± 0.99	1.7 ± 1.06
T1-1	10	1.6 ± 1.34	2.3 ± 1.58
T1-2	10	1.5 ± 1.43	2.3 ± 1.64
T2-1	10	0.9 ± 1.10	1.3 ± 1.43
T2-2	10	0.8 ± 1.14	1.2 ± 1.48
Control-1	10	0 ± 0.00	0 ± 0.00
Control-2	10	0 ± 0.00	0 ± 0.00

[a] Cultured in 5.5 mM glucose during the whole culture period.
[b] Cultured in 16.5 mM glucose day 3–4 post infection.

$p < 0.016$. Islets infected with the A isolate differed from islets infected with the T2 isolate day 3 post infection $p < 0.025$ and day 7 post infection $p < 0.009$. There was no difference in that respect between islets infected with the A isolate and islets infected with the T1 isolate, indicating that these two strains caused most islet destruction. There was no difference in degree of islet destruction between islets infected with the E or the T1 isolate day 3 post infection, day 7 post infection islets infected with the T1 isolate differed from islets infected with the E ($p < 0.063$). No difference was seen between islets infected with the E or the T2 isolate at any time point post infection. The effect of infection with the T1 isolate on the appearance of the islets differed from that of islets infected with the T2 isolate. Paired samples statistics also revealed that the most destructive isolate day 7 post infection was obtained from the T1D twin, Adrian, $p < 0.046$. Variations with regard to CPE, islet degradation and viral replication were also dependent on the islet donor.

The frequencies of dead cells, both in islet and detached from the islets, in wells infected with any of the four isolates did not differ from that of the control day 3 post infections. At day 7 post infection the percentage of dead cells in wells infected with the A isolate was 39% ($n = 8$), with the E isolate 29% ($n = 8$), with the T1 isolate 39% ($n = 9$), with the T2 isolate 38% ($n = 8$) and in the uninfected controls 17% ($n = 9$). This shows that islet cultures infected with any of the four isolates contained more dead cells than the control islets day 7 post infection ($p < 0.05$). Islets infected with the isolate from the healthy twin boy contained less dead cells than islets cultures infected with any of the other three isolates.

Islets infected with the different strains were stained for apoptosis but there were no clear differences between islets infected with the different isolates or between infected and uninfected islets using this method. A few apoptotic cells could be detected in almost all islets examined.

Electron microscopy of infected and uninfected islets 3 days post infection revealed no major differences in morphology between infected and uninfected islets. In Fig. 4A, cells from an uninfected control islet can be seen, in the centre of the photograph a β-cell can be seen characterized by the insulin containing

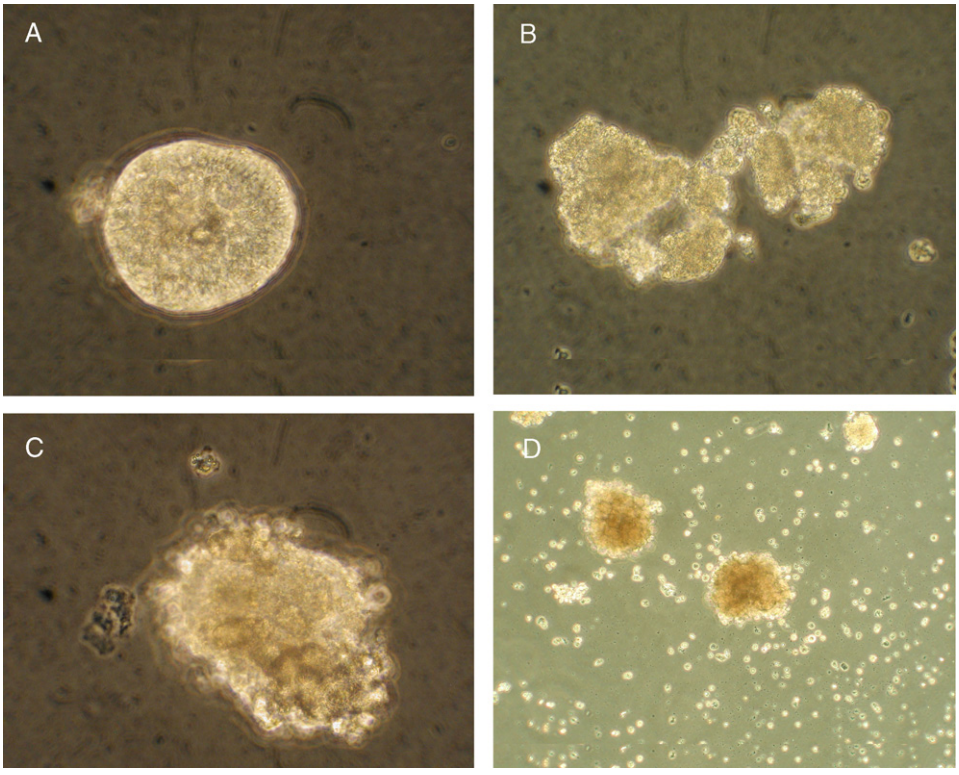

Fig. 3. Degree of islet degradation in human pancreatic islets infected with the isolates A or E, and in uninfected controls. (A) Uninfected human islet displayed no degradation of the islets. (B) Islets infected with the E isolate 3 days post infection. (C) Islets infected with the A isolate 3 days post infection. (D) Islets infected with the A isolate 6 days post infection (magnification ×40).

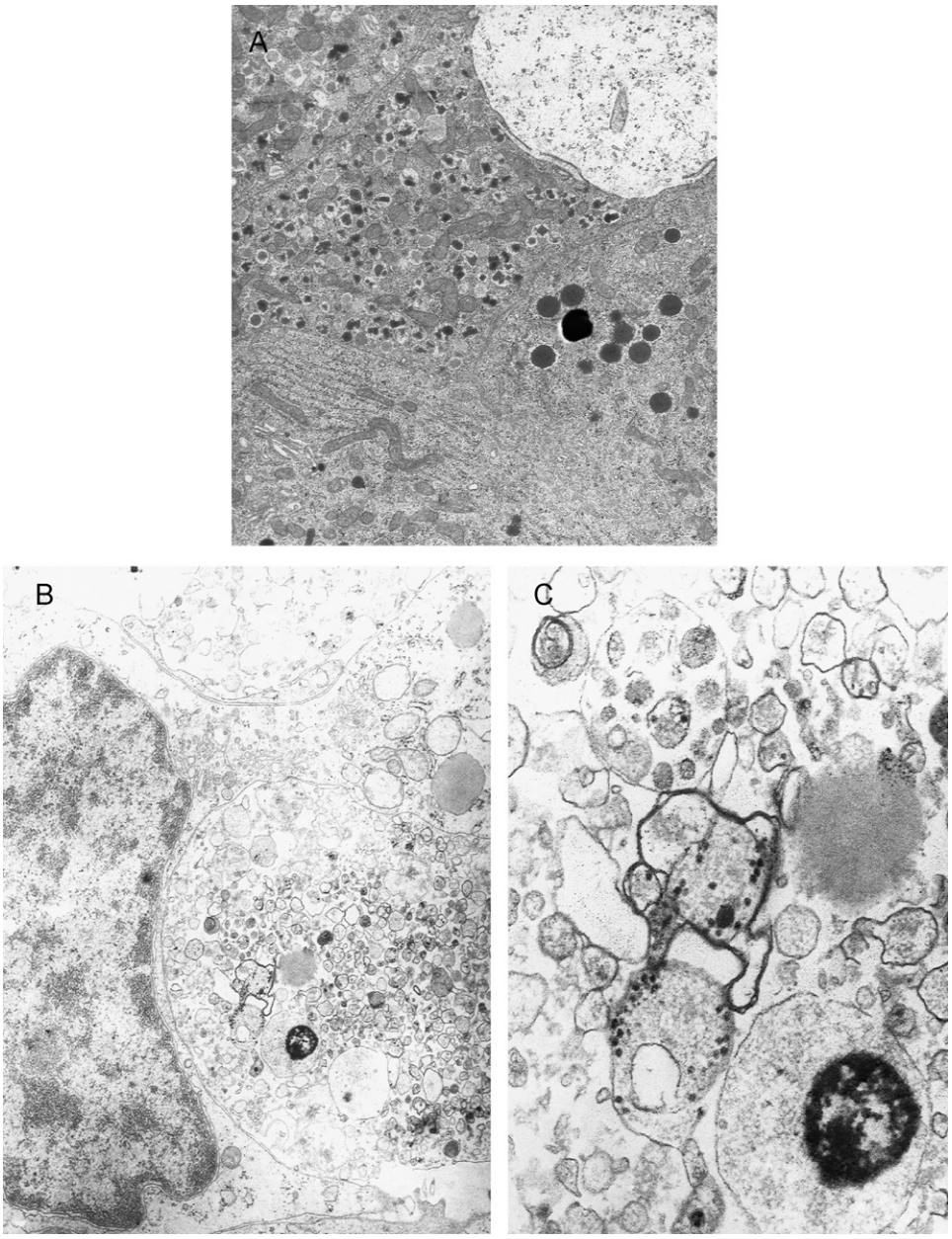

Fig. 4. Ultra structural changes and progeny virus production in infected human β-cells. Uninfected human islets cultured for 6 days (A) magnification 20,000×. In islets infected with the A isolate the cells revealed indications of apoptosis as well as necrosis such as perinuclear chromatin condensation. In β-cells from such islets virus-induced reorganization of intracellular membranes and vesicle formation could be seen (B) magnification 20,000×. Progeny virus (size ≈30 nm) can be seen in the virus replication complexes (C) magnification 80,000×.

granules in the cytoplasm. To the right parts of a α-cell can be seen.

A hallmark for EV infection is the reorganization of ER and other intracellular membranes to generate clusters of vesicles that serve as the sites for RNA replication. Such replication complexes could be seen in islet cells infected with three of the isolates (A, E and T1) day 6 post infection. In addition, the nuclei of islet cells infected with these isolates showed chromatin condensation, necrotic and/or apoptotic cells were also seen. In Fig. 4B and C replication complexes induced by the infection with the A strain can be seen, in addition in higher magnification viral particles of about 30 nm is visible inside some of the vesicles (Fig. 4B). None of this could be seen in uninfected islet cells (Fig. 4A).

Together, the EV specific staining of the infected human islets, the increased viral titres and the presence of virus particles in the β-cells, clearly shows that these EV isolates reveal tropism for β-cells in vitro.

The quality of the islets was excellent, the islet isolation resulted in a purity of 50–80%, and from cultures with this purity the islets used in this study were handpicked. We evaluated islet function using a dynamic perfusion system in which

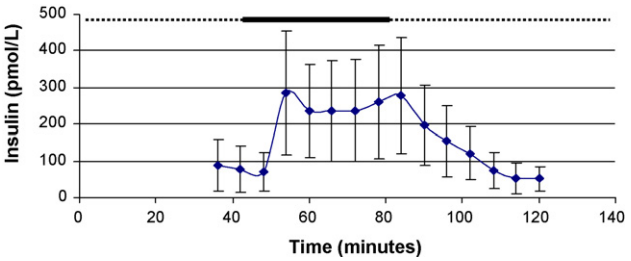

Fig. 5. Dynamic insulin release. Isolated islets ($n = 20$) were perfused with 1.67 (dotted line) and 16.7 mM (bold line) glucose as indicated in the figure. Data are presented as means ± S.E.M ($n = 4$).

Table 3
Increased insulin secretion into the culture media in response to high glucose (6.5 mM) during 24 h

Samples	n	Mean value mU/l ± S.D.	Median/(range)
Adrian	7	1.8 ± 1.0	2.2/(0–2.9)
Eric	7	1.9 ± 1.3	2.4/(0–3.6)
T1	7	2.8 ± 3.9	1.2/(0.3–11.1)
T2	6	1.3 ± 0.7	1.2/(0.4–2.5)
Control	7	4.4 ± 3.4	3.6/(0.8–10.3)

insulin secretion was assessed after stimulation with glucose. As can be seen in Fig. 5, the islet batches responded to the glucose challenge, although the variation between donors was high.

The ability to secrete insulin in response to glucose stimulation differed between islets infected with the A isolate and control islets day 3 post-infection ($p < 0.04$) (Wilcoxon signed tanks test). All infected islets differed from that of the uninfected control islets in that respect ($p < 0.03$) (independent samples t-test) day 3 post infection. As can be seen in Table 3, uninfected islets increased their insulin secretion 4.4 times when cultured in high glucose while the infected islets only could increase their secretion with 1.3–2.8 times. There were no differences in DNA content between infected islets and un-infected controls at this time point post infection indicating that all wells contained the same number of cells.

To summarize, all EV isolates could replicate in isolated human islets and there were no major effects on the islets appearance day 3 post infection compared to the controls. Despite that, β-cell function was hampered in all infected islets when compared to uninfected islets. Infection with virus isolated from the T1D boys affected the insulin release more than infection with any of the other isolates. Day 7 post infection all virus infected islets were more disrupted than the uninfected controls, and islets infected with the A isolate were most disrupted. At this time point post infection electron microscopy also revealed virus replication complexes in the cytoplasm of β-cells.

4. Discussion

We have isolated three EV strains from T1D patients and one relative at time of clinical presentation of the disease. It has been shown before that T1D can aggregate in families suggesting that genetic predispositions are risk factors for T1D. However, only 10% of children diagnosed with T1D have an affected family member and among first-degree relatives of individuals with T1D, the risk of developing the disease is 5–6%, thus the genetic susceptibility can only partly explain why an individual develops T1D. Such family clustering would also strongly indicate that an infection caused or triggered the disease. Simultaneous onset of T1D in two members of a family has been described earlier. In that report they describe the onset of T1D of a pair of twins within 14 days and in addition both of them had encountered an EV infection (Echovirus 6) (Smith et al., 1998). Our finding together with a few others shows that EV can be isolated at onset of T1D and this suggests a cause relationship between some infections with EV and T1D.

The absence of autoantibody against GAD65 of IgG class in both boys with T1D (A and T2) in the acute serum and a somewhat increased antibody level in the convalescent serum from the twin boy (A) seemed to indicate that there was no induction of autoimmunity by these infections. However, when a GAD65 assay detecting also IgM antibodies was used, both T1D boys had high levels of such antibodies already in the acute serum sample suggesting that the EV infection did cause release of islet-intracellular proteins resulting in an autoimmune reaction. However, it cannot be excluded that an existing autoimmune reaction facilitates the viral infection results in at the clinical presentation of the T1D.

We have studied the effect of infection with four EV strains isolated from T1D patients at clinical presentation of T1D on human islet cells in vitro for the first time. All isolates replicated in isolated human islets although a clear rise in virus titers was only detected 3–6 days post infection. A possible explanation for this late titer increase might be the change of culture medium day 3 and 4 post infection. However, exceptions to this late titer increase was seen in a few experiments and it has been shown earlier (Frisk and Diderholm, 2000; Yin et al., 2002b), that the titer rises obtained when human islets were infected with strains of CBV-4 were much higher. The reason for this discrepancy is not known, possible explanations might be that the CBV-4 strains have been passaged several times in cell cultures, whereas the isolates was passaged only twice or it could also depend on differences with regard to the donor. Another possible explanation could be that the islets used for infection with the CBV-4 strains were obtained from the Central Unit of Beta-Cell Transplant, Brussels, where islet isolation was carried out as described previously (Keymeulen et al., 1998), and the islets used in this study were obtained from the Transplant Unit at Uppsala University, Uppsala, Sweden. One known difference between islets from Brussel and islets from Uppsala is that the former were cultured for longer periods before infection.

Three of the isolates also induced reorganization of ER and other intracellular membranes to generate clusters of vesicles as sites for RNA replication. In some β-cells condensed chromatin was seen suggesting apoptotic cells. This was also seen in a few cells in the uninfected controls indicating that the apoptosis was not caused by the infection. The rearrangement of the intracellular membranes, a hallmark for EV infection, and the presence of virus-induced vesicles containing virus like particles of approximately 30 nm in size, clearly shows that the isolates could replicate in the insulin producing cells. The induction of

such vesicles has earlier been shown to affect the secretory pathway of infected islets (Lamphear et al., 1995). In a β-cell this could affect the insulin secretion.

In our study we could not detect any differences in the number of cells dying of apoptosis when we compared infected islets with uninfected, even though apoptotic cells were present in both, the inter-donor variation was larger. Cell damage and/or cell death due to EV infections might be an effect of the host's defence mechanisms. Both apoptotic and necrotic cell death have been described as a result of EV infections of human islet cells (Roivainen et al., 2000) and other human cells (Agol et al., 1998). The aggressive necrosis leads to a rapid lytic cell death while apoptosis is a controlled elimination of dying cells. During low dose infection of isolated human islets with EV the dynamics of both apoptosis and necrosis differs dramatically from that seen in systems with high dose infections (Rasilainen et al., 2004). This points toward a more prominent role for apoptosis as a contributor to β-cell death during a slowly progressing or a persistent infection (Yin et al., 2002b). However, even though our isolates seems to establish a more slowly progressing infection the number of apoptotic cells in infected islets did not differ compared to in uninfected control islets. It has been shown before that different EV differ in their ability to induce CPE and/or islet destruction in isolated human islets (Frisk and Diderholm, 2000; Yin et al., 2002b; Rasilainen et al., 2004) with the use of other strains of EV. The most interesting study in this respect is one by Paananen et al. (2003), when they compared the effect of infection with two prototype strains of echovirus 9 with one echovirus 9 strain that had been isolated from a child with T1D at onset. The latter induced a higher degree of cytolysis in islet cells suggesting that EV that cause/trigger T1D cause more destruction/cytolysis of the islet cells. In our study, the strain isolated from the diabetic twin (A) was clearly more destructive to human islets than the strain isolated from the non-diabetic twin (E). Many studies also show that even if some normally very lytic EV is able to infect isolated human islets the course of the infection is much slower in this system and the amount of dead cells is less suggesting that they in human pancreatic islets cause a slow progressing infection.

In agreement with the above described slow progression of the infection with these strains, are the results regarding the viability 3 days post infection were no differences between infected and uninfected islets was found. Thus, the majority of the islets cells were still alive both in infected and uninfected islets. However, 7 days post infection the number of dead cells were higher in islets infected with any of the isolates and these results are also in line with our results showing a higher degree of islet destruction at that time point post infection. Of note, the majority of islet cells in the infected islets were still alive even at this time point, clearly showing that the replication is much slower in these cells. In other cells, Hela and GMK, infections with all four strains caused rapid cell death (not shown) suggesting that these isolates only in the islet cell system have displayed a slower replication cycle. Our finding that these EV, having a well established cytolytic potential, cause a prolonged infection in human islet cells is supported by the findings in a recent publication (Bopegamage et al., 2005). They showed that also in vivo, in a mouse model, EV replicated in the islets without causing cell death. Induction of type 1 IFNs is not likely the cause of this slower replication cycle since it has been shown that IFN-α and IFN-β mRNA is not upregulated in human islets infected with other strains of EV (Olsson et al., 2005; Ylipaasto et al., 2005).

Earlier studies (Frisk and Diderholm, 2000) have shown that the ability to respond to high glucose by secreting insulin was only hampered in islets infected with a very lytic strain of CBV-4. In our study the islets infected with any of the isolates showed an impaired ability to secrete insulin response to high glucose. Islets infected with the A and the T2 isolate were most affected in this respect. The virus isolated from the two boys at clinical presentation of T1D revealed clear tropism for the insulin producing cells, both of them had IgM antibodies against GAD65; this might suggest that the virus infection triggered the autoimmunity.

5. Conclusion

All four isolates from the T1D families belonged to the genera EV (echovirus 21 and coxsakievirus B5) and all of them could infect isolated human islets. All four caused islet destruction and a higher rate of cell death than seen in the controls 7 days post infection. In addition, three of them also caused rearrangement of cellular membranes in the β-cells. The most destructive virus was the A isolate, and it originated from the twin boys with T1D, the least destruction was the isolate from his healthy brother. The A isolate, and to some extent the T2 isolate, could also as early as 3 days post infection affect the β-cells ability to release insulin in response to high glucose clearly indicating the diabetogenic property of these two isolates. Even though these isolates caused cell death, the course of the infection is best described as a slow progressing infection in human islet cells, supporting the idea of a long period of β-cell destruction before the onset of the disease.

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