IN VITRO INFECTION OF MOUSE PANCREATIC ISLET CELLS WITH COXSACKIE VIRUSES

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Summary. – We have demonstrated the ability of 4 standard coxsackie viruses (B4, B5, A7, and A9) and one fresh isolate (A7) from a newly diabetic child with homologous serological response, to infect *in vitro* grown mouse pancreatic islet cells. Up to the 9th day after infection the multiplication of viruses in the cells was proved using virus titration and immunofluorescence test. Isolated pancreatic cells proved to be a suitable model for detailed studies of experimental infection of pancreatic cells with coxsackie viruses.

Key words: coxsackie viruses; pancreatic islet cells; *in vitro* infection; insulin-dependent diabetes mellitus

Introduction

Insulin-dependent diabetes mellitus (IDDM) is considered to be a polyetiological disease with increasing prevalence in many European countries (Banatwala, 1987). Epidemiological and experimental evidence indicates that infection of humans and animals with several viruses is involved in the pathogenesis of pancreatic beta cell damage and even destruction. The event is in general regarded as the beginning or a triggering factor of the autoimmune process leading to the clinical onset of IDDM. This, most probably an organ-specific autoimmune disease is often diagnosed in the context of polyendocrine autoimmune process or in combination with other organ-specific disease (e.g. thyreoditis). The direct lysis of the beta cells owing to the productive viral infection is only in few cases the main pathogenetical mechanism (Numazaki *et al.*, 1989). The decisive process in the IDDM pathogenesis is considered to be mediated by a cellular immune reaction triggered by a number of environmental factors. Viral infection in a genetically susceptible individual is one of the most often recognized factors.

Viruses of different taxonomical groups are known to infect and replicate in the pancreatic islet cells *in vitro* (Jenson *et al.*, 1980; Jenson *et al.*, 1984; Numazaki *et al.*, 1989; Vuorinen *et al.*, 1992; Yoon *et al.*, 1978, 1981). The seasonal distribution of the onset of IDDM and seroepidemiological studies often implicate coxsackie B viruses in the etiology of IDDM (Banatwala *et al.*, 1985; Muir *et al.*, 1990). The possible role of coxsackie A and ECHO viruses in this process was suggested by Frisk *et al.* (1992).

Detailed studies of the cell infection require a well defined *in vitro* system displaying the highest possible degree of resemblance to the *in vivo* system used for similar studies. The aim of our present study was to compare the replication of coxsackie A7 virus recently isolated from the stool of a newly diagnosed diabetic child with standard coxsackie A and B viruses in mouse pancreatic cell clusters *in vitro*.

Materials and Methods

Cell culture. Green monkey kidney (GMK) cell line was maintained in MEM with 10% calf serum and antibiotics. It was used for passages and titration of the viruses, and for the control immunofluorescence tests.

Pancreatic islet cells were isolated from white albino 10 week-old male mice using our modification of the method described by Cambell *et al.* (1991). Pancreases from overnight fasted mice were removed into 10 mmol/l HEPES buffered Krebs-Ringer bicarbonate solution (HKRB) pH 7.2 with 1 mg/ml bovine serum albumin (BSA) and 1 mg/ml D-glucose. After cutting the pancreases into 8 pieces the suspension was shaken for 25 mins at 37 °C with 0.8 mg/ml colllagenase (SIGMA, type XI) or 0.05% trypsin. The tissues were washed twice in HKRB and slowly passed through 24 gauge needle, centrifuged (800 × g, 10 mins, 4 °C) and

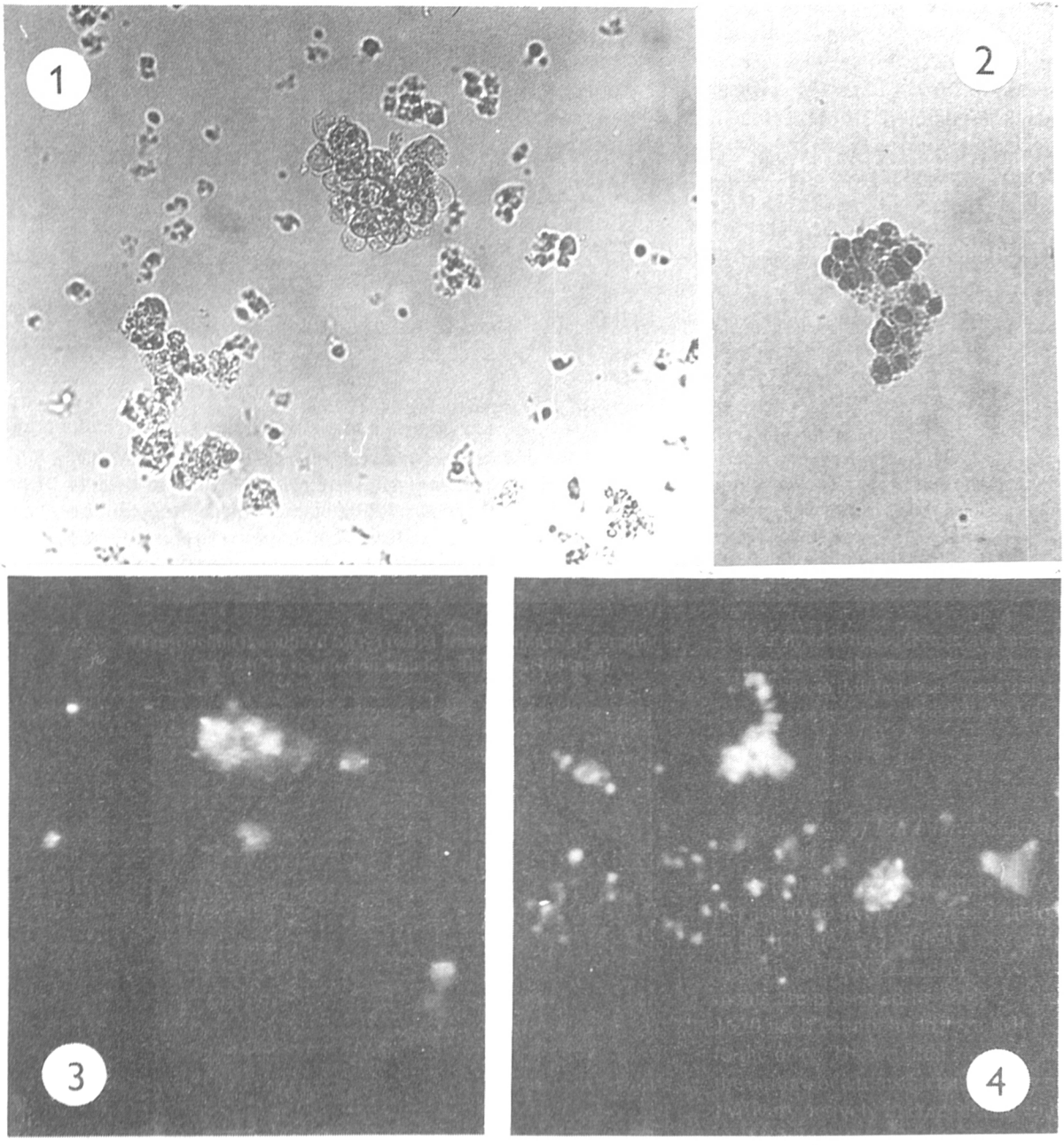

Fig. 1
Free floating islet cell clusters 1 day after isolation
Fig. 2
Fixed cell clusters stained with haematoxylin-eosin
Fig. 3
Infected pancreatic cells 2 days p.i. showing partially fluorescing cells
Fig. 4
Infected pancreatic cells 5 days p.i. showing fluorescing cells

the pellet was resuspended in HKRB. The isolated pancreatic cells were centrifuged through Ficoll 400 (SIGMA) gradient with densities 1.085, 1.075, 1.065 and 1.055 g/ml. The cell clusters in final concentration of about 160 clusters per 1 ml were plated out in Limbro 24 well-plates (Alpha Laboratories), 96 well-microtiter flat-bottom plates (Greiner), plastic Petri dishes (60 mm diameter, Gama, s.p.) and glass Miller tissue culture bottles (25 cm^2). Cells were cultivated in Leibovitz 15 (SERVA) or RPMI (SIGMA) medium, both enriched with 10% foetal calf serum, 10 mmol/l HEPES, antibiotics and 5×10^{-5} mol/l mercaptoethanol. Slides

were prepared from floating cells for haematoxylin-eosin staining and for staining by the Milenkovo-Ivičič method (Čunderlíková and Balážová, 1990).

Viruses. The standard coxsackie virus strains B4, B5, A7, A9, and the virus recently isolated from the stool of a newly diagnosed diabetic child and identified as coxsackievirus A7, were used.

Cell culture experiments. Limbro plates and Petri dishes were infected with coxsackie viruses A7 and A9 (2×10^9 $TCID_{50}$ or 2×10^7 $TCID_{50}$ per plate), B5 (2×10^{10} $TCID_{50}$ or 2×10^7 $TCID_{50}$ per plate) and B4 (2×10^4 $TCID_{50}$ or 2×10^2 $TCID_{50}$ per plate). The reason of such low infection doses of B4 virus was our inability to grow this virus to titers above $10^4 - 10^5$ $TCID_{50}$/ml. Samples for virus titration were collected at days 1, 2, 3, 4, 5, 6, 7 and 9 p.i. freeze-thawed 3 times and after clarification (centrifugation 3,000 × g, 30 mins) the supernatants were titrated. The thermal stability of the virus was controlled by incubating an identical dose of given virus in medium without cells. Samples were taken at the same intervals and titrated.

For immunofluorescence tests the floating infected and control (uninfected) cell clusters were pippetted out on days 1, 2, 4, 6 and 7 p.i., washed 3 times in PBS. Smears were made, dried and fixed on slides with cold acetone for 10 mins. The fixed cells were incubated at 37 °C for 30 mins with rabbit antiviral antibodies at 1:20 and 1:200 dilutions. After washing in PBS the slides were incubated with swine antirabbit conjugate (FITC, SEVAC Prague), washed again and observed under the OPTON Axiophot Photomicroscope.

Virus titration. All virus samples were titrated in 96 well-microtiter plate cultures of GMK cells using 100 µl of a tenfold dilution per well. CPE was read on day 5 p.i. The titers ($TCID_{50}$ per 0.1 ml) were evaluated by the standard method.

Results

After Ficoll separation the mouse pancreatic islet cells floated at the density interphase 1.055, 1.065 g/ml. Trypsinization completely separated the clusters into individual cells, whereas collagenase treatment did not. Hence we preferred the latter procedure. After seeding on the plastic or glass tissue culture vessels, the clusters became attached or remained floating. The comparison of growth in the two culture media (L_{15} and RPMI 1640) showed good adaptation of cells to L_{15} without enrichment with amino

Table 1. Growth of coxsackie B4 virus in pancreatic cell clusters

Virus inoculum	Virus titre (log $TCID_{50}$ per 0.1 ml) on day p.i.									
	0	1	2	3	4	5	6	7	8	9
2×10^4 $TCID_{50}$	4.2	2.5	1.2	1.5	1.8	3.2	ND	0	ND	0
Control[a]	4.0	3.1	1.4	1.4	0	0	ND	0	ND	0
2×10^2 $TCID_{50}$	2.0	2.0	1.2	1.4	0	2.5	ND	2.1	ND	2.2
Control[a]	2.1	1.2	1.2	1.2	0	0	ND	0	ND	0

[a]Virus inoculum incubated in the medium only under the same experimental conditions.
ND – not done.

Table 2. Growth of coxsackie B5 virus in pancreatic cell clusters

Virus inoculum	Virus titre (log $TCID_{50}$ per 0.1 ml) on day p.i.									
	0	1	2	3	4	5	6	7	8	9
2×10^{10} $TCID_{50}$	10.1	1.5	1.2	1.5	2.4	3.4	ND	0	ND	0
Control[a]	10.1	1.3	1.3	1.3	0	0	ND	0	ND	0
2×10^7 $TCID_{50}$	7.2	1.5	1.2	1.2	0	1.5	ND	2.4	ND	2.4
Control[a]	7.2	1.0	0	0	0	0	ND	0	ND	0

[a]Virus inoculum incubated in the medium only under the same experimental conditions.
ND – not done.

Table 3. Growth of coxsackie A9 virus in pancreatic cell clusters

Virus inoculum	Virus titre (log $TCID_{50}$ per 0.1 ml) on day p.i.									
	0	1	2	3	4	5	6	7	8	9
2×10^9 $TCID_{50}$	9.2	8.3	4.4	3.2	3.0	2.6	ND	0	ND	0
Control[a]	9.2	8.4	3.2	3.2	1.2	0	ND	0	ND	0
2×10^7 $TCID_{50}$	7.2	4.5	2.2	2.6	1.1	2.5	ND	3.2	ND	3.2
Control[a]	7.2	4.9	2.2	1.1	1.1	0	ND	0	ND	0

[a]Virus inoculum incubated in the medium only under the same experimental conditions.
ND – not done.

Table 4. Growth of coxsackie A7 virus in pancreatic cell clusters

Virus inoculum	Virus titre (log $TCID_{50}$ per 0.1 ml) on day p.i.									
	0	1	2	3	4	5	6	7	8	9
2×10^9 $TCID_{50}$	9.1	4.4	2.3	2.6	3.2	2.6	ND	0	ND	0
Control[a]	9.1	4.2	2.2	1.2	0	0	ND	0	ND	0
2×10^7 $TCID_{50}$	7.1	4.4	2.0	1.2	0	0	ND	3.5	ND	1.4
Control[a]	7.1	4.0	1.2	0	0	0	ND	0	ND	0

[a]Virus inoculum incubated in the medium only under the same experimental conditions.
ND – not done.

Table 5. Growth of coxsackie A7 virus (stool isolate) in pancreatic cell clusters

Virus inoculum	Virus titre (log $TCID_{50}$ per 0.1 ml) on day p.i.									
	0	1	2	3	4	5	6	7	8	9
2×10^9 $TCID_{50}$	9.2	8.4	3.2	4.6	3.6	1.5	ND	0	ND	0
Control[a]	9.1	8.4	3.4	3.2	1.2	0	ND	0	ND	0
2×10^7 $TCID_{50}$	7.2	5.5	1.2	1.2	0	1.5	ND	4.5	ND	1.4
Control[a]	7.1	6.2	1.2	1.2	0	0	ND	0	ND	0

[a]Virus inoculum incubated in the medium only under the same experimental conditions.
ND – not done.

acids and vitamins (data not shown). Fig. 1 and Fig. 2 show floating and fixed cell clusters.

A significant fall of the virus titer at 37 °C in the controls probably due to thermal inactivation was observed on day 2 and 4, respectively, depending on the introduced virus amount (Table 1-5). The highest inactivation was recorded in case of coxsackie B5. In cells infected with high dose of the given virus, the titer of virus reached maximum on day 5 and 6 p.i. When a low dose of virus was used, the highest titer was reached on day 7 – 9 p.i. The main morphological change in the clusters observed after infection was the loss of the typical margin and later on the destruction of cells.

Immunofluorescence assays with antisera untreated with the mouse pancreatic cells showed significant nonspecific positivity in controls, which was totally overcome by pretreating the diluted sera with the pancreatic cells overnight at 4 °C. The presence of infectious virus in the pancreatic cells was hence invariably proved. The serum dilution 1:20 gave better results than the 1:200.

Low infecting doses 2×10^2 $TCID_{50}$ for B4, and 2×10^7 $TCID_{50}$ for the other viruses used showed capability of infecting pancreatic cells gradually, as concluded from the number of intensively and partially fluorescing cells observed in single clusters. As it is seen in Fig. 3, partial fluorescence was observed on days 1 and 2 p.i. in clusters infected with low doses of all the viruses tested. Infection with standard coxsackie B4 showed bright fluorescence on day 3 p.i. whereas with standard strains of coxsackie B5, A7, A9 and with freshly isolated A7 complete fluorescence was observed from day 4 p.i. When the high dose of the infecting virus was used, the cells showed bright intensive fluorescence right from day 1 p.i. (data not shown).

All antisera used showed specific reaction with their respective viruses in GMK cells following the same procedure; there was no non-specific positivity of antisera untreated with GMK cells observed .

Discussion

The role of viral infection in the pathogenetic process of the IDDM has been suggested firstly on the basis of epidemiological studies showing seasonal incidence (Gamble *et al.*, 1969). Later the serological data (Banatwala, 1987; Muir *et al.*, 1990) indicated that this pathological process most often coincided with the coxsackie B4 virus infection. Attention has been directed mostly to the coxsackie B group of viruses and there is much convincing evidence in case reports (Yoon *et al.*, 1979). The overlooked role of coxsackie A and ECHO viruses was first questioned by Frisk *et al.* (1992), who have proven the presence of IgM antibodies against enteroviruses other than coxsackie B4 in newly diagnosed diabetics by RIA. Our recent experience showed that coxsackie A7 and A9 viruses were prevalent among the virus types isolated from stool of children with recently diagnosed IDDM.

In the present study we tried to compare the ability of standard coxsackie viruses (B4, B5, A7, and A9) and the coxsackie virus A7 isolated recently from a newly diagnosed diabetic child with homologous serological response to infect mouse pancreatic cells *in vitro*. We used islet cell clusters prepared by using collagenase XI. Disintegration of the islets into monodispersed single cell suspension was observed when trypsin was used in the isolation technique. This observation agrees with the results of Peakman *et al.* (1994) though they have used EDTA as a chelating agent in adition to trypsin. Our results show that all the viruses tested caused a lytic infection of pancreatic cells. Literature data show that different viruses like mumps, rubella, herpes simplex type 2, cytomegalovirus, and coxsackie B4 and B3 are capable of infecting pancreatic cells *in vitro*, causing corresponding changes in the insulin production and alteration of HLA class molecule presentation with rapid destruction of infected cells (Parkkonen *et al.*, 1992; Vuorinen *et al.*, 1992; Yoon *et al.*, 1978).

Pour *et al.* (1993) have described a multilabelling histochemical technique in which all four different endocrine cells (A, B, C and D) can be demonstrated, and offers an ideal picture of the relative distribution of endocrine cells in different species. We have shown the presence of B cells in the isolated cell clusters by the Milenko-Ivič method for demonstration of A and B cells (Čunderlíková and Balážová, 1991), and comparing with the microscopic picture in cross sections of healthy mouse pancreas. Though we did not assay the insulin production we did demonstrate the rise in titers of the infecting viruses, especially the titers of the freshly isolated A7, showing the ability of virus replication in the pancreatic islet cells. We have no explanation for our unfeasibility to obtain higher titers of coxsackievirus B4 or for the extreme heat instability of coxsackie B5 virus.

The fluorescence assays showed that though the virus was not detected in medium during earlier stages of infection, viral antigen was demonstrated in the cytoplasm of infected cells from the 1st day p.i. There was a marked increase of viral antigen and the number of fluorescence cells in individual clusters within the following days. We did not observe any reduction of fluorescence as Vuorinen *et al.* (1991) did in cell clusters 7 days p.i. with coxsackie B3 virus. Our observation could be due to the high infective doses used.

The non-specific fluorescence observed in uninfected pancreatic cells could probably be due to several common antigen epitopes shared by the coxsackie viruses and the pancreatic beta cells (e.g. 64 K protein), but further studies are required to confirm this assumption. We have proven that the coxsackie A viruses are capable of infecting the clusters of pancreatic islet cells, replicating in them and successively damaging them with the same intensity as coxsackie B4 and B5 viruses do. Further studies are required to elucidate the possible differences among standard laboratory strains and the new isolates of the coxsackie viruses.

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