

Chapter 17

Viruses in the Human Pancreas

S.J. Richardson, A. Willcox, A.J. Bone, N.G. Morgan, and A.K. Foulis

Abstract There is extensive epidemiological evidence highlighting a possible aetiological role for enteroviral infection in type 1 diabetes. Direct evidence of the presence of enterovirus in the pancreatic islets of type 1 diabetics is, however, limited, due mainly to the paucity of samples from patients diagnosed recently with the disease. This chapter summarises the evidence implicating enteroviral infection in the human pancreas in type 1 diabetes and considers both factors indicating the presence of virus (viral capsid protein, electron microscopic visualisation of viral particles or expression of viral RNA) and the host response to a viral infection (a “viral footprint”). The relationship of these two indicators of viral infection with two apparently different forms of type 1 diabetes (autoimmune versus fulminant) is discussed. It is hypothesised that differing host responses, and perhaps different genetic variation among the viruses involved, may determine whether an enteroviral infection of beta cells causes (1) A rapid lytic cell death—characteristic of fulminant type 1 diabetes and neonatalcoxsackievirus infection, (2) A persistent infection which evokes an autoimmune reaction to beta cells, eventually resulting in their destruction—autoimmune type 1 diabetes, (3) A persistent infection with little host response and little damage.

Overall, it is concluded that the evidence for viral involvement is persuasive but that there is still a long way to go in order to elucidate the precise role of enteroviral infections in the aetiology of type 1 diabetes.

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Mounting epidemiological evidence has implicated viruses, particularly (but not exclusively) enteroviruses, in the pathogenesis of type 1 diabetes (Andreoletti et al. 1997; Clements et al. 1995; Coutant et al. 2002; Lonnot et al. 2000; Moya-Suri et al. 2005; Nairn et al. 1999; Richardson et al. 2011; Sarmiento et al. 2007; Schulte et al. 2010; Tauriainen et al. 2011; Yin et al. 2002). However, it is unclear whether viral infection plays a role in the initial development of beta cell autoimmunity or if it accelerates the progression of a pre-existing autoimmune reaction. Indeed, both possibilities may be true depending on the circumstances. In this chapter, we review the evidence implicating viral infection within the pancreas as a causative factor in human type 1 diabetes.

Type 1 diabetes results from destruction of insulin-secreting beta cells in the islets of Langerhans and can occur in two forms. Most commonly, an autoimmune reaction proceeds over a protracted time course leading to the onset of clinical symptoms when ~75% of beta cells have been destroyed. By contrast, a second form of the disease has been described, principally in Japanese populations, in which the process of beta cell destruction occurs much more quickly, leading to “fulminant” type 1 diabetes. In both cases there is increasing evidence that enteroviral infection may underlie the process of beta cell destruction, but the role of the virus appears to be quite different in these two forms of the disease (Dotta et al. 2007; Richardson et al. 2009; Shibasaki et al. 2010; Tanaka et al. 2009).

In order to examine the relationships between enteroviral infection and the pathogenesis of type 1 diabetes, it is important to conduct studies of the target organ (i.e. the pancreas). Such studies have revealed that an early event within the pancreatic islets in autoimmune diabetes is the expression of interferon-alpha by insulin-secreting beta cells (Foulis et al. 1987b). Interferon-alpha expression is closely associated with Class I MHC hyperexpression by the endocrine cells of the affected islet, a hallmark feature of type 1 diabetes (Bottazzo et al. 1985; Foulis et al. 1987a; Pujol-Borrell et al. 1986). Later there is an infiltrate into the islet of immune cells (insulinitis), dominated by cytotoxic CD8⁺ T lymphocytes, with smaller numbers of B lymphocytes, CD4⁺ cells and macrophages (Willcox et al. 2009). Beta cells are destroyed within the inflamed islet and, following their loss, the inflammatory cell infiltrate disappears, leaving an insulin-deficient islet containing glucagon-secreting alpha cells, somatostatin-secreting delta cells and pancreatic polypeptide-secreting PP cells, which have not been harmed (Foulis and Stewart 1984). The whole process in the pancreas, from the first to last beta cell being destroyed, is thought to take many months or even years in autoimmune diabetes.

By contrast, in fulminant diabetes, there is a diffuse infiltrate of inflammatory cells within the pancreas resulting in exocrine inflammation (and clinical acute pancreatitis) as well as islet inflammation. The predominant infiltrating cell type in the islets in fulminant type 1 diabetes is the macrophage, closely followed by CD8⁺ T cells (Shibasaki et al. 2010; Tanaka et al. 2009), and the inflammatory process occurs rapidly with most beta cells (and many alpha cells) being destroyed in a matter of days or weeks (Shibasaki et al. 2010; Tanaka et al. 2009). Within 1 month, the insulinitis declines and, after this time, evidence of immune cell infiltration is rarely detected in pancreas biopsy samples.

The presence of interferon-alpha in beta cells in autoimmune type 1 diabetes (Foulis et al. 1987b) and in fulminant type 1 diabetes (Aida et al. 2011) is consistent with the hypothesis that the beta cells might be infected by a virus, as viral infection stimulates beta cells to secrete this cytokine (Chehadeh et al. 2000). Equally, the appearance of massive rapid destruction of the beta cells in fulminant diabetes led some to hypothesise that this was due to a widespread lytic infection by a virus (Foulis et al. 1988). Examination of the literature reveals, however, only limited evidence of direct infection of pancreatic beta cells in patients with type 1 diabetes. At first sight, this appears problematic to the viral hypothesis but, in reality, the paucity of data relates to a still more fundamental problem; namely that pancreatic tissue obtained from patients diagnosed recently with type 1 diabetes is available in only very limited quantity. By contrast, tissue from patients with longer disease duration can be obtained more readily, but this is less profitable to study since, as described above, the pancreas becomes largely depleted of beta cells over the course of time and many of the clues which might hint at the cause of their destruction are lost. Despite these constraints, evidence has been marshalled which supports a viral aetiology for type 1 diabetes.

In the late 1970s, two case studies were published which documented the development of acute-onset diabetes in each of two children who had been infected with a coxsackievirus of the B4 serotype (CVB4) (Gladisch et al. 1976; Yoon et al. 1979). Gladisch et al. (1976) confirmed the presence of virus in the pancreas using FITC-labelled CVB antibody and they also noted extensive insulinitis and lysis of islet cells. In the second study, Yoon et al. prepared pancreas homogenates from a diabetic patient and inoculated mouse, monkey and human cell cultures with these extracts. They were then able to isolate and type an enterovirus and found that, again, this was classified as the CVB4 serotype. As in the case studied by Gladisch et al. (1976), analysis of the pancreas revealed evidence of islet inflammation and lysis of beta cells. When used in subsequent mouse studies, the virus isolated by Yoon et al. (1979) was capable of infecting beta cells and causing islet inflammation. This was accompanied by beta cell necrosis and the development of hyperglycaemia in infected mice (Yoon et al. 1979).

Such studies prompted others to search for evidence of the presence of enteroviruses in the pancreases of more patients with type 1 diabetes. In a very comprehensive immunohistochemical analysis of formalin-fixed paraffin-embedded pancreases by Foulis et al. in 1990, 88 patients with recent-onset type 1 diabetes were studied. The investigators sought evidence for the presence of the highly conserved enteroviral capsid protein, VP1, but failed to detect any positive staining among the samples (Foulis et al. 1990). This outcome was especially unexpected given that the technique used was able to detect enteroviral VP1⁺ cells in the heart and pancreas of neonates who had died of culture proven coxsackie viral myocarditis, which suggested a sensitivity for the immunohistochemical technique similar to that of viral culture from autopsy tissue. This group went on to extract DNA from the samples of diabetic pancreases and performed PCR looking for viral-specific sequences. They also looked for evidence of enteroviral RNA by *in situ* hybridisation (Foulis et al. 1997). Amplification of extracted DNA from 47 of the patients with primers designed to detect Epstein–Barr virus or cytomegalovirus did not reveal any positive signals. Non-radioactive *in situ* hybridisation with enteroviral probes revealed infection in

coxsackie-infected neonatal pancreas, but no positive signal was observed in the 29 pancreas samples of diabetic patients in which good RNA preservation was established by the detection of insulin mRNA. Subsequently, however, the use of a radioactive enteroviral-specific *in situ* hybridisation probe did reveal the presence of enteroviral RNA in 4 out of 65 diabetic pancreas samples (Ylipaasto et al. 2004), suggesting that detection of virus in autopsy diabetic pancreases may be possible with more sensitive techniques.

Recently, new antibodies with improved detection efficiency for enteroviral VP1 (notably the 5D8/1 clone marketed by Dako), coupled with the development of heat-induced antigen retrieval (HIER) techniques for immunohistochemistry (which enables antigens that were previously masked to be revealed), have improved the sensitivity of virus detection in fixed archival tissues. Dotta et al. (2007) reported evidence of enteroviral VP1 in the pancreases of two recent-onset type 1 diabetes patients and in a third patient who had undergone whole pancreas graft. The immunohistochemical evidence of VP1 staining was supported by the isolation of a virus (once again belonging to the CVB4 serotype) from the pancreas of one of the patients studied. The VP1 staining was largely confined to beta cells and viral particles were observed in the cytoplasm of these cells when examined under the electron microscope. More importantly, it was revealed that the viral isolate was capable of infecting human islets cultured *in vitro* and that this resulted in an impairment of insulin secretion (without any change in islet insulin content). This implies that enteroviral infection can lead to functional impairment of beta cells under conditions where the virus does not induce specific beta cell cytotoxicity. In summarising these important findings, it is also worth noting that the virus isolated by Dotta et al. had a high degree of homology with a laboratory reference strain dating from a much earlier period, which has led some to question its provenance (Tracy et al. 2010).

The advent of improved techniques for enteroviral detection in formalin-fixed paraffin-embedded tissue allowed our group to revisit the original collection of recent-onset type 1 diabetic pancreases studied by Foulis et al. (Richardson et al. 2009). Whereas these had previously been thought to be negative for viral protein, we found evidence of enteroviral VP1 immunopositivity in the pancreas of 44 of 72 (61%) of patients. This compared with a total of only 4 positive endocrine cells detected in the pancreas of 3 of 39 (7.7%) non-diabetic paediatric cases. Serial sections showed that the VP1 staining was restricted to insulin-containing islets (ICIs) (insulin-deficient islets being negative), and dual immunofluorescence demonstrated that VP1 was confined to beta cells within these islets (Richardson et al. 2009; Willcox et al. 2011).

Virally infected cells can be expected to mount a specific pattern of molecular responses, the function of which is to minimise the impact of the infection on the cell by, for example, shutting down host protein synthesis. Alteration of the expression of certain markers in a cell may therefore act as a “footprint”, indicating the likely presence of a virus within the cell. One such marker, the inducible pathogen recognition receptor, protein kinase R (PKR) was found to be frequently present in VP1⁺ islets (Richardson et al. 2009), and more recent work has confirmed that VP1 and PKR co-localise in the beta cells of patients with type 1 diabetes (Richardson, unpublished observations, Fig. 17.1). Interestingly, the level of VP1 expression is

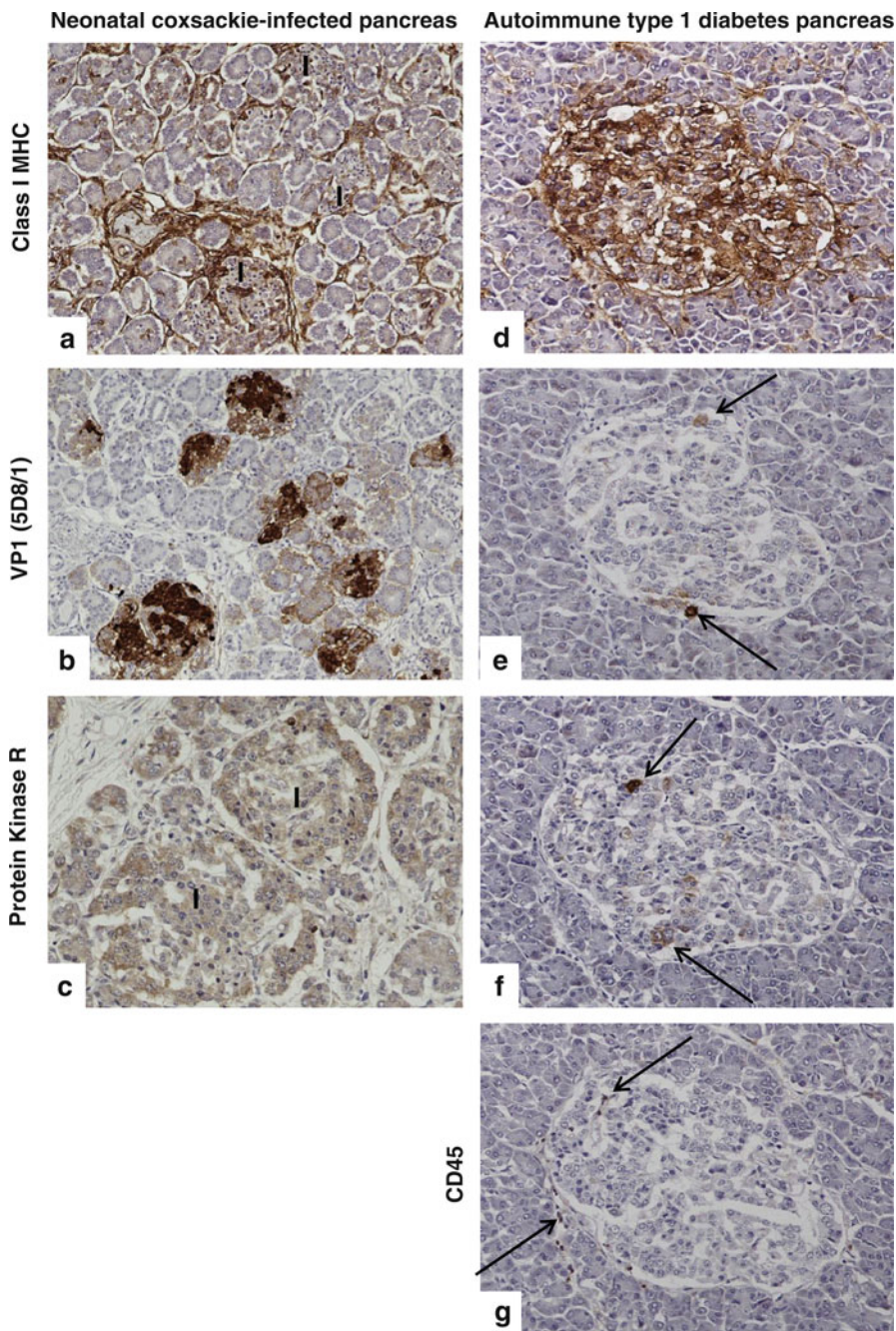


Fig. 17.1 Immunohistochemical staining for Class I MHC (**a** and **d**), VP1 (mAb 5D8/1; **b** and **e**) and PKR (**c** and **f**) in neonatal coxsackie-infected pancreas (**a–c**) and serial sections of an autoimmune type 1 diabetes pancreas (**d–f**). Further staining for CD45 (**g**) is demonstrated in autoimmune type 1 diabetes. Although both the neonatal coxsackie-infected pancreas and the autoimmune type 1 diabetes pancreas express enteroviral VP1, the islet-specific hyperexpression of Class I MHC and the beta cell selective up-regulation of PKR only occur in autoimmune type 1 diabetes. Endothelial cell and lymphocyte-specific Class I MHC staining is observed in the coxsackie-infected pancreas (**a**), but the endocrine cells remain largely unstained

Table 17.1 A comparison of pancreas pathology between neonatal coxsackie infection, fulminant diabetes and autoimmune type 1 diabetes

	Neonatal CVB infection	Fulminant diabetes	Autoimmune T1D
Endocrine cell loss	Alpha and beta cells	Alpha and beta cells	Beta cells
Insulinitis	+	100% +++ but by 1 month negative	++ but selected islets only
Predominant immune cells	Unknown	Macrophage > CD8	CD8 > B cells > Macrophage > CD4
Class I MHC in islets	No hyperexpression	Little or no hyperexpression	Hyperexpression
Evidence of islet VP1 staining	+++	+++	Occasional positive cells
IFN alpha expression by beta cells	++	+	++

much lower in patients with autoimmune type 1 diabetes (Fig. 17.1e) than in those with acute systemic enterovirus infection (Fig. 17.1b). In the autoimmune type 1 cases, VP1 strongly correlates with PKR expression (Fig. 17.1e–f; Richardson unpublished results), which is unlike the situation in the acutely infected pancreas where more VP1⁺ cells are observed and there appears to be no correlation with PKR (Fig. 17.1b, c).

Interferon-alpha and hyperexpression of Class I MHC are not present in the islets of non-diabetic controls but are each observed at clinical onset and years after the onset of autoimmune type 1 diabetes, and these markers may form part of a “viral footprint” (Bottazzo et al. 1985; Foulis et al. 1987a; Oikarinen et al. 2008) present in infected islets. However, only a small number of the beta cells within such islets also express enteroviral VP1, suggesting that there may be an underlying persistent enteroviral infection in cells that express little or no VP1 protein, but which can still drive the expression of interferon-alpha and the hyperexpression of Class I MHC. In support of this, enteroviruses can establish persistent infections in human tissues and, when this happens, it is thought that the virus exists in a different but stable molecular form, possibly as double-stranded RNA (Cunningham et al. 1990; Tam and Messner 1999). Under these circumstances VP1 is less likely to be expressed (Klingel et al. 1992), but it seems probable that the occasional islet cells which do express VP1 may represent only the “tip of the iceberg” amongst numerous adjacent beta cells that harbour a more persistent infection.

In fulminant type 1 diabetes, high levels of expression of VP1 were detected in the islets at early time points following disease onset suggesting that the induction of this form of diabetes may result from an acute lytic infection of islet cells leading to damage of the surrounding tissue and promoting a generalised inflammatory response. Thus in this regard, fulminant type 1 diabetes has strong similarities to the acutely infected neonates who died of viral myocarditis (Table 17.1, Fig. 17.1). It is important to emphasise, however, that the number of fulminant diabetes patients examined for VP1 expression remains small with viral protein having been detected

in three of the three cases studied (Aida et al. 2011; Tanaka et al. 2009) while enteroviral RNA was found in one of the three cases (Shibasaki et al. 2010); so further studies are necessary to confirm these findings.

It's All in the Order

As noted previously, it is not known whether an enteroviral infection precedes the onset of autoimmunity or whether it accelerates the disease process once autoimmunity is established. The latter appears to be the case in the NOD mouse model, where a threshold of established insulinitis must be present in order that enteroviral infection can hasten the onset of diabetes (Drescher et al. 2004). In humans the immunopathological evidence seems to suggest that the order is subtly different as islets from patients with recent-onset autoimmune type 1 diabetes can be found that are completely devoid of immune cell infiltration, but with evidence of hyperexpression of Class I MHC, expression of interferon-alpha and VP1 protein (Foulis et al. 1987b; Richardson et al. 2009). The detection of VP1 protein in islets in the absence of any signs of insulinitis in an autoantibody positive case has also been documented (Oikarinen et al. 2008). Therefore, more research is required in humans to determine whether a viral infection is the “chicken or the egg” in terms of immune-mediated beta cell destruction.

There is still a long way to go in order to elucidate the precise role of enteroviral infections in the aetiology of type 1 diabetes. It appears though that different host responses, and perhaps different genetic variants among the viruses involved, may determine whether an enteroviral infection of beta cells causes (1) rapid lytic cell death, (2) a persistent infection which evokes an autoimmune reaction to beta cells, eventually resulting in their destruction, or (3), a persistent infection with little host response and little damage done.

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