

**The prevalence of enteroviral vp1 immunostaining in pancreatic islets in human type 1 diabetes**

S. J. Richardson, A. Willcox, A. J. Bone<sup>1</sup>, A. K. Foulis<sup>2</sup>, N. G. Morgan

Institute of Biomedical and Clinical Sciences, Peninsula Medical School, Tamar Science Park,

Plymouth, PL6 8BU

<sup>1</sup>School of Pharmacy and Biomolecular Sciences, University of Brighton, Moulscroomb, Brighton,

BN2 4GJ

<sup>2</sup>Department of Pathology, Royal Infirmary, Glasgow, G4 OSF

Corresponding author: Professor Noel Morgan

Institute of Biomedical and Clinical Sciences

John Bull Building

Peninsula Medical School

Tamar Science Park, Plymouth

PL6 8BU

Tel: 44 (0)1752 437412

Fax: 44 (0)1752 517846

E-mail: noel.morgan@pms.ac.uk

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## **Abstract**

### *Aims/ hypothesis*

Evidence that the beta-cells of human patients with type 1 diabetes can be infected with enterovirus is accumulating but it remains unclear whether such infections occur at high frequency and are important in the disease process. We have now assessed the **prevalence** of enteroviral vp-1 capsid **staining** in a large cohort of autopsy pancreases of recent-onset type 1 diabetic patients and a range of controls.

### *Methods*

Serial sections of paraffin-embedded pancreatic autopsy samples from 72 recent-onset type 1 diabetes patients and up to 161 controls were immunostained for insulin, glucagon, enteroviral vp1, double-stranded RNA activated protein kinase R (PKR) and MHC class I.

### *Results*

Vp1 immunopositive cells were detected in multiple islets of 44/72 young recent-onset type 1 diabetic patients, compared with a total of only 3 islets in 3/50 neonatal and paediatric normal controls. Vp1 **staining** was restricted to insulin containing beta-cells. Among the control pancreases vp1 immunopositivity was also observed in some islets from 10/25 type 2 diabetic patients. A strong correlation was established between islet cell vp1 positivity and PKR production in insulin-containing islets of both type 1 and type 2 diabetic patients, consistent with a persistent viral infection of the islets.

### *Conclusion/ Interpretations*

**Immunoreactive** enteroviral vp1 is commonly found in the islets of recent-onset type 1 diabetes patients, but only rarely in normal paediatric controls. Vp1 immunostaining was also observed in some islets of type 2 diabetes patients, suggesting that the phenomenon is not restricted to type 1 diabetes patients.

*Keywords: Type 1 diabetes, type 2 diabetes environmental triggers, enterovirus, insulinitis, islets of Langerhans, beta cells, MHC, PKR*

**Abbreviations:** CVB – Coxsackie B virus; MHC – major histocompatibility antigen; PKR – double-stranded RNA activated protein kinase R; Vp1 – enteroviral capsid protein vp1;

## **Introduction**

It has been suggested that environmental factors may be involved in triggering autoimmunity, islet inflammation (insulinitis) and beta-cell death in type 1 diabetes. Much of the evidence supporting the possible involvement of viruses as initiators of type 1 diabetes in man is circumstantial, as highlighted in a recent review [1]. However, in support of this proposal, antibodies against common Coxsackie B enteroviruses (CVB) are known to occur more frequently in the serum of recent-onset type 1 diabetics when compared to controls [2] and enteroviral RNA is more prevalent in the peripheral blood [3-9] and serum [10] of such patients.

These observations are consistent with a possible viral aetiology for type 1 diabetes (at least in some patients) but direct evidence to support this hypothesis has been more difficult to obtain. Previously a strain of CVB4 was isolated from the pancreas of a recent-onset type 1 diabetic patient and shown to be capable of inducing diabetes in mice [11]. More recently, the presence of enteroviral RNA in the islets of four recent-onset type 1 diabetes cases has been revealed by *in situ* hybridisation [12], thereby confirming that the islets of patients with type 1 diabetes can harbour a persistent viral infection. In confirmation of this, the enteroviral capsid protein, vp1, was detected by immunohistochemistry in the beta-cells of 2 of 5 patients with type 1 diabetes, and in a sixth diabetic patient who had received a recent pancreatic transplant [13]. Examination by electron microscopy showed the presence of virus particles within the islet beta cells of the cases with demonstrable vp1, and subsequently a strain of CVB4 was isolated from the pancreas of one of these patients. This virus was capable of infecting normal human islets *in vitro*, resulting in their functional impairment [13].

In view of these findings, we considered it important to establish the prevalence of immunoreactive vp1 staining in the islets of a much larger cohort of recent onset type 1 diabetes cases. In addition, we have studied the frequency of vp1 immunopositivity in the islets of a wide range of relevant controls, including a selection of type 2 diabetes patients, to determine the specificity of the finding. We have also examined the islets of these cases to establish whether an antiviral response is mounted.

It has been shown that enteroviral infection of skeletal muscle cells both *in vitro* and *in vivo* resulted in production of double stranded RNA (dsRNA) within cells [14]: In keeping with this observation double-stranded RNA activated protein kinase R (PKR) was markedly upregulated in islets infected by Coxsackie virus B5 *in vitro* [15]. Similarly, islets infected by Coxsackie virus B produced interferon-alpha *in vitro* [16] and this has been associated with hyperexpression of class I MHC by islets [17]. We have therefore attempted to correlate the staining of enteroviral vp1 within islets with both PKR staining and class I MHC hyper-expression. Finally, we have assessed whether vp1 staining occurs independently of insulinitis.

## Methods

### *Subjects*

Formalin fixed paraffin embedded pancreatic autopsy tissue from 72 patients with recent onset type 1 diabetes mellitus was used in the study. The collection of these cases and the histological findings in the pancreases have been described previously [18] and full ethical permission was obtained for their use. The cases were from many different hospitals and had been fixed in either buffered formalin, formol saline, formol saline with added mercuric chloride, or Bouin's fixative. There had been no standardisation of fixation time. The cases were selected such that nearly all of the pancreatic sections studied contained residual insulin-positive islets. The cohort consisted of 41 female and 31 male patients with a mean age of  $12.65 \pm 1.1$  years (range 1 to 42 years) and a mean time since diagnosis of  $8.2 \pm 4.1$  months; (range 0-6 years). Control autopsy tissues, fixed in a similar variety of fixatives, were 5 culture proven Coxsackie virus-infected neonatal pancreases and hearts; 11 normal neonatal pancreases (<1 month); 3 normal neonatal hearts; 39 normal paediatric pancreases (age range 6

weeks-17 years); 11 pancreases from cystic fibrosis patients with a mean age of  $8.1 \pm 1.7$  years (age range 2-19 years); 69 normal adult pancreases and 25 pancreases from adult patients with type 2 diabetes.

#### *Immunohistochemistry*

Serial sections (4 $\mu$ m) were cut from each case and mounted on glass slides coated in (3-Aminopropyl)-triethoxysilane (Sigma, Poole, Dorset, UK). Sections were processed and labelled using a standard immunoperoxidase technique for paraffin sections. With the exception of glucagon, insulin and MHC class 1, all other antigens were unmasked by heat-induced epitope retrieval. Sections to be labelled with anti-PKR and anti-CD45 were heated in 10mM citrate buffer pH 6.0 while those to be labelled with DAKO anti-enteroviral vp1 (5D8/1; DAKO, Glostrup, Denmark), polyclonal anti-vp1, or anti-Pan-enterovirus (9D5; Millipore, Livingston, UK) were heated in 1mM EDTA, pH 8.0. See Supplementary Table (online) for details of antibody dilutions and sources.

Primary antibodies were applied for 30 min at room temperature (except for anti-Pan-enterovirus (9D5) and anti-PKR which were incubated overnight at 4°C) and Dako REAL™ Envision™ Detection System used for antigen detection. Some slides were processed in the absence of primary antibody or with isotype control antisera to confirm the specificity of labelling. Slides were analysed by light microscopy.

#### *Immunofluorescence staining*

To determine the islet cell subtypes infected by enterovirus, double immunofluorescence staining was performed. Anti-insulin, anti-glucagon and anti-vp1 reactivity were detected using an Alexa Fluor 488-conjugated anti-guinea pig antibody, an Alexa Fluor 488 anti-rabbit antibody and an Alexa Fluor 568 conjugated anti-mouse antibody respectively (Invitrogen, Paisley, UK). Sections were mounted using Vectashield medium (Vector Labs, Burlingame, Ca, USA) and were observed using a Zeiss LSM510 Meta confocal microscope.

## *Statistics*

Statistical analysis was performed either by the Chi squared test or Students t-test as appropriate and results were considered significant when  $p < 0.05$ .

## **Results**

### ***Vp1 staining in the islets of recent-onset Type 1 diabetic patients***

In order to confirm the utility of the DAKO enteroviral antibody used previously to identify vp1 immunoreactivity in the pancreas [13, 19] and intestine [20] of patients with type 1 diabetes, we initially examined its reactivity in formalin-fixed, paraffin embedded, heart and pancreas samples taken from 5 neonates having fulminant, fatal Coxsackie B infection. Cardiac myocytes and many pancreatic islets from these samples contained cells which were strongly immunopositive for vp1 (Figure 1a and Figure3a). By contrast, none of the islets examined in normal pancreas sections recovered from 11 non-diabetic neonates, nor the myocytes in hearts of neonates without myocarditis, were immunopositive for vp1.

Probing of the pancreases from our cohort of 72 recent-onset type 1 diabetes patients with the DAKO anti-vp1 antibody revealed the presence of small numbers of intensely stained cells in multiple islets from the majority of cases (Figure 1b-c). These immunopositive islet cells were readily distinguishable from the surrounding unstained endocrine cells (which formed the majority in all cases) suggesting that the antibody did not label an endogenous islet cell antigen. To facilitate quantitative analysis, a case was deemed positive for vp1 when at least one intensely stained endocrine cell was present in an islet within any given section. Using this criterion, 44 of the 72 (61%) recent-onset type 1 diabetes cases were positive for enteroviral vp1 antigen within the pancreas (Figure 1g) and most contained multiple immunopositive cells in a range of islets. Comparison of the age and sex of the type 1 diabetes patients between the vp1-positive and vp1-negative cases failed to reveal any significant differences. However, the duration of diabetes tended to be lower in the vp1-positive group ( $2.32 \pm 0.8$  months vs  $16.5 \pm 8.0$  months;  $p = 0.06$ ).

Occasional islet cell vp1 immunopositivity was detected in the pancreases of 3 of 39 (7.7%) non-diabetic paediatric cases (Figure 1e, g) but this amounted to a total of only 4 endocrine cells among the entire islet population examined. This was significantly lower than that observed in the patients with type 1 diabetes ( $X^2=29.71$ ,  $p<0.001$ ). A series of 11 paediatric cystic fibrosis pancreases was also examined for vp1 immunopositivity to determine whether the viral protein is found commonly in patients with another pancreatic inflammatory disease. Of the 11 pancreases examined from patients with this disease, 2 contained multiple vp1 positive islets similar to those seen in the type 1 diabetes patients (not shown). Importantly, it was discovered upon subsequent examination of the case histories of these patients, that the two with vp1 immunopositivity in the islets, also had diabetes. None of the remaining (vp1 negative) cystic fibrosis cases had diabetes.

#### ***Vp1 immunostaining is restricted to insulin-containing islets***

In the pancreas in recent onset type 1 diabetes approximately 60% of the islets are insulin deficient and the remainder contain residual insulin secreting beta cells [18]. An analysis was made of the distribution of vp1 positive cells between insulin deficient islets (IDI) and insulin containing islets (ICI) in serial sections from 10 patients with frequent vp1 positive endocrine cells. A total of 342 ICIs were analysed and 269 (78.7%) were found to be positive for vp1. By contrast, only 16 of 611 (2.6%) IDIs showed evidence of vp1 within the plane of the section (Figure 2a). This finding suggests that beta cells **contain** the viral protein. A more direct approach to assessing this was double immunofluorescence staining for either insulin or glucagon together with vp1 (Figure 2b). This revealed that staining of the viral antigen was restricted to a minority of insulin-positive cells. These results were verified in 5 separate cases in which an average of 32 islets were examined per section.

The presence of CD45<sup>+</sup> immune cells was also examined to determine whether a relationship existed between vp1 immunopositivity and the extent of insulinitis in type 1 diabetes patients. A total of 200 vp1 positive ICIs were studied (from 7 patients) and 123 (61.5%) of these showed no evidence of inflammation (i.e. they were negative for CD45).

### ***Verification of islet vp1 staining with other enteroviral antisera***

To confirm that the DAKO antiserum was detecting enteroviral vp1 in the islets of type 1 diabetes patients, the staining pattern was compared with that obtained using a second, polyclonal antiserum raised against vp1 (designated Kdf), and an additional pan-enteroviral monoclonal antibody, not specifically directed against vp1 (clone 9D5). Initially these antisera were tested in normal and Coxsackie-infected neonatal human heart to verify their specificity and sensitivity. This revealed that similar regions of the tissue were stained with each antibody in infected tissue (examples for DAKO and Kdf are shown in Fig 3) and that staining was absent from normal control hearts. Under optimal conditions, the DAKO antiserum appeared to be the most sensitive of the three, since staining was more intense and extensive with this antibody than with the others. It also gave more consistent results on tissues fixed in a variety of fixatives.

Comparison of staining with the three antisera applied to serial sections of pancreas is presented in Figure 4 (a-f) together with a quantitative analysis from 11 patients (Table 1). The images show islets from two different patients stained with either the DAKO vp1 (Figure 4a, e), Kdf (Figure 4b, f) or 9d5 (Figure 4c) antibodies and demonstrate that intensely positive islet cells could be detected in the same islets with these reagents. Of the 11 patients studied in detail, 1 **Bouin's fixed case** had islets stained by all 3 antisera and at least a further 3 had positive immunostaining in ICI with two antibodies. The DAKO antiserum appeared most sensitive since it stained one or more of the ICI in all 11 cases. No IDI were stained with any of the antisera.

### ***Correlation between islet vp1 staining and additional markers of viral infection***

In the next series of studies, serial sections of pancreas were stained with antibodies to insulin, vp1, PKR and MHC-1 in 6 vp1 positive type 1 diabetes patients to determine whether vp1 **staining** correlated with other markers of viral infection and autoimmunity. Representative insulin containing islets from one of these patients are shown in Figure 5a and a representative insulin-deficient islet is also shown for comparison. PKR staining correlated with vp1 immunopositivity in 154 of 177 (87.0%) ICIs analysed in the type 1 diabetes patients (Figure 5a and b) whereas it was not detected in

any islets of 5 non-diabetic age-matched control pancreases. PKR staining was only rarely observed in insulin deficient islets. Islet cell MHC-1 hyperexpression was detected in all of the type 1 diabetes cases analysed and this correlated with the presence of insulin (Figure 5a). All vp1 positive islets were found to hyper-express MHC-1, but so did many vp1 negative insulin-containing islets. MHC-1 was not hyper-expressed in any neonatal or paediatric control pancreases.

### ***Vp1 staining in sections of pancreas from Type 2 diabetic patients and from non-diabetic adults***

The study was extended to include pancreas samples recovered post mortem from 69 normal adults and 26 patients with type 2 diabetes. Islet endocrine cell immunopositivity for vp1 (DAKO antiserum) was observed in 9 of 69 (13.0%) normal pancreases (Figure 6ai and b). Of 25 type 2 diabetic patients analysed, 10 (40%) contained a range of islets having intensely positive cells (Figure 6aii and b;  $X^2=7.89$ ,  $p<0.01$  vs normal pancreases).

A more detailed comparison of the frequency of vp1 positive islets in 10 type 1 and 6 type 2 diabetic pancreases as well as 7 non-diabetic adult pancreases was performed (Fig 6c). The cases were chosen at random among pancreases which had vp1 positive islets. 70/251 (27.9%) islets contained vp1 positive endocrine cells in the 6 type 2 diabetes pancreas sections studied whereas only 15/210 (7.1%) islets from 7 normal adult pancreas contained equivalent cells (Figure 6C;  $X^2=32.81$ ,  $p<0.001$ ). This compared with 269/342 (78.7%) vp1 positive insulin containing islets in the 10 type 1 diabetes cases studied in detail ( $X^2=152.31$ ,  $p<0.001$ ; vs type 2 diabetes). In the islets of patients with type 2 diabetes islets that were vp1 positive, PKR immunopositivity was also often detected (Figure 5a and 6d). Hyper-expression of MHC-1 was not seen in any islet from type 2 diabetes patients (Figure 5a) nor in non-diabetic adult pancreases.

Finally, particularly in some adults, focal pancreatic centroacinar cells in the exocrine tissue and, in certain cases, smooth muscle cells in vein walls, were stained positively by the DAKO anti-vp1 serum (Figure 6aiii-iv). Such staining was occasionally seen, but much less frequently, in the neonatal and

paediatric pancreases of both diabetic and non diabetic patients, and it was never observed with the kdf antiserum in any case studied.

## Discussion

Circumstantial evidence has implicated enteroviral infection in the aetiology of type 1 diabetes but direct support for the hypothesis that islet cell enterovirus infection is a common occurrence in the disease has been more difficult to obtain [1]. However, it was recently reported that the enteroviral capsid protein, vp1, was detectable in a range of beta-cells in 2 of 5 recent onset type 1 diabetic patients and in another diabetic patient who had received a pancreatic transplant, but not in 26 non diabetic controls [13]. In order to verify and extend this finding, we have analysed the prevalence of pancreatic islet vp1 immunopositivity in 72 recent-onset type 1 diabetic patients, most of whom had died at clinical presentation of diabetic ketoacidosis, and in a range of controls. The results reveal a prevalence of islet vp1 immunopositivity of ~ 61% in type 1 diabetic patients, with almost no equivalent staining in the pancreases of neonatal and paediatric non diabetic controls. Double immunofluorescence studies showed that vp1 staining was confined to beta-cells. Thus, the proposal that vp1 immunopositivity may be common in the insulin-secreting cells of type 1 diabetic patients is supported by this more extensive analysis.

An important consideration is the identity of the antigen labelled by the DAKO vp1 antiserum. In the literature the specificity of the DAKO vp-1 antiserum has been assessed by comparing immunohistochemical and enteroviral RNA detection techniques. For example, in a study of heart biopsies of 70 patients with dilated cardiomyopathy, enteroviral RT-PCR testing was positive in 25 samples. Seventeen of these had focal staining of cardiac myocytes by DAKO vp1 antiserum while all of the RT-PCR negative samples were vp-1 negative on immunohistochemistry. All 45 normal control hearts were negative with both techniques [21]. Additionally, RT-PCR for enterovirus was positive in salivary gland biopsies in 7 of 8 patients with primary Sjogren's syndrome but not in 16 normal salivary glands. In confirmation of this, ductal, acinar and infiltrating inflammatory cells stained positively with DAKO anti-vp-1 in the Sjogren's cases but not in the controls [22].

We verified that the DAKO vp1 antibody labelled human cardiac myocytes in neonates infected with a number of different Coxsackie B viruses *in vivo*, whereas non-infected neonatal heart was immunonegative. However, in common with others [19,23] we also noted that the DAKO antiserum can, under certain conditions, label tissues in which vp1 production is not expected. In particular, our preliminary work revealed that, when applied to formalin fixed, paraffin-embedded, sections of Coxsackie-infected neonatal mouse organs, this antibody stained some tissues which were reported to be negative for viral RNA by *in situ* hybridisation [24]. Such tissues included smooth muscle, bone, dental and hair follicle epithelium, pancreatic duct and centroacinar cells. Moreover, these tissues were also stained by the antibody in uninfected neonatal mice. Thus, while it is clear that the DAKO antibody is a sensitive agent for labelling enteroviral vp1 in a range of tissues in mouse and man, it is also evident that it can label non-infected cells, including, in the pancreas, vascular smooth muscle and centroacinar cells. However, it should be emphasized that in uninfected neonatal mice and neonatal children the antibody did not stain pancreatic endocrine cells. Thus, in the context of islets of Langerhans, the DAKO antibody stained endocrine cells only in control tissues known to be infected by virus.

Polyclonal antisera raised against enteroviruses have been shown to cross react with hsp-60 [25] and the tyrosine phosphatase, IA-2 [26]. However, it seems unlikely that the DAKO vp-1 antiserum recognised either of these proteins in the beta cells in this study. IA-2 is present in alpha cells as well as beta cells while the vp-1 staining was seen in a minority of beta cells, but not alpha cells. The expression of hsp-60 has been studied in these diabetic samples before [27] and there was no equivalent staining of occasional beta cells as described here for vp-1.

Because of the potential for non-specific immunoreactions with the DAKO antiserum and possible doubts about the origin of the virus isolated previously [13, 28] we used two additional antisera raised against enteroviral proteins to analyse the presence of enterovirus in sections of pancreas. With each

of these antisera, specific enteroviral immunopositivity was first demonstrated in infected neonatal human hearts. Both antisera stained endocrine cells in insulin containing islets, which correlated with immunopositivity detected with DAKO anti-vp1 in some, but not all, type 1 diabetic patients tested.

In an earlier study [13] two additional techniques were used to verify that vp1 immunostaining in diabetic islets represented virus infection. Firstly, virus particles were observed by electron microscopy, confined to beta-cells, in the same cases as had demonstrable vp-1. Secondly, a strain of CVB4 was isolated from one of the pancreas samples and shown to be capable of infecting normal human islets *in vitro*, leading to impaired glucose-stimulated insulin secretion [13]. Hence, the authors directly correlated vp1 immunopositivity, as revealed by the Dako antiserum, with the presence of active virus in at least one patient. While this evidence appears persuasive, others [28] have cast doubt on the origin of the virus strain, by suggesting that the reported RNA sequence analysis implies possible contamination with a laboratory strain.

Since most of the previous studies [13,21,22] were conducted using fresh biopsy tissues, or tissues removed at the time of organ donation, the authors were able to employ EM, RT-PCR or *in situ* hybridization techniques to confirm the validity of the vp1 staining. These options were not open to us since the pancreatic material from patients with diabetes in the present study was collected almost entirely retrospectively from autopsies performed hours after the death of patients. In previous work [29] attempts were made to detect enteroviral RNA by *in situ* hybridization in this collection and no evidence of infection was found. The poor quality of RNA in formalin fixed, paraffin embedded autopsy pancreas, probably the result of early RNA degradation in a tissue rich in RNase, was exemplified in that study by the fact that mRNA for insulin could be detected by *in situ* hybridization in only 29 of the 75 pancreases studied, even though all had insulin containing beta cells as shown by immunohistochemistry. In support of this, very recent work has also failed to detect viral RNA by *in situ* hybridisation in an autopsy pancreas where evidence of viral protein production was detected by immunohistochemistry [19]. Thus no attempt was made in the present study to repeat the search for enteroviral RNA by *in situ* hybridisation or RT-PCR. Rather, we looked at the production of another

protein that is up-regulated in response to enteroviral infection, protein kinase R [30] and found a strong concordance between PKR immunopositivity and that for vp1 in the islets both type 1 and type 2 diabetic patients. This is arguably the best evidence in the present study that detection of vp1 by the DAKO antibody in beta cells is indicative of a persistent enteroviral infection.

While staining of vp1 correlated with PKR immunostaining in the islets of patients with type 1 diabetes it was not seen in many of the insulin containing islets where there was hyperexpression of class I MHC on islet endocrine cells. This latter phenomenon has been shown to be intimately associated with interferon-alpha production by beta cells [17] and is a phenomenon unique to type 1 diabetes [31]. Thus, vp1 staining by beta cells does not appear to be directly associated with secretion of interferon-alpha and it is unclear why interferon-alpha is produced by beta cells in those islets which hyperexpress class I MHC [26] but have no demonstrable viral capsid protein.

In experimental acute enteroviral myocarditis in mice, it has been reported that viral capsid protein was most easily detectable by immunohistochemistry under conditions when there was an excess of positive strand viral RNA over negative strand viral RNA within infected cells. By contrast, if the infection continued into a chronic phase, then the number of positive and negative enteroviral RNA strands per cell became equivalent and capsid protein was less readily demonstrable [32]. On this basis, it is conceivable that a molecular form of virus, that is not readily detectable by immunohistochemistry for vp1, may exist as a chronic infection in many of the beta cells in type 1 diabetes and that, occasionally, production of the whole virus increases, leading to capsid protein synthesis in a small number of beta-cells. If this hypothesis is correct, then our results imply that the molecular form of virus detectable by vp1 immunostaining is also the form that most potently induces PKR production, whereas interferon-alpha may be induced by both molecular forms.

Extending the current analysis from paediatric patients to adults revealed additional unexpected features. Most surprisingly, 40% of the adult type 2 diabetes cases displayed focal staining for vp1 in their islets. This intense focal staining pattern was indistinguishable from that seen in the islets of

patients with type 1 diabetes (though it occurred much less frequently in type 2 diabetes) which might suggest that immunopositivity for vp1 occurs as a consequence of diabetes. Alternatively, these results may indicate that enteroviral infection plays an important role in the pathology of beta-cell loss and malfunction in both type 1 and type 2 diabetes.

The latter proposition is consistent with emerging evidence that type 2 diabetes may be associated with islet cell inflammation [33] although it must also be noted that there is no induction of autoimmunity in type 2 diabetes. Nevertheless, our present finding of production of PKR in consort with vp1 in the islets of patients with type 2 diabetes is consistent with an active response by infected endocrine cells to the presence of virus in this disease. Since non-cytopathic infection of beta-cells by enterovirus leads to decreased glucose-induced insulin secretion *in vitro* [13] it is possible that, in a person with obesity and increased insulin resistance, in whom the demand for insulin is raised, such an infection may contribute to beta-cell dysfunction and the development of type 2 diabetes, even if not associated with autoimmunity. Our findings could also suggest a similar role for enterovirus infection in precipitating clinical diabetes in some patients with cystic fibrosis.

Vp1 staining in islets was found in approximately 10% of non diabetic controls of all ages. This is consistent with studies showing that 4-5% of normal children had enteroviral RNA in their serum [4, 7, 8] and 11.8% had enteroviral RNA present in faecal samples [34,35]. In addition, it has also been shown that a range of enteroviruses can infect human islets *in vitro* [12,36,37], which is consistent with the pancreas being quite frequently colonised during an enteroviral infection.

This study has extended recent work [13] to reveal the prevalence of vp1 positivity in beta cells in type 1 diabetes. The results are consistent with the hypothesis that enteroviral infection of beta cells at a young age, in patients with a genetic predisposition to type 1 diabetes, might precipitate autoimmunity to beta cells. The islet reaction to that infection would include production of PKR, interferon-alpha secretion by beta cells and abnormal production of MHC molecules in islets, leading to the type 1 form of the disease. Infection by a similar virus, in a person with a different genetic

background, might be associated with PKR production, but if it did not precipitate interferon alpha-production by beta cells, or any abnormalities of MHC expression in islets, this infection would not be associated with autoimmunity, and in the great majority of patients would lead to no clinical disease. However, in patients with an already compromised metabolic situation, as a result of insulin resistance, it may help precipitate the development of type 2 diabetes. Further studies, including viral RNA detection techniques on fresh, organ-donation pancreas samples from patients with recent onset disease, are indicated to confirm the presence and identity of enterovirus strains causing diabetes.

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**Table 1 Analysis of islet staining with 3 anti-enteroviral antisera on 11 cases of type 1 diabetes**

<b>Number of insulin-containing islets stained by</b>					
<b>Number of ICI</b>	<b>Any antiserum</b>	<b>Any two antisera</b>	<b>Dako 5D8/1</b>	<b>Kdf</b>	<b>9D5</b>
50	12 (24%)	5	7	0	10
31	5 (16%)	0	3	2	ND
18	3 (17%)	0	3	0	ND
31	9 (29%)	5	7	7	ND
28	4 (14%)	0	4	0	0
21	21 (100%)	19*	18	20	19
2	2 (100%)	0	2	0	ND
26	18 (69%)	0	18	0	ND
36	12 (33%)	0	12	0	ND
23	3 (13%)	0	3	0	ND
16	11 (18%)	0	11	0	0

**ND – not determined \*18islets stained by 3 antisera**

Supplementary Table (to be available only in electronic format): Antibody details and dilutions

<b>Antigen</b>	<b>Dilution</b>	<b>Species</b>	<b>Manufacturer</b>
<b>Human Glucagon pAb</b>	1/350	Rabbit	Dako Cytomation, UK
<b>Swine Insulin pAb</b>	1/400	Guinea-pig	Dako Cytomation, UK
<b>Human CD45 mAb</b>	1/750	Mouse	Dako Cytomation, UK
<b>Human MHCI pAb</b>	1/200	Rabbit	Jacques Neefjes
<b>Enterovirus vp1 (5D8/1) mAb</b>	1/500-1000	Mouse	Dako Cytomation, UK
<b>Polyclonal vp1</b>	1/400	Rabbit	R.Kandolf
<b>Human PKR mAb</b>	1/350	Rabbit	Abcam, UK
<b>Enterovirus mAb 9d5</b>	1/10	Mouse	Chemicon

## Figure legends

Figure 1. Immunohistochemical analysis of enteroviral vp1 staining in human pancreatic islets. Pancreas sections from a Coxsackie-infected neonatal child (a) and two different recent-onset type 1 diabetic patients (b, c) were stained for vp1. A serial section of the islet in (c) has also been immunostained for insulin (f) and shows that most beta cells do not produce vp1. Vp1 immunoreactivity was not observed in islets from the majority of paediatric cases (d). Only 4 positively stained islets cells were observed in 3 of 39 paediatric cases, one such cell is shown by the arrow in (e). The percentage of cases from each subject group that were found to have at least 1 intensely stained cell within an islet is shown in (g). The number of positive cases/ total number of cases is quoted above each respective bar.

Figure 2. Enteroviral vp1 production is restricted to insulin-containing beta cells. (a) Analysis of serial sections stained for vp1, insulin and glucagon was used to determine the percentage of insulin-containing islets (ICIs; black bars) and of insulin-deficient islets (IDIs; white bars) that stained positively for vp1. The results presented are from 10 recent-onset type 1 diabetic cases which had vp1 positive endocrine cells; a total of 342 ICIs and 611 IDIs were analysed. Of these 269 and 16 were vp1 positive respectively. (b) Confocal microscopic analysis of representative islets reveals that vp1 (red; panels b,c,e,f) co-localised with insulin (green; panels a,c) but not with glucagon (panels d,f). Those stained in yellow are double-positive cells (panels c,f).

Figure 3. Comparison of the staining patterns obtained in Coxsackie-infected neonatal heart with two antisera raised against vp1. Sections of Coxsackie-infected neonatal human heart were stained with either the DAKO vp1 5D8/1 antibody (a) or the Kdf vp1 antibody (b).

Figure 4: Comparison of the staining patterns obtained in human type 1 diabetic pancreas with three different antisera raised against enteroviral antigens. Representative islets from two different type 1

diabetic pancreases, a-d, e-f, were stained with DAKO vp1 antibody (a,e), kdf vp1 (b,f), the anti-enteroviral antibody 9d5 (c) and insulin (d).

Figure 5. Examination of vp1 immunopositivity, production of PKR and hyper-expression of MHC class I in the islets of recent-onset type 1 diabetic patients and a patient with type 2 diabetes. (a) Two representative islets from recent-onset type 1 diabetic patients and one from a patient with type 2 diabetes were stained for insulin, vp1, protein kinase R (PKR) and MHC Class I (MHCI). The upper panel shows an insulin-containing islet (ICI) and the middle panel an insulin-deficient islet (IDI) from a patient with type 1 diabetes (T1D), while the lower panel shows an islet from a type 2 diabetic patient (T2D). In the lower panel note that the same cells within the islet stain with vp1 and PKR on these adjacent sections. (b) Detailed analysis of 177 insulin-containing islets (ICIs) from 6 representative vp1 positive type 1 patients demonstrates that vp1 is associated with PKR production in 154 (87%) of these ICIs.

Figure 6. Immunostaining of vp1 in pancreas sections from patients with type 2 diabetes. Representative islets from a normal adult (Ai) or a patient with type 2 diabetes (Aii) were stained for vp1. In these cases, centroacinar (Aiii) and smooth muscle A(iv) cell staining was also observed. (B): analysis of the percentage of cases from each subject group that have at least 1 intensely stained cell within an islet. (C): detailed analysis of the frequency of vp1 immunopositivity within insulin-containing islets in patients with type 1 diabetes (n=10), type 2 diabetes (n=6) and normal adults (n=7). (D): detailed analysis of PKR and vp1 production in up to 50 randomly selected ICIs from 9 Type 2 diabetic patients (339 islets; black bars) and 9 normal adult (403 islets; white bars).