Expression of the enteroviral capsid protein, VP1, in the islet cells of patients with type 1 diabetes is associated with induction of protein kinase R and down-regulation of Mcl-1

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Running Title: VP1, PKR and Mcl-1 expression in the islet cells in type 1 diabetes

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Main Text Word Count: 3998 Abstract Word Count: 250

Number of tables: 2 (2 Online Supplementary)

Number of figures: 6

Key words: enterovirus; immunocytochemistry; apoptosis; islet autoimmunity; anti-viral response.

Abstract

Aims/hypothesis: Immunohistochemical staining reveals that the enteroviral capsid protein, VP1, is present at higher frequency in the insulin-containing islets of patients with recent onset type 1 diabetes than in controls. This is consistent with epidemiological evidence suggesting that enteroviral infection may contribute to the autoimmune response in type 1 diabetes. However, immunostaining of VP1 is not definitive since the antibody widely used to detect the protein (Dako 5D8/1) might also cross-react with additional proteins under some conditions. Therefore, we sought to verify that VP1 immunopositivity correlates with additional makers of viral infection.

Methods: Antigen immunoreactivity was examined in formalin-fixed, paraffin-embedded, pancreases from two different collections of type 1 diabetes and control cases; a historical collection from the UK and the nPOD cohort (USA).

Results: VP1 immunoreactivity was present in ~20% of insulin-containing islets of both cohorts under stringent conditions but was absent from insulin-deficient islets. VP1 expression was restricted to beta cells but only a minority of these expressed the antigen. The innate viral sensor, protein kinase R (PKR) was up-regulated selectively in beta cells that were immunopositive for VP1. The anti-apoptotic protein, Mcl-1, was abundant in beta cells that were immunonegative for VP1 but Mcl-1 was depleted in cells expressing VP1.

Conclusions/interpretation: The presence of immunoreactive VP1 within beta-cells in type 1 diabetes is associated with a cellular phenotype consistent with the activation of antiviral response pathways and enhanced sensitivity to apoptosis. However, definitive studies confirming whether viral infections are causal to beta-cell loss in human diabetes are still awaited.

Introduction

Considerable circumstantial evidence has accumulated to suggest that enteroviral infection of pancreatic islet beta cells may contribute to the development of autoimmunity in some patients with type 1 diabetes [1-4]. This is important since, if verified in a majority of cases, it may provide a means to minimise the future development of type 1 diabetes in susceptible individuals via a targeted vaccination program.

The evidence implicating enteroviral infection of pancreatic beta cells has arisen from a variety of sources including the successful culture of live enteroviral strains from within the pancreases of individuals with type 1 diabetes and the capture of electron microscopic images showing the generation of viral arrays within the beta cells of patients [5, 6]. Additionally, the common enteroviral capsid protein, VP1, has been detected immunologically in the islet cells of patients with recent onset type 1 diabetes, at much higher frequency than in age-matched controls [5, 7]. Arguably, this latter evidence is the more persuasive for several reasons. In particular, it has been noted that the enteroviral strains isolated during pancreas culture are of uncertain provenance [8] and the electron microscopic evidence of viral particle assembly is derived from only a very small number of cases [5]. By contrast, enteroviral VP1 was detected at high frequency in the islets of more than 60% of patients with recent onset type 1 diabetes in a large UK cohort [7]. Importantly, however, it must also be recognised that the immunolabelling of enteroviral VP1 in islet cells remains equivocal since the specificity of the principal antibody employed in these studies (Dako 5D8/1) has been questioned (although its ability to detect enteroviral VP1 is not in debate) [9]. Furthermore, the presence of viral antigen is detected much more readily than viral RNA in islet cells under most conditions. Therefore, it is important that additional evidence is gained to evaluate further the hypothesis that the immunodetection of enteroviral VP1 in the islet beta cells of patients with recent onset type 1 diabetes is associated with an underlying viral infection.

In the present study we have addressed this issue in two ways. Firstly, we have made use of a second (much more modern) collection of pancreas samples from within the JDRF nPOD (Network of Pancreatic Organ donors with Diabetes) program [10] and have compared the staining patterns obtained using the 5D8/1 anti-VP1 serum in these samples with that obtained previously in the historical UK cohort. This is important since the UK samples were collected between 30-50 years ago and were harvested at autopsy in random locations across the UK, where entirely different methods of recovery and fixation were employed [11]. By contrast, the nPOD program was established much more recently (the collection began in 2007) in a geographically distinct region (the USA) and uses standard operating procedures to ensure consistency of recovery and processing of the samples [10]. Secondly, we have evaluated whether, in both cohorts, the detection of VP1 at the level of individual islet cells under stringent conditions, is directly correlated with additional markers consistent with the establishment of an anti-viral response. In particular, we have monitored the expression of two further proteins, protein kinase R (PKR) and myeloid cell leukemia sequence-1 (Mcl-1) in concert with VP1. PKR is an enzyme responsible for the activation of antiviral cascades within cells and is known to be induced during enteroviral infection of cells [12, 13]. Mcl-1 is an anti-apoptotic protein which is subject to rapid turnover in cells such that it is degraded quickly during the translational arrest that ensues following viral infection [14]. Accordingly, we present a detailed analysis of the correlations between the immunodetection of VP1, induction of PKR and the level of Mcl-1 in individual beta cells in patients with type 1 diabetes.

METHODS

Subjects. Pancreases recovered from 6 patients with recent onset type 1 diabetes mellitus (disease duration ≤ 18 months) and one with type 1 diabetes for 12 years were selected from within a UK cohort used previously [7]. The specimens were studied with ethical approval and had been variously fixed in buffered *p*-formaldehyde, un-buffered formol saline or Bouin's fixative. They were all paraffin-embedded. 5 of the 7 pancreases were removed at autopsy and two were recovered at the time of organ donation (D4 and D5). The patients were typically between 1-18 years old but one was 42y, raising the overall mean to 19.0±5.0y (ESM Table 1). A second cohort of 17 type 1 diabetes mellitus pancreases (mean age: 25.7±2.9y) and 12 age-matched non-diabetic controls (mean age: 27.9±7.1 years) was obtained through the JDRF Network for Pancreatic Organ Donors with Diabetes (nPOD) program (Table 1 and ESM Table 2) [10].

Immunohistochemistry. Sections (4μm; mounted on glass slides coated in (3-Aminopropyl)-triethoxysilane; Sigma, UK) were processed and labelled using a standard immunoperoxidase technique [7, 15, 16]. With the exception of insulin, all antigens were unmasked by heating in 1mM EDTA (Sigma, UK) buffer pH8.0, in a pressure cooker in a microwave oven on full power (800W) for 20 min, followed by 20 min of cooling at room temperature. Primary antibodies were applied as detailed in ESM Table 3 and Dako REAL™ Envision™ Detection System (Dako, UK) 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.

Combined immunofluorescence. For co-localisation studies, anti-insulin and anti-VP1 immunoreactivity were detected using an AlexaFluor® 488-conjugated anti-guinea pig

antibody and an AlexaFluor® 568 conjugated anti-mouse antibody respectively (Invitrogen, Paisley, UK). To determine if the enteroviral VP1 protein co-localised with either PKR or Mcl-1 in beta cells, primary antibodies were incubated as described in Online Supplementary Table 2. The primary antibodies were detected with relevant goat secondary antibodies conjugated to AlexaFluor® 488 or 568 (Invitrogen) or with goat anti-guinea-pig DyLight 405 (Stratech, UK). Control sections were stained with relevant primary and secondary antisera to confirm that no cross-reactivity was detected. Sections were mounted in Vectashield hard-set mounting medium (Vector Laboratories, Peterborough, UK) under glass cover slips. Images were captured using a Nikon Eclipse 80*i* microscope (Nikon, Surrey, UK) and overlaid using NIS-Elements BR 3.0 software (Nikon) to study the relative localisation of each antigen. Sections directly adjacent to those stained with the combined method were stained with an anti-glucagon antibody (rabbit; Dako) or an anti-insulin (guinea-pig; Dako) using a standard immunoperoxidase technique in order to determine total islet numbers in the sections and to distinguish insulin containing islets (ICIs) from insulin deficient islets (IDIs).

RESULTS

Enteroviral VP1 is frequently detected in islets of type 1 diabetes patients from both the UK and nPOD cohorts. Previous analysis of the UK cohort had revealed the expression of the enteroviral VP1 protein in the insulin-containing islets (ICIs) of 44 of 72 (61%) recent-onset type 1 diabetes patients. This compared with only 3 of 50 paediatric and neonatal controls (Figure 1) [7]. The recent-onset type 1 diabetes pancreas samples collected within the UK cohort are unique in that they represent about half of all such cases described worldwide and because they come from individuals with short duration of disease. However, they were harvested between 30-50 years ago and were fixed using non-standardised protocols [11]. We considered it important, therefore, to establish whether enteroviral VP1

staining is also present in a different cohort, collected more recently, from a geographically different population, using more standardised protocols. The JDRF nPOD collection provided this opportunity [10]. A comparison of the samples available within these two cohorts is shown in Table 2 and it is noteworthy that the donor with the shortest duration of disease among the nPOD samples had been diabetic for 1 year. Moreover, the mean disease duration among this group was approximately 12 years. This compares with an average disease duration of less than 1 year in the UK cohort (Table 2).

Initial experiments using an enteroviral VP1 antibody (5D8/1) were performed to determine the optimal concentration for routine use which allowed clear immunodetection of VP1 with minimal background staining. 17 type 1 diabetes nPOD cases and 12 non-diabetic controls were then immunostained (Table 1 and ESM Table 2). Among the nPOD controls, only 1 case was found in which a combined total of more than 5 individual islet cells were immunopositive for VP1 in the entire pancreas section. Accordingly, this value was chosen to represent the threshold limit to define immunopositivity for VP1 in control samples. By definition, then, 1 of 12 (8.3%) non-diabetic controls was judged to be immunopositive for VP1 using this criterion (Figure 1). By contrast, multiple intensely vp-1 positive islet cells were observed in many insulin-containing islets (ICI) in 8 of the 17 nPOD type 1 diabetes cases. Of the remaining 9 cases, 7 were immunonegative for insulin. Hence, expression of VP1 was detected at well above the threshold level in the islet cells of 8 of the 10 nPOD cases (80%) in which ICI were present (Figure 1 and Table 1). Representative VP1+ ICIs for both cohorts are shown in Figure 1A and B and the data are presented graphically in Figure 1C.

The absolute number of enteroviral VP1+ islet cells is elevated in organ donor cases by comparison with those recovered at autopsy. Quantification of the frequency of VP1+ ICIs in 7 VP1+ nPOD cases and 7 VP1+ UK cases was performed by counting the total number of

islets containing one or more VP1+ cells within a given section. Analysis of both cohorts revealed that 65 of 227 (28.6%) islets from within the nPOD cases contained VP1+ cells; this compared with 77 of 374 (20.6%) islets within the UK cohort (Figure 2A, Table 1 and Supplementary Table 1). Therefore the frequency of VP1 immunopositive ICIs was similar between the two cohorts. However, when the absolute number of VP1+ cells within any given islets was studied and compared between the two cohorts, initial evidence implied that this number might be elevated in the nPOD cases (Figure 1). Therefore, in order to assess this more formally, a minimum of 20 randomly selected VP1+ islets were imaged from among the nPOD organ-donors (nPOD OD), UK organ-donors (UK OD) and UK post-mortem (UK PM) cases. The total number of endocrine cells contained within the imaged islets was recorded and correlated with the number of VP1+ endocrine cells. This revealed a significant increase in the number of individual VP1 positive endocrine cells present within any given islet among the organ donor samples (nPOD OD – 5.52±0.90%; UK OD – 5.10±0.87%; Figure 2B) compared to the post-mortem cases (1.76±0.32%; $p<0.001$). Therefore the frequency of VP1+ islet cells seen in organ donors was similar in both cohorts (whether from the USA or the UK).

Enteroviral VP1 co-localises with insulin in both the UK and the nPOD cohort. The observation that VP1+ islet cells were confined only to those nPOD cases in which ICIs were also present, implies that VP1 might be preferentially localised within beta cells (as was reported previously for the UK cohort [7, 17]). In order to confirm this, 3 of the nPOD VP1+ cases were stained for the presence of both VP1 and insulin using dual immunofluorescence. A total of 31 VP1+ ICIs were identified. Overlaying the fluorescent images (Figure 3) revealed that VP1+ immunopositivity always co-localised with insulin. A similar situation pertained within the UK cohort. Interestingly, it was noted anecdotally, that the intensity of

insulin staining appeared to be reduced in the VP1+ beta cells when compared to surrounding VP1- beta cells.

Enteroviral VP1 co-localises with PKR in patients with type 1 diabetes. Enhanced expression of the pathogen recognition receptor PKR has been observed in VP1+ islets within the UK recent-onset type 1 diabetes patients [7] although it was not established whether VP1 and PKR are preferentially localised within the same cells. To study the relative localisation of VP1 and PKR, dual immunofluorescence staining was performed in pancreas samples from both the UK and the nPOD cohorts. Within the 7 UK type 1 diabetes patients, 374 of 866 islets (43.1%) contained cells that were immunopositive for insulin and VP1 was detected in 77 of these. Dual immunofluorescence staining confirmed that the VP1+ cells always co-expressed protein kinase R. A representative image of one such islet is presented in Figure 4. Similar co-localisation of VP1 and PKR within individual beta cells was also observed in the 31 VP1+ islets of the nPOD type 1 diabetes cases (Figure 4). PKR expression in surrounding cells tended to be slightly elevated with respect to background in the nPOD cases when compared to the UK cohort, where it was only the VP1+ cells which displayed clear immunoreactive PKR expression.

Mcl-1 is present in beta cells but its expression is reduced in those cells containing VP1. PKR exists in both active (phosphorylated) and inactive (dephosphorylated) forms but it may be misleading to infer changes in specific protein phosphorylation patterns by immunological means in FFPE tissue due to potential changes arising during tissue fixation and processing. Therefore, we employed an alternative approach to assess the activity status of PKR in the islet cells of T1D patients. This was based on the understanding that the anti-apoptotic protein Mcl-1 is subject to rapid turnover in cells and that its levels decline quickly under conditions when protein translation is attenuated [18]. Since an important consequence of the activation of PKR is to inhibit protein translation in response to viral infection (via

phosphorylation of eIF2α) we reasoned that Mcl-1 levels should decline in cells where PKR becomes activated. Therefore, the expression of Mcl-1 was evaluated in human FFPE pancreatic tissue and, more specifically we examined the intensity of immunostaining of Mcl-1 in VP1+ cells (which, as shown above, always have elevated PKR) within the islets of the type 1 diabetes cases. Immunoperoxidase staining of non-diabetic control pancreas revealed intense staining of a subset of islet endocrine cells (Figure 5) confirming the presence of Mcl-1 within certain islet cells. Dual immunofluorescence labelling revealed that these were beta cells (Figure 5). Importantly, the intensity of staining of Mcl-1 was markedly reduced (and was frequently undetectable) in those islet cells which were immunopositive for VP1. Example images are shown in Figure 6. To verify these data, 152 individual VP1+ islet cells were examined across a total of 8 different type 1 diabetes cases and scored for the presence of Mcl-1. Mcl-1 immunostaining was reduced (or was undetectable) in all cells expressing VP1, whereas Mcl-1 was stained much more intensely in adjacent, VP1-immunonegative, beta cells.

DISCUSSION

Previous studies have shown that, in patients with recent-onset type 1 diabetes, a proportion of islets contain cells in which the enteroviral capsid protein, VP1, can be detected by immunocytochemistry [5, 7]. This conclusion was first drawn by Dotta et al. based on studies of 5 patients with type 1 diabetes from Italy [5] and was substantiated by the analysis of a much larger cohort of samples collected from within the UK. These had been harvested from patients (mainly children) who died between 30-50 years ago and, among whom, the mean duration of diabetes was 8 months [7]. In the present work, it is shown that pancreas samples from a third group of patients with type 1 diabetes (who died within the USA and were collected since 2007) also contain islets that stain positively for enteroviral VP1. A group of relevant age-matched controls was employed to verify the selectivity of immunolabelling in

each cohort and the results revealed that, while VP1 is occasionally detected in the islets of non-diabetic individuals, it is found much more abundantly in the cells of patients with type 1 diabetes. As such, these data support the earlier conclusion that enteroviral infection of islet cells may be a characteristic feature of patients with type 1 diabetes in the Western world [7, 19]. The current study also reveals that this feature is seen not only in patients with recent-onset disease but also in a group with longer-standing type 1 diabetes, since the mean duration since diagnosis of diabetes in the nPOD samples analysed, was almost 12 years. This, in turn, reinforces the view that the nature of the infection occurring in the islet cells in type 1 diabetes is unusual in that it may persist over long periods rather than developing as an acute, lytic infection that is more typical of enteroviruses [19]. The results also show that both the historical UK collection and the nPOD samples represent suitable material for analysis of this infection in detail.

The deduction that the enteroviral infection is persistent, may offer an explanation for our observation that the enteroviral capsid protein, VP1, is found in relatively few of the total number of islet cells available within each patient. As such, it has been proposed that detection of VP1 may be simply the "tip of an iceberg" in which many more cells harbour a latent infection and that, occasionally, this is manifest by the activation of viral protein synthesis within defined endocrine cells [19]. Mechanisms by which enteroviruses can persist in a latent form without causing large-scale cell lysis have been described [20-24] and we have confirmed that cultured mammalian cells which harbour such persistent infections *in vitro* are not routinely immunopositive for VP1 (SJ Richardson & NM Chapman; unpublished observations). However, the mechanism by which viral persistence is achieved in type 1 diabetes has not been defined and must await the final isolation and characterisation of the relevant viral serotype(s).

A second important consideration arising from the present work is that immunodetection of VP1 should be undertaken only after rigorous optimisation of the staining pattern in pancreas samples. The most widely employed and robust antiserum available currently for immunodetection of VP1 in formalin fixed, paraffin embedded tissue samples is the 5D8/1 monoclonal serum (Dako). However, we have noted that, in non-diabetic and diabetic pancreas samples from both the UK and nPOD cohorts, this serum can generate intense positivity in a wide range of endocrine and exocrine cells when employed at low dilution [7]. By contrast, when titrated to high dilution, the majority of this staining is lost and only occasional single, strongly immunopositive cells remain. In both cohorts these were exclusively beta cells, as judged by the pattern of co-staining with antisera to islet hormones. This is consistent with the earlier finding that beta cells express surface receptors capable of mediating enteroviral entry [25, 26].

It is of interest that the dilution of clone 5D8/1 required for optimal immunostaining of nPOD samples was much higher (1:2000) than that which proved optimal for the UK cohort (1:500) and this may reflect the better tissue preservation of the former samples. In support of this, it was noted, too, that the mean number of VP1 immunopositive cells per islet (>5%) was greater in those samples recovered from organ donors (where tissue preservation was at a premium) compared with those collected at autopsy (<2%; where tissue preservation is less controlled). This finding held true both among the older UK cohort and within the more recent nPOD samples, such that the mean number of VP1 immunopositive cells per islet was similar in both organ donor groups (5.1 vs 5.5% respectively). This could be taken to imply that the frequency of enteroviral infection of beta cells has not changed dramatically over time or with geographical location between these two groups.

A further critical issue is the identity of the immunoreactive protein labelled by the anti-VP1 serum in the samples studied here. It is well-accepted that clone 5D8/1 binds to enteroviral

VP1 encoded by a wide range of viral serotypes, but there remain hints in the literature that it may also bind to additional proteins (albeit with lower affinity) under certain conditions [9, 27]. Indeed, as indicated above, we find that, at low dilution, the serum stains multiple cells (within and outside islets) in both control and type 1 diabetes patients [7] but that this issue is resolved at higher dilution. Nevertheless, it cannot be formally excluded that an additional (non-viral) target is labelled even under the more stringent conditions. However, if this is the case, then we now show that this response only occurs in cells which display certain critical additional features. In particular, we have studied samples from both the UK and nPOD cohorts and find that, remarkably (and without exception) beta cells which are immunopositive for VP1 also show intense immunostaining of the pathogen recognition receptor, PKR. This implies that the total amount of PKR protein is enhanced in these cells, presumably via induction of mRNA synthesis and translation. Previously, we had provided evidence (using serial sections examined by immunohistochemistry) that the same islets are stained positively by both antisera [7] but the present work now reveals that this staining is co-localised within the same individual beta cells. Hence, it can be concluded that these specific cells are mounting a response to an insult that is directly associated with VP1 production. Since enteroviral infection has been shown to markedly up-regulate PKR expression in islet cells at the mRNA level [12, 13], the precise conjunction of the two phenomena monitored here, adds weight to the conclusion that VP1 expression is representative of underlying enteroviral infection. Having made this point, however, it must also be accepted that, in principle, the expression of PKR might also be induced by other (non-viral) stressors in beta cells. However, if this is the case, then it still remains true that the anti-VP1 serum must be capable of selectively labelling a unique subset of beta cells which are experiencing atypical stress.

To advance these arguments further, we also investigated the status of Mcl-1 expression in islet cells in type 1 diabetes. Mcl-1 is an anti-apoptotic member of the Bcl-2 protein family, which due to unique motifs in its N-terminal domain, is subject to rapid turnover under normal conditions [18]. Therefore, in order to maintain adequate levels of Mcl-1 within the cell, the protein must be synthesised and replenished at a rate which is at least equal to its rate of degradation. Under conditions when PKR is activated, the translation initiation factor eIF2α becomes phosphorylated, leading to translational arrest and thereby to a reduction in the rate of Mcl-1 synthesis [14, 28]. As a result, Mcl-1 levels are expected to decline. This scenario has been confirmed recently in *in vitro* studies where it was shown directly that treatment of cultured beta cells with the viral dsRNA mimetic, poly-I:C (which will activate PKR) led to rapid loss of Mcl-1 from the cells [14].

In the present analysis, a similarly striking relationship was observed since, of 152 individual VP1 immunopositive beta cells studied in detail (across 8 different cases) all showed loss of immunoreactive Mcl-1 by comparison with those cells immediately adjacent to them. Since we have proven that VP1 expression is invariably correlated with PKR induction in beta cells in type 1 diabetes, these results also reveal that the PKR must have become activated under these conditions and that the cells had entered an anti-viral state in which bulk protein translation is arrested. As such, all of these features are fully consistent with the concept that viral infection underlies the response. If this is not the case and a specific subset of beta cells are responding to an unrelated stimulus (which, by chance, is identified uniquely, and with high affinity, by the anti-VP1 serum) then this stimulus must also be capable of causing the selective up-regulation and activation of PKR in only a small proportion of the available beta cells, thereby driving the loss of Mcl-1. Irrespective of the initiating mechanism, it should be emphasised that the loss of Mcl-1 is likely to render the cells more responsive to the cytotoxic

actions of pro-inflammatory cytokines present within the islet milieu and that this could then play a role in their ultimate demise.

On the basis of the data reported here, we suggest that the presence of immunoreactive VP1 within beta-cells in human type 1 diabetes is associated with a cellular phenotype that could be indicative of viral infection and enhanced sensitivity to apoptosis. However, definitive studies confirming whether viral infections are causal to beta-cell loss in type 1 diabetes are still awaited.

Acknowledgements

This research was performed with the support of the Network for Pancreatic Organ Donors with Diabetes (nPOD), a collaborative type 1 diabetes research project sponsored by the Juvenile Diabetes Research Foundation International (JDRF). Organ Procurement Organizations (OPO) partnering with nPOD to provide research resources are listed at www.jdrfnpod.org/our-partners.php. The work was supported by funding from the [European Union's] Seventh Framework Programme PEVNET [FP7/2007-2013] under grant agreement number 261441 and from a Diabetes Research Wellness Foundation Non-Clinical Research Fellowship to S.J.R.

S.J.R. was responsible for study conception and design, acquired, analysed and interpreted data and drafted the manuscript. P. L. acquired and analysed data and edited the manuscript. A.J.B. contributed to the study design and revised the manuscript. A.K.F. collected samples, contributed to study design and conception and revised the manuscript. N.G.M. was responsible for study conception and design, interpreted data and drafted the manuscript. All authors approved the final version. The authors have no duality of interest to declare.

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TABLE 1: nPOD Type 1 diabetes patients and non-diabetic controls

Donor	T1D/ Non-diabetic	Age	Duration of T1D	Block	VP1 status	Insulin
nPOD 6026	T1D	22.4	14 years	PanBody-01	Negative	No ICIs
nPOD 6031	T1D	39	35 years	PanBody-01	Negative	No ICIs
nPOD 6038	T1D	37.2	20 years	PanBody-01	16 ICIs with multiple VP1+ cells	98 ICIs
nPOD 6039	T1D	28.7	12 years	PanBody-01	Negative	No ICIs
nPOD 6040	T1D	50	20 years	PanTail-01	Negative	No ICIs
nPOD 6046	T1D	18.8	8 years	PanBody-01	6 ICIs with multiple VP1+ cells	14 ICIs
nPOD 6051	T1D	20.3	13 years	PanTail-01	4 ICIs with multiple VP1+ cells	4 ICIs
nPOD 6052	T1D	12	1 year	PanTail-01	5 ICIs with multiple VP1+ cells	10 ICIs
nPOD 6063	T1D	4.4	3 years	PanBody-01	Negative	No ICIs
nPOD 6069	T1D	23	7 years	PanBody-01	Negative	ICIs
nPOD 6070	T1D	22.6	7 years	PanBody-01	23 ICIs with multiple VP1+ cells	61 ICIs
nPOD 6081	T1D	31.4	15 years	PanBody-02	Negative	Multiple ICIs
nPOD 6084	T1D	14.2	4 years	PanBody-01	8 ICIs with multiple VP1+ cells	34 ICIs
nPOD 6088	T1D	31.2	5 years	PanHead-03	3 ICIs with multiple VP1+ cells	6 ICIs
nPOD 6113	T1D	13.1	1 year	PanBody-01	Negative	No ICIs
nPOD 6121	T1D	33.9	4 years	PanOTHER-04	Numerous ICIs with multiple VP1+ cells[a]	Multiple ICIs [a]
nPOD 6141	T1D	36.7	28 years	PanBody-04	Negative	No ICIs

[a] The number of VP1+ islets and ICIs were not analysed in this case due to the unusual morphology of the tissue.

TABLE 2: A comparison of the two cohorts from the UK and nPOD

	UK Cohort	**nPOD Cohort**
Number of cases	72	17
Mean Age	12.7±1.1y	25.7±2.9y
Age Range	1-42y	4-50y
Mean Time since diagnosis	8.2±4.1mths	11.9±2.3y
Range Time since diagnosis	0-6y	1-35y
Geography	Scotland, England and Wales	USA
Sample Collection	1959-1983	2007 onwards
Autopsy/ Organ Donors	Autopsy and organ donors	Organ Donors
Sample Processing	Variable fixation types and times	Strictly controlled

FIGURE LEGENDS

FIG. 1. Representative immunohistochemical images of enteroviral VP1 expression in the (a). UK (D4) and (b). nPOD cohorts (6052-01). (c). The proportion of islets staining intensely positive for VP1. White bars – non diabetic controls; hatched bars – type 1 diabetes cases with insulin-containing islets (ICIs); black bars – type 1 diabetes cases containing only insulin-deficient islets (IDIs).

FIG. 2. a: Detailed analysis of the proportion of ICIs with VP1+ cells in 166 ICIs from the nPOD cohort (black bars) and from 374 ICIs from the UK cohort (white bars). b: Analysis of the percentage of total endocrine cells that are VP1+ in UK post-mortem (PM) cases (white bars; n=21 islets), UK organ donor (OD) cases (black bars; n=20 islets) and nPOD OD (hatched bars; n=25 islets).

FIG. 3. Photomicrographs of representative islets from the nPOD (upper panel; 6052-01) and UK (lower panel; D4) cohorts reveals that VP1 (red; c, d) co-localises with insulin (green; a, b). Double positive cells are stained yellow and are visible in e and f. Nuclear DAPI staining is shown in blue in the merged images (e and f).

FIG. 4. Photomicrographs of representative islets from the nPOD (upper panel; 6084-01) and UK (lower panel; D4) cohorts reveals that VP1 (green; a, b) co-localises with PKR (red; c, d). Double positive cells are stained yellow and are visible in e and f. Nuclear DAPI staining is shown in blue in the merged images (e and f).

FIG. 5. A. Light microscopic image of Mcl-1 expression in the islet of a non-diabetic control demonstrates intense staining of a subset of islet endocrine cells. b-d. Fluorescence microscopic analysis of a representative islet from a UK case reveals that Mcl-1 (red; c) co-

localises with insulin (green; b). Double positive cells are stained yellow and are visible in d. Nuclear DAPI staining is shown in blue in the merged images (d).

FIG. 6. Photomicrographs of a representative islet from a UK case reveals that the VP1+ cells (green staining outlined in yellow); a) have reduced Mcl-1 expression (red; b) when compared to surrounding beta cells that are shown to express high levels of Mcl-1 (purple in merged image (c). Autofluorescent red blood cells are indicated with asterisks (*).