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A search for evidence of viral infection in pancreases of newly diagnosed patients with IDDM

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Summary Techniques were developed to look for evidence of viral infection in formalin-fixed paraffin-embedded autopsy pancreatic tissues from patients who had died of recent-onset insulin-dependent diabetes mellitus. DNA extracted from 47 pancreases in which good DNA preservation was confirmed was analysed by a polymerase chain reaction for Epstein-Barr virus and by a nested polymerase chain reaction for cytomegalovirus. Histological sections from 29 pancreases in which there was good RNA preservation were tested for the presence of enterovirus and Epstein-Barr virus using in situ hybridization techniques. Seventy-five pancreases were analysed immunohistochemically for the presence of mumps virus. None of these viruses could be detected in any of the diabetic pancreases studied. Control studies suggested that the techniques employed were as sensitive as culture done at the time of autopsy. Pancreas was available for study in 9 infants who had died of myocarditis; enterovirus was demonstrable in islets in 5 of these cases. An acute or persisting infection in the pancreas at the time of clinical onset of insulin-dependent diabetes by any of the 4 virus included in this study seems unlikely. [Diabetologia (1997) 40: 53–61]

Keywords Insulin – dependent diabetes mellitus, virus, enterovirus, myocarditis, mumps virus, Epstein-Barr virus, cytomegalovirus.

The most dramatic evidence for a role of viral infection in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) was the culture of a coxsackievirus B_4 from the autopsy pancreas of a 10-year-old boy who died of newly diagnosed diabetes [1]. Following inoculation of the viral isolate into mice, the animals developed islet endocrine cell necrosis and diabetes. Virus could be demonstrated in the murine insulin containing beta cells. Previously, an untyped coxsackievirus B had been demonstrated by immunofluorescence in the pancreas of a 5-year-old child who had died of IDDM and myocarditis 3 weeks after cardiac surgery [2]. There has also been supporting serological evidence linking enteroviral infection with IDDM. Coxsackievirus B specific IgM responses were detected in 29–33 % of recently diagnosed diabetic patients, but only in 5–8 % of normal control subjects [3].

Other viruses have been implicated in the aetiology of IDDM. Mumps infection resulted in the transitory appearance of islet cell antibodies in 50 % of infected children [4] and these antibodies persisted in some of those patients infected with mumps who also had subclinical pancreatitis [5]. Patients with congenital rubella have a considerably increased incidence of clinical diabetes [6], the disease tending to occur in those patients who also carry the HLA related genetic predisposition [7]. Analysis of DNA from peripheral blood lymphocytes showed evidence of cytomegalovirus (CMV) infection in 22 % of newly diagnosed IDDM patients but only in 2.6 % of control subjects [8]. Islet cell membranes from newly diagnosed diabetic pancreases co-cultured with

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Abbreviations: IDDM, Insulin-dependent diabetes mellitus; CMV, cytomegalovirus; EBV, Epstein-Barr virus.

peripheral blood lymphocytes from normal donors preferentially stimulated V beta 7-positive T-cell clones out of 24 V beta T-cell receptor families tested, suggesting the presence of a superantigen in the islet cell membranes [9]. Acute infectious mononucleosis caused by Epstein-Barr virus (EBV) is associated with selective over-expression of V beta 7 and V beta 6.1-3 T cells [10] and patients with newly diagnosed diabetes have been shown to have an abnormality of EBV specific immune responses [11]. These results raised the possibility that EBV infection in diabetic islets could cause a superantigen effect.

Evidence has accumulated to show that IDDM is a disease of slow evolution, with islet cell antibodies being present many years before clinical diagnosis [12]. Thus, the concept of a one hit cytopathic event by a virus causing beta-cell necrosis and diabetes has recently lost favour. However, many of the viruses thought to have a putative role are known to be capable of causing persistent or latent infection in man. The observation that beta cells in IDDM expressed interferon-alpha, a cytokine known to be induced by viral infection, supported the possibility that these cells harboured such persisting viruses [13].

The aim of the present study was to develop techniques for demonstrating the presence of candidate diabetogenic viruses in fixed archival autopsy pancreas that were as sensitive as viral culture of fresh autopsy tissue. If this could be done, an assessment could be made of how unusual was the culture of a virus from the pancreas of a patient with newly diagnosed IDDM [1]. Specifically, experiments were done to look for the presence of CMV, coxsackievirus B, Mumps virus and EBV.

Subjects, materials and methods

Selection of cases. Previous studies from this laboratory have involved the collection of formalin-fixed paraffin-embedded pieces of autopsy pancreas from patients with IDDM. Many of these patients died at the initial clinical presentation of their disease [14]. All 75 pancreases selected for the present study had residual beta cells but further selection of cases was required for the different studies looking for CMV, EBV and coxsackie viruses, as described below.

CMV study. A nested polymerase chain reaction (PCR) technique was used to detect CMV (see below). DNA was extracted from single 10 μm pancreatic sections and only those cases where the single copy β-globin gene was amplifiable in a separate PCR reaction were included. Thus, cases with poor preservation of tissue DNA were excluded. Of a total of 75 pancreases from patients with IDDM 47 satisfied these criteria. The age range of the patients was 6 months to 42 years (mean 11.9 years), the sex ratio was 27 female to 20 male, the duration of disease was known to be less than 1 year in 35 patients and insulitis was present in 42. Also included in the study were 41 normal autopsy pancreases (9 from adults and 32 from children), tissues from 5 patients with clinical CMV infection and autopsy pancreas from 10 patients who had died from AIDS, 3 of which had occasional CMV-infected cells demonstrable on routine immunocytochemistry.

Coxsackievirus study. In situ hybridization was employed for this part of the study and only those diabetic pancreases in which insulin mRNA and poly A mRNA tails could be detected by appropriate in situ hybridization methods were included. This ensured that only cases where there was good preservation of RNA in the tissues were studied. Pancreatic tissue from 29 of the 75 cases of IDDM fulfilled these requirements. The duration of disease was known to be less than 1 year in 20 and insulitis was present in 25. The age range was 1–42 years with an average of 12.9 years and 10 patients were male. Other tissues examined in this study were a range of organs, including heart, from 14 children age range 3 days–10 months), who had died of acute myocarditis. Here there were 12 males and 2 females and a coxsackievirus B was cultured either in life or at autopsy in 9 cases. In the remainder, either no culture was done, or the result was not available.

Mumps virus study. An immunocytochemical method was used to detect mumps virus. Protein preservation, as witnessed by immunocytochemical demonstration of insulin, was present in all 75 diabetic pancreases collected. The age range of the patients was 6 months to 42 years with an average of 11.8 years. There were 45 females and 30 males and 56 patients were known to have been clinically diabetic for less than 1 year. The maximum duration of disease was 12 years and insulitis was present in 62 cases. Other tissues examined included a variety of autopsy organs from 3 children (age range 4–16 years, 2 males, 1 female) who had died of mumps. Two of these patients had died of encephalitis and one of laryngeal oedema.

EBV studies. Two different methods were used – a PCR technique looking for viral DNA and an in situ hybridization technique to detect viral RNA. The PCR study examined the same 47 diabetic pancreases which were used in the CMV study, i. e. those pancreases where the β-globin gene was amplifiable. In situ hybridization for EBV RNA was performed on the same 29 diabetic pancreases as those used in the coxsackievirus study. These pancreases had well-preserved mRNA as described above. Two positive controls for EBV infection were included in each study. The first was a surgically removed formalin-fixed paraffin-embedded tonsil from a young man with a diagnosis of infectious mononucleosis. The second was a post-cardiac transplant lymphoproliferative lesion present in the lung of a 57-year-old male. The material studied had been removed at an autopsy performed 24 h after death.

Laboratory methods

CMV study. The protocol used in this study has been described previously [15]. Briefly, tissue sections 10 μm thick were cut into sterile sample tubes ensuring no cross contamination between specimens. The wax was removed with xylene and alcohol and the sample dried under vacuum for approximately 10 min. The resulting tissue was digested overnight at 37 °C in 100 μl of digestion buffer containing 500 μg/ml proteinase K and then boiled for 10 min [16]. A single PCR reaction was performed with 10 μl of the resulting digest.

For the detection of CMV, DNA was amplified by PCR using a "Gene Amp" Kit (Perkin Elmer Cetus, Emeryville, Calif., USA). Specific oligonucleotide primers were synthesised by Oswel DNA Services, (University of Southampton, UK) to

a highly conserved region of the intermediate-early gene of CMV [17, 18]. The initial pair of primers, (CMVA1 and CMVA2) (Table 1), amplified a 167 base pair product. The second set of internal primers, (CMVB1 and CMVB2) (Table 1), produced a 125 base pair product. In both rounds of amplification initial denaturation was carried out at 94 °C for 8 min followed by cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C and 1 min elongation of 72 °C. The first round of amplification consisted of 40 cycles. Four per cent of the product was then subjected to a second round of 30 cycles with the internal primers. We ran 50 % of the final reaction mixture on a 2.5 % agarose gel and visualised by ethidium bromide staining and transillumination in ultraviolet light.

Only samples in which the β-globin gene could be detected were included in the CMV study (see above). Oligonucleotide primers (BG1 and BG2, Table 1) were synthesized to the β-globin gene (Oswel) [19] and these amplified a 110 base pair product. The following cycling parameters were used: denaturation at 94 °C for 5 min followed by 50 cycles of 1 min denaturation at 94 °C, 30 s annealing at 60 °C and 1 min elongation at 72 °C and finally 1 cycle of extension at 72 °C for 5 min. The conditions for visualisation of the product were as used for the CMV technique.

Great care was taken to avoid contamination by using sterile techniques and reagents. In each PCR run there was a positive control (1 μl from a proteinase K digestion of a known positive CMV infected autopsy pancreas and 5 μl from a proteinase K digestion of normal tonsil for β-globin) and a negative control (distilled water).

In an effort to further test the sensitivity of the system for the detection of CMV, a single "owl's eye" CMV infected cell was microdissected from a section of infected neonatal pancreas along with a small amount of adjacent exocrine tissue (Fig. 1). DNA from this sample was extracted and amplified in the standard manner.

Coxsackievirus study. Details of the in situ hybridization protocol have been published previously [20, 21]. Cocktails of 6, 30 mer DNA nucleotides were used as probes in the technique. Five of these were complementary to sequences in the 5′ noncoding region of the virus and the sixth complementary to sequences 7213-7184. The probes were labelled at the 3′ end using the homopolymer tailing method, incorporating digoxigenin 11-dUTP using terminal deoxynucleotide transferase [20]. In previous studies these probes have been shown to detect coxsackievirus B1–5 in paraffin-embedded tissue sections of infected neonatal murine heart.

The basic method for the in situ hybridization technique in our laboratory has been described [22], but slight variations were used in this study. Following dewaxing and rehydration the slides were pre-treated in 2 × Salt sodium citrate at 70 °C for 30 min rather than Triton X for 15 min as previously described. The coxsackievirus probe cocktail was used at a concentration of 0.1 ng/μl on 2 serial sections predigested with proteinase K at 20 and 30 μg/ml respectively at 37 °C for 30 min. Hybridization was allowed to occur overnight at 42 °C. An alkaline phosphatase conjugated sheep anti-digoxigenin (Boehringer Mannheim UK, Lewes, East Sussex, UK) was then applied and the reaction visualised using nitroblue tetrazolium and bromo-chloro-indolyl-phosphate (NBT, BCIP) (both Sigma, Poole, UK) dye solution as substrate [22]. Sections were treated with RNA-ase at a concentration of 100 μg/ml for 2 h at 37 °C prior to hybridization of confirm that target sequences were RNA. Two positive controls were used in all runs. The first consisted of formalin-fixed spinal cord and intercostal muscle tissue from a 3-day-old mouse infected at birth with coxsackievirus B_3. The second was a heart from a 13-day-old male infant who had died of acute myocarditis. Culture of urine in life had grown coxsackievirus B_4, and the autopsy had been performed two and a half days after death. Two additional controls were done on all pancreatic sections to check the preservation of mRNA in the tissue sections. A digoxigenin labelled 20 mer poly dT probe (Oswel) to detect mRNA poly A tails was hybridized at 37 °C overnight at

Table 1. Sequence of the primers used in PCR tests

CMV primers	
CMVA1	5′ TGG CAC GGG GAA TCC GCC T 3′
CMVA2	5′ GGT AGG CGT GTA CGG TGG G 3′
CMVB1	5′ GCA GAG CTC GTT TAG TGA ACC G 3′
CMVB2	5′ ATC CGC GTT CCA ATG CAC CGT T 3′
EBV primers	
$EBVW_1$	5′ CAC TTT AGA GCT CTG GAG GA 3′
$EBVW_2$	5′ TAA AGA TAG CAG CAG CGC AG 3′
β-globin primers	
BG1	5′ CAA CTT CAT CCA CGT TCA CC 3′
BG2	5′ ACA CAA CTG TGT TCA CTA GC 3′

Sequence of the probes used in the in situ hybridization study

Insulin
TTG TTC CAC AAT GCC ACG CTT CTG

Arginine vasopressin
GGC CCG TCC AGC TGC GTG GCG TTG CTC

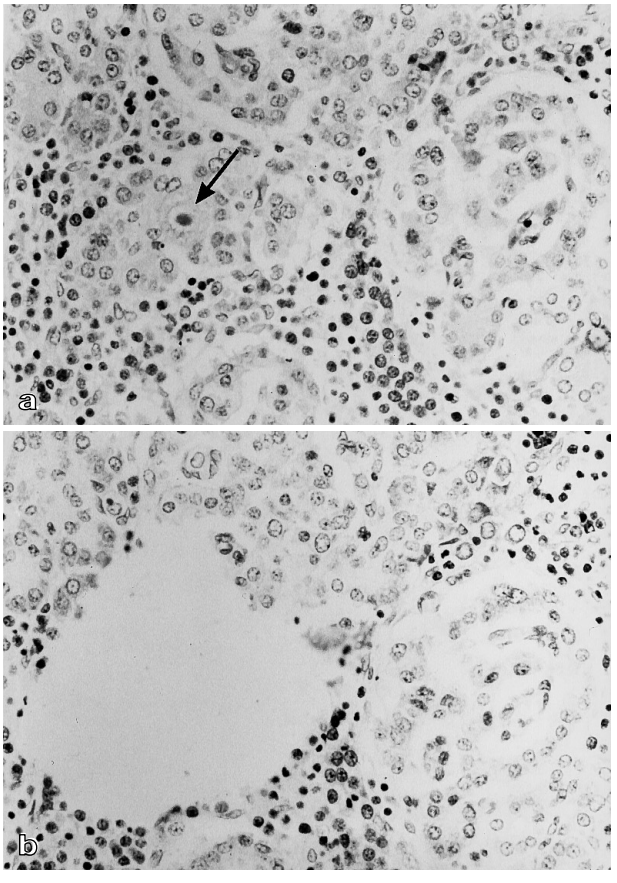

Fig. 1 a, b. Cytomegalovirus infection in the pancreas of a child. A single infected cell present in the top photograph (**a**) (arrow) was microdissected out from an adjacent section (**b**) (Toluidine Blue × 600)

a concentration of 1 ng/μl. Sections in this control were pre-treated with proteinase K at 20 μg/ml. A 24 mer alkaline phosphatase labelled probe to insulin mRNA was synthesized (Oswel) (Table 1) and applied to sections at a concentration of 1 ng/μl in buffer without formamide, overnight at 37 °C. Sections here were pre-treated with proteinase K at 20 μg/ml. Only those cases of diabetes where there was positive staining with both these probes were included in the study (see "selection of cases").

All sections were also analysed using a non-relevant probe (to arginine vasopressin). This 27 mer probe (Oswel) (Table 1) was applied under identical conditions to the coxsackieviral probe cocktail following proteinase K (20 μg/ml) pretreatment. All sections were analysed by two independent observers.

Mumps virus study. We screened 19 murine monoclonal antisera to mumps viral proteins for their ability to detect mumps viral proteins by immunocytochemistry in infected rhesus kidney tissue culture cells which had been formalin fixed and paraffin embedded. Six of the antibody clones were directed towards the mumps fusion protein, 6 towards the nucleocapsid protein, 4 towards the polymerase protein and 3 towards the membrane protein. These clones have been described previously [23, 24]. One of these (NP2132), which detects a nucleoprotein epitope present on all strains of mumps virus tested [24], was capable of detecting virus in this system when used a dilution of 1 in 400. This antibody was subsequently used to screen sections of diabetic pancreas and mumps infected control tissues. Sections were dewaxed and rehydrated and a 1 in 400 dilution of the antibody placed on the sections for an overnight incubation at 4 °C. A standard indirect immunoperoxidase technique was used where the second antibody was peroxidase conjugated goat anti-mouse serum (Dako, High Wycombe, UK). Diaminobenzidine was used as the subsequent substrate. For each test case a second section was analysed where normal mouse serum, diluted 1 in 400 was substituted for the monoclonal antiserum. Formalin-fixed mumps infected rhesus kidney cells were used as a positive control in all runs. Sections were analysed by 2 independent observers.

EBV study

i) *PCR technique.* The method used was that described in detail by Coates et al. [25]. Briefly, extraction of DNA was identical to that described in the CMV study. Details of the primers used ($EBVW_1$ and $EBVW_2$, synthesized by Oswel) are given in Table 1. These flanked a 153 base pair of the Bam H1 'W' internal repeat sequence of the EBV genome. Each amplification cycle consisted of a denaturation step at 94 °C for 1 min, primer annealing at 60 °C for 30 s and extension at 72 °C for 1 min. Cycles were preceded by 2 min at 94 °C to ensure full denaturation of the target DNA. A two-staged procedure was performed in which extracted DNA samples were subjected to an initial 25 cycles of amplification. A small aliquot was then removed and added to fresh amplification reagents. Samples were then subjected to a further 25 cycles of amplification. The conditions for visualising the product were as used for the CMV technique.

ii) *In situ hybridization technique.* For this part of the study a kit provided by Dako (Code No. Y017) was used. The technique used five 30-mer oligonucleotides labelled with the hapten fluorescein isothiocyanate (FITC). These were visualised using the Dako in situ hybridization Detection Kit (Dako Code No. K0074) in which an alkaline phosphatase conjugated anti-FITC antiserum is applied and the reaction visualised with NBT, BCIP solution. The procedures described with the kits were followed without modification. Negative controls in which the oligonucleotides were omitted were run for each case. The deoxyribo-oligonucleotides used in the kit are complementary to the 2 nuclear EBER RNAs encoded by the EBV which are highly expressed in both latent and active infection.

Table 2. Cytomegalovirus study: results of PCR analysis

Tissue	Nn. of cases	Cytomegalovirus positive
Normal autopsy paediatric pancreas	32	0
Normal autopsy adult pancreas	9	0
Autopsy AIDS pancreas	10	3
Tissues from patients with known cytomegalovirus infection	5	3
IDDM pancreases	47	0

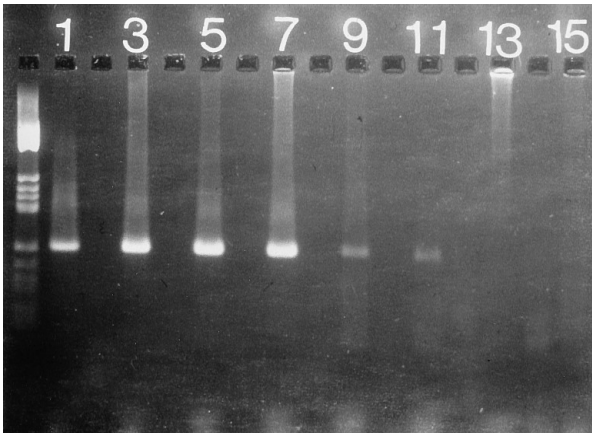

Fig. 2. Agarose gel stained by ethidium bromide and viewed under ultraviolet light. DNA was extracted from a single 10 μm section of the CMV control pancreas, diluted and amplified by PCR. In lane 1 the dilution of extracted DNA was 10^{-1} in distilled water prior to amplification, in lane 3, 10^{-2}; in lane 5, 10^{-3}; in lane 7, 10^{-4}; in lane 9, 10^{-5}; in lane 11, 10^{-6}; in lane 13, 10^{-7}. Reaction product is still visible in lane 11

Results

CMV Study. As can be seen from Table 2 no evidence of CMV was found in the diabetic pancreases. This raises the question of the sensitivity of the technique. Dilution experiments were performed on DNA extracted from a single 10-μm section of formalin-fixed autopsy pancreas of a neonate who died of disseminated CMV. The section adjacent to the one analysed contained 52 infected cells, as judged by standard immunocytochemisty. Extracted DNA could be diluted to 1 part per 1 million parts of distilled water and still give a positive signal after PCR (Fig. 2). DNA was extracted from the micro dissected "owl's eye" cell

(Fig. 1) and the CMV sequence was still amplifiable after 500-fold dilution. All 3 AIDS pancreases which had immunocytochemically identifiable infected CMV cells were correctly found to be positive as were the tissues from 3 of the 5 cases of clinical CMV infection. In the case of the 2 negative samples, the patients had been treated for the infection and the tissue analysed (lung) had no demonstrable CMV infected cells on immunocytochemical analysis.

Coxsackievirus study. None of the pancreases from patients with IDDM had demonstrable coxsackievirus RNA. All pancreases studied had well-preserved mRNA (insulin mRNA and poly A). Evidence of coxsackieviral infection was readily discernible in 13 out of 14 hearts of the infants who died of myocarditis (Table 3, Fig. 3). In these cases the pancreas was infected by virus in 5 out of 9 cases and islets were particularly affected (Fig. 4). Only occasional acinar cells stained positively. Brown fat, usually sampled next to the adrenal, was also frequently positive. In the infants who had died of myocarditis all tissues not mentioned in Table 3 were negative, including samples of lung and brain. All pancreatic sections were stained using the probe to arginine vasopressin and no positive cells were identified. When sections of the positive control heart were treated with RNA-ase prior to hybridization no staining was observed following the standard procedure.

Mumps virus study. Pancreases from all 75 cases of IDDM were negative. In the salivary glands sampled from the children with mumps infection there was striking intralobular duct necrosis with accumulation of debris in duct lumens. An intense chronic inflammatory cell infiltrate was present which was clearly centred on the intralobular ducts. Immunocytochemistry demonstrated mumps virus in the ductal epithelial cells and the intraduct debris in 2 of the 3 cases (Fig. 5) both of whom had had infection for a short time (1–3 days). In the third patient there was clear histological evidence of duct epithelial regeneration and no positive duct cells were present. Death in this case occurred 7 days after the onset of clinical mumps infection. Salivary gland acinar cells were negative in all cases.

Pancreas was available in the 3 cases of mumps infection and a single small area of exocrine pancreatic necrosis was present in the patient with mumps of 7 days duration. No virus was demonstrable in any pancreatic tissue sampled.

EBV study. Diabetic pancreases studied by both the PCR and in situ hybridization techniques were uniformly negative. Both positive control cases were positive by both techniques, although there was considerable autolysis in the tissue section of the autopsy post-transplant lymphoproliferative disorder (Fig. 6).

Table 3. Results of coxsackievirus study

Disease	Tissue	No. tested	No. positive
Infantile myocarditis			
	Heart	14	13
	Pancreas	9	5
	Brown fat	9	5
	Adrenal cortex	10	2
	Liver	9	1
	Skeletal muscle	6	1
	Anterior pituitary	1	1
IDDM			
	Pancreas	29	0

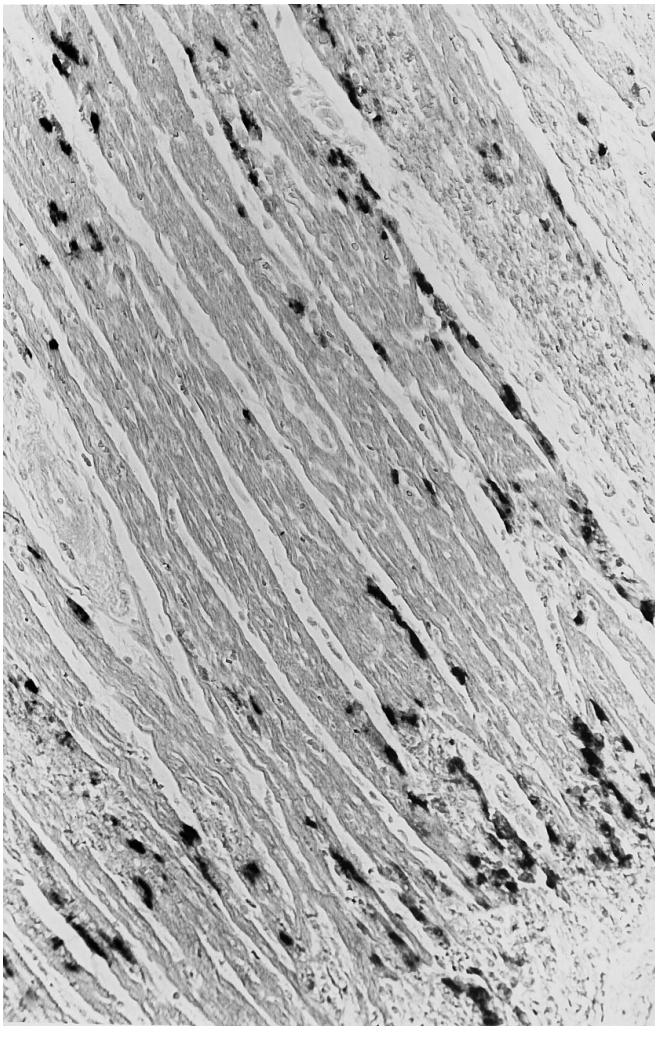

Fig. 3. Coxsackie virus B4 infection in the heart of a 13-day-old infant. Numerous cardiac myocytes contain enteroviral RNA (in situ hybridization for coxsackie virus × 150)

Discussion

Deaths from IDDM at clinical presentation are very rare and attempts at viral culture at autopsy are seldom reported. The aim of the present study was to use techniques on stored autopsy pancreases which

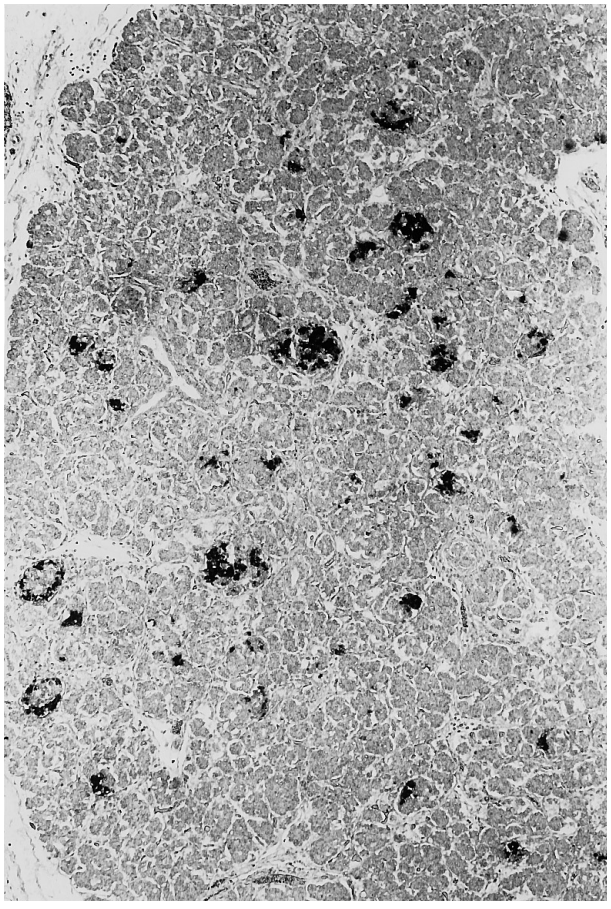

Fig. 4. Coxsackie virus B3 infection in the pancreas of a 9-day-old infant. There is tropism of the virus to islets of Langerhans (in situ hybridization for coxsackievirus × 60)

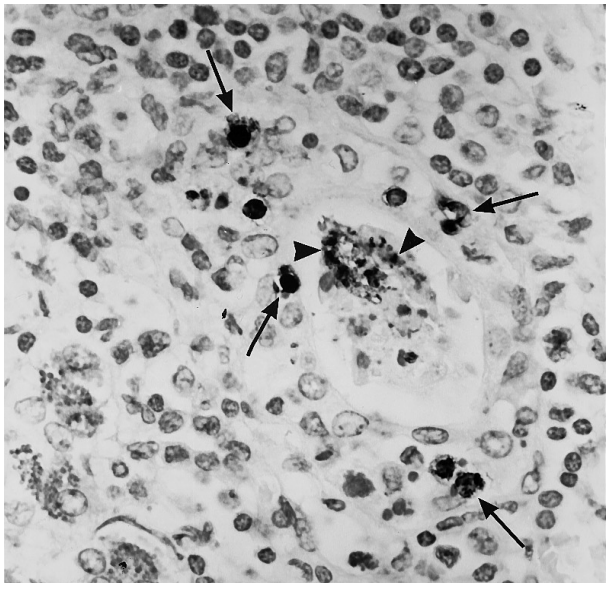

Fig. 5. Mumps infection of salivary gland in a 16-year-old girl. Here there is a small salivary duct surrounded by inflammatory cells. Necrotic debris is present in the duct lumen. Virus is present in numerous ductal epithelial cells (arrows) as well as the luminal debris (arrowhead). (Indirect immunoperoxidase with monoclonal antibody NP2132 × 600)

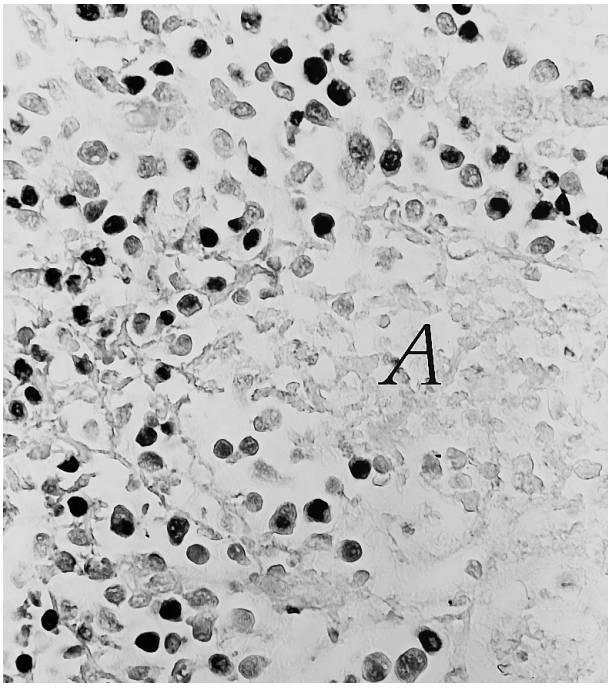

Fig. 6. Post-transplant lymphoproliferative lesion in autopsy lung. EBV can be demonstrated in these cells in spite of the presence of widespread autolysis (A). (in situ hybridization for EBV × 375)

are as sensitive as viral culture in detecting virus, to determine whether diabetogenic viruses could have been cultured from the pancreases at their respective autopsies had culture been attempted.

In the event that no evidence for either CMV, coxsackievirus, EBV or mumps virus could be found in the diabetic pancreases studied, the question is raised as to how appropriate the techniques used really were. The PCR method employed to identify CMV appeared to be exquisitely sensitive, being able to detect evidence of 52 infected cells in a section of pancreas following a 10^6 dilution of extracted DNA. Coxsackieviral RNA was demonstrable in the heart by in situ hybridization in 13 out of 14 infants with myocarditis and positive staining was easily seen even in autopsies done several days after death (Fig. 3). The patient in whom no infected cells were seen was aged 10 months. Diagnosis had been made in life from a stool culture and no viral culture was done at autopsy. Coxsackie B virus was cultured from the heart at autopsy in 7 of the infants in the myocarditis study and virus was demonstrable in the heart by in situ hybridization in each case. No attempt was made to culture mumps virus at the autopsies of the 3 positive control children who had died of that infection, the diagnosis having been made clinically. However, immunocytochemistry clearly demonstrated positive staining for virus in those salivary ductal cells which exhibited a cytopathic effect. Both techniques used in the EBV part of the study were capable of

detecting virus in partially autolysed autopsy tumour tissue.

A reasonable conclusion from the present study, therefore, is that had pancreatic viral culture been done at the time of autopsy on the 47 diabetic patients in the CMV study, the 29 patients in the coxsackie study, the 75 patients in the mumps study and the 47 patients in the EBV study, these respective viruses would not have been cultured.

Other studies, of smaller numbers of patients, also failed to detect evidence of viral infection in diabetic pancreases [26–28]. What therefore is the explanation for the positive culture of a coxsackievirus B4 in the autopsy studied by Yoon et al. [1]? That child had a family history of IDDM. He presented with ketoacidosis but also developed clinical signs of meningitis. During life there was a rising serological titre to the virus cultured at autopsy, which makes it unlikely that the virus was a laboratory contaminant. However, only one of two attempts to culture virus from the pancreas was successful and virus could not be demonstrated by immunofluorescence in the pancreas. Immunofluorescence did identify the virus in the brain stem of the patient. The histology of the pancreas appears to have been typical of IDDM ([1] and Gepts W personal communication) with insulin-deficient islets and insulitis but little evidence of the dramatic beta-cell necrosis which characterises coxsackieviral pancreatitis in infants [29]. IDDM appears usually to be a disease of slow evolution with evidence of endocrine dysfunction, and presumably beta-cell loss, months or even years before clinical presentation [12]. It is therefore possible that this child had already lost a critical number of beta cells through the typical autoimmune process and that the clinical presentation of diabetes was provoked by a severe coxsackieviral infection, accompanied by viraemia, affecting primarily the brain, i. e. the virus may not have been primarily responsible for the beta-cell loss in this child.

If an acute viral infection is unlikely as a cause of IDDM, would the techniques used here be able to detect a low-grade persistent infection? The PCR technique for detection of CMV was the same as that used by Hattersley et al. [15]. Although they were unable to detect CMV in the autopsy pancreases of their patients with NIDDM, they could detect the virus in samples found to be positive in the study by Lohr and Oldstone [30]. In this latter study samples were positive in the absence of inflammation or classical histological features of CMV infection. Foulis et al. [29] found no immunohistochemical evidence of the VP-1 coxsackieviral protein in IDDM pancreases. It was argued then that this capsid protein may only be expressed significantly in a lytic infection of cells as opposed to a persistent infection. This was the rationale for using an in situ hybridization technique in the present study, since nucleic acid would still be expected to be present in a non-cytopathic infection. The question of enteroviral persistence in human pathology is unresolved with several studies suggesting that it does occur in idiopathic dilated cardiomyopathy [31, 32] and chronic inflammatory muscle disease [33] but there have been doubters [34, 35]. These studies have been done on fresh tissue biopsies of heart and skeletal muscle and this clearly is a more ideal starting point than autopsy pancreas, as examined here. As there is little evidence that PCR for enterovirus offers a more sensitive method of detecting enterovirus in autopsy tissues [21], the question of whether the inability to detect enterovirus in the present study is a false negative must currently remain an open one.

An alternative strategy to detect persisting enteroviral infection in diabetic patients has recently been employed [36]. Serum of newly diagnosed diabetic children was examined for the presence of enteroviral RNA, using PCR. Nine of 14 diabetic patients had positive samples compared to 2 of 45 non-diabetic children. Sequencing of the amplified nucleic acid showed a high degree of similarity with published sequences of coxsackieviruses B3 and B4. These potentially important findings await confirmation.

It has frequently been argued that if there was immunological cross reactivity between a virus and a beta-cell antigen then an immune response to eliminate the virus could cause continuing damage to beta cells. Such cross reactivity has been suggested for enterovirus and glutamic acid decarboxylase, the islet autoantigen [37, 38], and CMV and a 38 kDa protein present in islet endocrine cells [39]. There is indeed a murine model of diabetes where coxsackieviral infection of the pancreas is followed by the appearance of anti 64 kDa antibodies and diabetes. The virus is not culturable after the first week [40, 41]. While antigen cross reactivity is a very plausible scenario, the immunopathology of the diabetic human pancreas does not appear to accord with it. A clear finding in both the prediabetic [42, 43] and diabetic [44] pancreas is that beta cells in many islets which are not inflamed express interferon-alpha [13]. This finding suggests a continuing abnormality of the beta cell (production of interferon-alpha) in the absence of any obvious immune attack. An immune response caused by a cross reaction between a non-persisting pathogen and an islet autoantigen would more likely cause insulitis without interferon-alpha expression by the beta cells.

The most obvious explanation for the finding that beta cells express interferon-alpha in IDDM is that they harbour a persistent viral infection. We have failed to detect any evidence of such infection in this study but the recent report that islet cells from IDDM pancreases may contain a superantigen [9] suggests that infections may be involved other than

those caused by the common viruses studied here. Retroviruses can act as superantigens and preliminary data suggest that a relevant retrovirus could be present in human IDDM pancreas [45].

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