Lack of islet neogenesis plays a key role in beta-cell depletion in mice infected with a diabetogenic variant of coxsackievirus B4

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Group B coxsackieviruses (CVBs) have a well-established association with type 1 diabetes but the mechanism of depletion of beta-cell mass following infection has not yet been defined. In this report we show that the major difference in pathogenesis between the E2 diabetogenic strain of CVB4 and the prototypic JVB strain in SJL mice is not in tropism for islet cells but in the degree of damage inflicted on the exocrine pancreas and the resulting capacity for regeneration of both acinar and islet tissue by the host. Both strains replicated to a high titre in acinar tissue up to day 3 post-infection (p.i.), while the islets of Langerhans were largely spared. However, the pancreases in the JVB-infected animals then regenerated and many small islets were seen throughout the tissue by day 10 p.i. In contrast, the acinar tissue in E2-infected mice became increasingly necrotic until all that remained by day 21 p.i. were large islets containing varying numbers of dead cells, caught up in strands of connective tissue. Surviving beta cells were found to synthesize little insulin, although islet amyloid polypeptide was detected and glucagon synthesis in alpha cells appeared normal or enhanced. Our results suggest that the key to CVB-E2-induced damage lies in the exocrine tissue and prevention of islet neogenesis rather than from direct effects on existing islets.

INTRODUCTION

Type 1 diabetes is caused by the loss of functional beta cells within the islets of Langerhans in the pancreas, resulting in insulin deficiency and hyperglycaemia. Under normal conditions, there is continual turnover of beta cells with a proportion of cells undergoing apoptosis due to senescence and replacement of these dying cells by both beta-cell replication and islet neogenesis, a process that involves differentiation of progenitor cells (Bonner-Weir, 2001). In fact, it has been calculated that, in adult rats, the percentage of replicating cells is around 2–3%, which extrapolates to complete beta-cell renewal in approximately 1 month (Finegood et al., 1995; Hellerstrom et al., 1988). However, as the differentiated beta cells have a limited capacity for replication, differentiation of progenitor cells also plays an important role in maintaining beta-cell mass (Bonner-Weir, 2001). The induction of type 1 diabetes could therefore be due to factors that limit beta-cell replication or neogenesis in addition to factors that induce beta-cell death, both contributing to the ‘dynamic instability’ concept described by J. Nerup and co-workers (Freiesleben De Blasio et al., 1999).

The prevailing view has long been that type 1 diabetes is an autoimmune disease with genetic susceptibility linked to HLA DR3, DR4 and DQβ alleles (Gale, 2001; Lipton et al., 1992; Nerup et al., 1976). In part due to lack of concordance in identical twins, an environmental trigger is also thought to be involved, which has been suggested to be a virus infection or in certain instances drugs or even diet (Couper, 2001; Yoon, 1995). A number of viruses have been linked to the onset of type 1 diabetes including rubella and mumps viruses (Yoon & Ray, 1985), but the strongest association is with enteroviruses and, in particular, members of the group B coxsackieviruses (CVBs) (Hyoty et al., 1998; Szopa et al., 1993; Ramsingh et al., 1997a). Evidence that CVBs have a role includes epidemiological data (Hyoty et al., 1998; Hovi, 1998) and individual case studies that have described patients who developed diabetes shortly after a documented infection with one of the CVBs (Toniolo et al., 1988). In particular, reports of patients who developed fatal diabetic ketoacidosis during a documented CVB infection and in whom the virus was detected in islets of Langerhans post-mortem (Iwasaki et al., 1985; Jenson et al., 1980) provide strong evidence for the ability of coxsackieviruses to damage the pancreas and cause diabetes. In addition, beta cells have been shown to be susceptible to coxsackievirus infection in vitro (Szopa et al., 1993; Roivainen et al., 2000) and various mouse strains have been reported to develop hyperglycaemia following infection with certain CVB isolates (Yoon et al., 1978; Szopa et al., 1993; See & Tilles, 1995).
Epidemiological evidence suggesting a role for CVBs in the pathogenesis of diabetes includes the detection of anti-CVB IgM, indicating recent infection, in up to 50 % of patients at time of onset of symptoms, compared with approximately 5 % of age-matched controls (Frisk et al., 1992). In addition, elevated T-cell responses to CVB antigen (Jones & Crosby, 1996) have been found and shown to display different specificities in patients who develop type 1 diabetes relative to patients who do not, potentially affecting virus clearance (Varela-Calvino et al., 2002). The most direct evidence, however, is the detection of CVB-specific RNA in serum by RT-PCR in 40–60 % of recently diagnosed patients with insulin-dependent diabetes (Clements et al., 1995; Andreoletti et al., 1997). Therefore, there is substantial evidence for the presence of coxsackieviruses but what is currently unclear is the underlying mechanism of pathogenesis. In the rare fatal cases, fulminant infection of the pancreas and consequent destruction of both exocrine and endocrine tissue occurs, but if coxsackieviruses have a wider role in type 1 diabetes, how does virus infection induce a slow depletion of beta cells consistent with the normal onset of this disease, where there is a prolonged subclinical period of months or years during which some islet function is preserved? Moreover, what is the role of autoimmunity, in the form of both autoreactive T cells and islet cell antibodies, which are a hallmark of the majority of cases of type 1 diabetes?

To address the question of how CVBs cause beta-cell depletion, we have undertaken a detailed study of the pathological events occurring in the pancreas following CVB infection of SJL mice, a mouse strain that has previously pathological events occurring in the pancreas following CVB infection of SJL mice, a mouse strain that has previously

**METHODS**

**Virus strains and stock preparation.** The JVB strain of CVB4 was obtained from the ATCC and the Edwards (E2) strain was provided by Margaret Cam, UBC. Virus stocks were prepared in Vero cell monolayers infected at 80–90 % confluence with an m.o.i. of 0-1 p.f.u. of JVB or E2 per cell. Cultures were harvested when maximum cytopathic effect was observed and the flask were freeze-thawed twice to lyse infected cells. The medium was then centrifuged at 2500 r.p.m. for 10 min at 4°C to pellet cellular debris. The supernatant virus stock was stored at -70°C and titrated by plaque assay. An aliquot of E2 strain was passaged multiple times through a beta-cell line, NIT 1 cells, cultured as described above, to produce the E2 stock used for the experiments described.

**Animal care and virus inoculation.** Five-week-old SJL/J male mice (n=6) were obtained from Jackson Laboratories and housed in a Level 3 containment facility at the BCRICWH. They were acclimatized for 1 week before inoculation intraperitoneally (i.p.) with 10⁵ p.f.u. of either CVB4 (JVB) or CVB4 (E2) diluted in PBS to a final volume of 0.2 ml per mouse. Control mice were injected with an equal volume of PBS. Mice were sacrificed on selected days between day 1 and day 95 post-infection (p.i.) and tissues (pancreas, heart, liver and spleen) as well as heart blood were collected. One half of each tissue was snap-frozen in dry ice and stored at -70°C for virus titration, while the other half was fixed in 4 % formaldehyde in sodium phosphate buffer, pH 7.4, for histopathological examination and immunohistochemistry. For mice that were supplemented with exocrine enzymes, each pellet was fortified with 150 µl of enzyme suspension containing 30 mg lipase, 50 mg amylase, 15 mg cellulase, 50 mg papain and 30 mg bromelain. Food intake was monitored daily by weighing the pellets provided and those remaining after 24 h.

**Serum glucose measurements.** Heart blood was obtained at sacrifice from all animals. In addition, another set of age-matched mice was inoculated as part of a longitudinal experiment to monitor blood glucose levels in individual animals over time (between days 1 and 95 p.i.). Blood was collected from the tail vein or the saphenous vein in a hind leg and serum glucose was measured by the glucose Trinder assay.

**Insulin ELISA.** The whole pancreas of selected mice was removed and homogenized in 1 ml of cold (−20°C) acid-alcohol homogenizing solution. The homogenate was centrifuged at 12 000 r.p.m. to remove tissue debris and the concentration of insulin measured using a sandwich ELISA, with streptavidin–HRP as secondary antibody and TMB as substrate. Absorbance was read at 450 nm using a microtitre plate reader (SpectraMax 190).

**Histopathological examination.** Tissues were fixed in 4 % PF at 4°C overnight and stored in 70 % ethanol until processing. They were embedded in paraffin, sliced into 3 µm sections with a Leica microtome and mounted on slides for immunohistochemical staining, in situ hybridization or histological staining with Masson’s trichrome. Regions of fibrosis and connective tissue deposition stained blue against a pink-purple background tissue stain. Necrotic areas in the pancreatic acinar tissue stained pale pink.

**Immunohistochemistry.** The sections of paraffin-embedded tissue were dipped in xylene and rehydrated in graded alcohols (100 %, 70 %, 50 % ethanol). Endogenous peroxidases were quenched with 3 % H₂O₂ and non-specific proteins were blocked with 2 % BSA in PBS. Primary antibodies for insulin (Dako), glucagon (Dako), pIAPP (provided by B. Verchere, Vancouver) or viral antigen (Chemicon) were diluted in blocking buffer and 25 µl was added per section. The slides were incubated at 37°C for 1 h in a humidified chamber, washed with PBS and then for 30 min with 100 µl HRP-conjugated secondary antibody diluted 1:500 in blocking buffer. After a further PBS wash, the DAB substrate was added for 10 min at room temperature. Sections were counterstained with haematoxylin (blue nuclear stain) and then mounted under coverslips.

**Immunofluorescent staining for insulin and Glut-2.** Tissue sections were treated with xylene and rehydrated in graded alcohols (100 %, 70 %, 50 % ethanol). Endogenous peroxidases were quenched with 3 % H₂O₂ and non-specific proteins were blocked with 2 % BSA in PBS. Primary antibodies for insulin (Dako), glucagon (Dako), pIAPP (provided by B. Verchere, Vancouver) or viral antigen (Chemicon) were diluted in blocking buffer and 25 µl was added per section. The slides were incubated at 37°C for 1 h in a humidified chamber, washed with PBS and then for 30 min with 100 µl HRP-conjugated secondary antibody diluted 1:500 in blocking buffer. After a further PBS wash, the DAB substrate was added for 10 min at room temperature. Sections were counterstained with haematoxylin (blue nuclear stain) and then mounted under coverslips.
In situ hybridization. The tissue sections were baked overnight at 60°C, deparaffinized using xylene and rehydrated in graded alcohols. The tissues were permeabilized using 0.2 M HCl, 2× SSC, 20 mM Tris/HCl (pH 7.4) containing 2 mM calcium chloride and 1 μg proteinase K ml⁻¹ and then quenched in 0.25% acetic anhydride containing 0.1 M triethanolamine. The slides were then dehydrated using graded alcohols. The hybridization solution (25 μl) containing 100 ng ml⁻¹ of the sense or antisense probes labelled with digoxigenin (Boehringer Mannheim) was added to each section, glass coverslips were placed and the slides were placed in a sealed humidified dish at 42°C overnight. Post-hybridization washes were performed overnight using 50% formamide, 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and 600 mM NaCl in a 56°C rocking water-bath, followed by several washes in 2× SSC. The slides were equilibrated in buffer 1 containing 0.15 M NaCl and 0.1 M Tris/HCl (pH 7.5) and blocked with 2% lamb serum. Development was carried out according to the Boehringer Mannheim instructions for digoxigenin-labelled probes. The slides were counterstained with eosin and were examined with a light microscope for a positive reaction indicated by a blue-black colour.

RESULTS

Histological damage to pancreas caused by JVB and E2

The histology of the pancreas was examined following infection of SJL mice with either the E2 or JVB CVB4 strain for 35 days p.i., in comparison with control tissue (Fig. 1A). Both virus strains infected the pancreas and caused acute tissue damage localized primarily to the acinar tissue on days 1 and 3 p.i. A proportion of islets in both JVB- and E2-infected animals showed signs of cytopathology but the majority appeared normal. Lymphocytic infiltration was observed throughout the pancreas and a few mononuclear cells could also be seen penetrating the occasional islet close to areas of dying cells on day 3 p.i. (see Fig. 1B for a higher magnification). However, most islets were notably intact despite being surrounded by infected acinar tissue. The major difference in pathogenesis was seen between days 5 and 10 p.i., when a substantial portion of the acinar tissue in JVB-infected mice regenerated, while in E2-infected animals, increased necrosis of the tissue was seen (Fig. 1A, l, o, r). In the JVB-infected animals, on day 5 there was a massive infiltration of small mononuclear cells into the exocrine pancreas, but rather than causing increased cell death, this influx occurred simultaneously with acinar regeneration (Fig. 1A, h, arrow). By day 10, we observed tiny clumps of cells with the staining characteristics of islet cells scattered within the acini (Fig. 1A, k, arrow). To confirm the identity of these cells, immunostaining for insulin (beta cells) and glucagon (alpha cells) was performed on JVB-infected tissues between days 1 and 10 p.i. Small foci and also single cells that contained one of these two markers were readily detected by day 5 p.i. Some insulin-positive cells originated in ductal tissue while others appeared in areas with abundant cellular infiltrates (Fig. 2A, b) or within acini (Fig. 2A, b and c). By day 10, small islet structures were seen scattered throughout the acinar tissue (Fig. 2A, C) and an assessment of the numbers of these showed that there were significantly more in JVB-infected tissue sections compared with the numbers in controls or E2-infected animals (Fig. 2B).

In marked contrast, in the E2-infected pancreata, far fewer infiltrating mononuclear cells were present on day 5, and by day 10 p.i. the exocrine tissue was almost completely necrotic. Small condensed islets (Fig. 1A, l, arrows) could be seen, together with large numbers of tubular structures comprising ductal cells, as well as a few infiltrating mononuclear cells. On days 21 and 35 p.i., the JVB-infected pancreata were indistinguishable from controls. However, E2-infected pancreata did not regenerate with increased time but continued to show complete ablation of the acinar tissue while surviving islets contained increasing numbers of dead and dying cells (Fig. 1A, o and r). No islet neogenesis was seen although ductal cells containing immunoreactive insulin were present (see Fig. 6A, d and g).

Presence of the Glut-2 glucose transporter protein outside islets in JVB-infected mice

Glut-2 is a beta-cell-specific glucose transporter protein and is highly expressed on the surface of beta cells, giving a honeycomb pattern to the islets on immunofluorescent staining (see Fig. 3). No staining of the acinar tissue is normally seen in adolescent or adult mice. However, during embryonic development, it has been shown that islet precursor cells in the pancreatic duct migrate into the tissue parenchyma and differentiate into cells that form islet tissue (Slack, 1995). These ductal cells express Glut-2, which is therefore believed to be a marker for both islet precursor cells and beta cells (Wang et al., 1995). In the JVB-infected pancreata between days 5 and 10 p.i., we observed widespread Glut-2 staining associated with the tubular or duct-like structures that were scattered throughout the exocrine tissue (Fig. 3, b and d) as well as within remaining islets. Co-staining for insulin identified clusters of one to five insulin-positive cells associated with the Glut-2-stained cells outside established islets. This suggests that these Glut-2-positive tubular structures are equivalent to the neogenic areas responsible for islet development that have been described by others (Bouwens, 1998; Wang et al., 1995). By day 21 post-JVB-infection, both the acinar tissue and islets were largely reconstituted and Glut-2 staining was again restricted to beta cells within the islets. The large number of these tubular structures that were Glut-2 positive indicates that these are the major source of progenitor cells for islet regeneration. However, we also observed single insulin- and glucagon-positive cells and clusters, not yet enclosed in recognizable islet structures, in areas with high mononuclear cell infiltration not obviously associated with ductal tissue (Fig. 2A, g).
Does the degree of virus replication correlate with the amount of damage to the pancreas?

To assess whether the difference in outcome of infection of SJL mice with the two strains was directly correlated with the extent of virus replication in the pancreas, the titres of each virus in pancreatic tissue was determined. These were compared with the serum titre, which provides an estimate of the quantity of virus circulating and available to infect the target tissues. Virus titres in spleen and heart were also measured to determine whether E2 has a particular tropism for pancreatic tissues (Fig. 4A). Although the mice were inoculated with equal amounts of virus, the E2 strain was found to be more virulent, giving yields 10- to 100-fold
greater than the JVB strain in all the tissues tested. Moreover, E2 virus replication continued for a longer period, with ongoing replication on day 5 in pancreas and until day 10 in heart, while in JVB-infected animals no infectious virus was detected after day 3 in these tissues. Immunohistochemical detection of viral antigens (Fig. 4B) and in situ hybridization for viral genome (Fig. 5, arrows) were carried out in order to localize virus within the pancreas. With both virus strains, on days 1 and 3 p.i., large amounts of viral proteins were detected in exocrine tissue while only a small number of islets were infected. A higher magnification of two of these infected islets (day 3 p.i.) showing widespread distribution of E2 or JVB antigen is shown in Fig. 4(B, e and f). At later times, no viral antigen was detected in islets of either JVB- or E2-infected mice. A considerable amount of viral genome of both JVB and E2 strains was also detected by in situ hybridization throughout the acinar tissue on days 1 and 3 p.i. but in only a few islets (Fig. 5, i and j). However, using this more sensitive technique, persistence of viral genome was detected in E2- but not in JVB-infected animals on days 21 and 35 p.i. and, in a subsequent experiment, up to day 150 p.i. (not shown). The genome was present in surviving islets and also in the tubular structures and fibroblast-like cells that represented the remnants of the exocrine tissue (Fig. 5, f and h, arrows). The negative-strand replicative intermediate of the viral genome was also detected, although infectious virus could not be isolated at these late times, raising the possibility that E2 genome persistence occurs as dsRNA. In agreement with this, we were able to detect the presence of dsRNA concentrated in inclusion-like bodies, using an antibody raised against poly(IC), in E2-infected tissues as late as day 150 p.i. (not shown).

**Are the surviving islets in E2-infected animals functional?**

To determine whether islet cells in E2-infected mice still produce insulin and glucagon, we performed immunohistochemical staining for these two pancreatic hormones

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**Fig. 1.** Histopathology of pancreas following infection with JVB or E2 strains of CVB. In (A), sections of pancreas obtained between days 1 and 35 p.i. from SJL mice injected with PBS or with 10^5 p.f.u. of JVB or E2 and stained with Masson’s trichrome are shown. Early virus-induced cytopathology of exocrine tissue together with a lymphocytic infiltration can be seen on days 1 and 3 p.i., but the islets are largely spared. At later times, the JVB-infected tissues regenerate while E2-infected pancreata show almost complete necrosis of acinar tissue leaving large misshapen islets and tubular structures caught up in connective tissue. In (B), a higher magnification of single islets at days 3 and 5 p.i. shows some acute damage and lymphocytic infiltration (arrows). In particular, a large number of infiltrating cells can be seen in JVB-infected pancreas on day 5 (c, arrow). Original magnification: (A) × 200; (B) × 400.
up to day 95 p.i. (Fig. 6A) in comparison with control and JVB-infected tissues (Fig. 2). On day 3 p.i., a proportion of both JVB- and E2-infected islets appeared degranulated, and between days 5 and 10, the small regenerating islets scattered throughout the acinar tissue in JVB-infected animals stained strongly for both insulin and glucagon (Fig. 2). In contrast, the islets that survived the acute stage of E2 infection did not regain full insulin staining (Fig. 6A, d, g and j) although glucagon was present in normal or enhanced amounts (Fig. 6A, i and l).
In order to quantify the levels of insulin in JVB- and E2-infected animals relative to controls, the total insulin content of pancreata from a group of mice ($n=5$) at selected timepoints up to day 55 was measured by ELISA assay (Fig. 6B). On day 3, the E2-infected mice contained reduced levels of insulin (approximately 50% of controls) and by day 10 this had further diminished to around 10%, remaining at around this level up to day 55. The JVB-infected pancreata also contained diminished levels of insulin on day 3 (70% of controls) and this was further reduced to approximately 50% of normal levels on day 10, remaining at 50–70% up to day 55. In subsequent experiments we found that total insulin in JVB-infected mice never returned to control levels but stabilized at between 60 and 80% that of the controls up to day 95, while following E2 infection the insulin levels remained at around 10–15% of the control group.

To determine whether the beta cells in E2-infected pancreata retained other functional properties, we also immunostained for islet amyloid polypeptide (pIAPP), a beta-cell-specific protein, which is normally secreted together with insulin. As shown in Fig. 6(A, b, e, h and k), pIAPP immunostaining was strongly positive up to day 95 indicating that
Fig. 3. (A) Glut-2 staining. The glucose-transporter protein (Glut-2), a surface marker for beta cells and their precursors, was detected by immunofluorescent staining using a rabbit polyclonal antibody to Glut-2 and a secondary antibody conjugated to a red Alexa-Fluor dye (see Methods). Positive beta cells show a honeycomb staining pattern and are found only in islets in both control and infected tissue. In addition, widespread staining, associated with the tubular structures that are found throughout the tissue, was seen in the regenerating JVB-infected pancreas, particularly on days 5–7 p.i. (B) Glut-2/insulin double-immunofluorescence. In sections that were stained for both Glut-2 and insulin, islet clusters were seen in close association with the Glut-2-positive ductal tissue.
Fig. 4. (A) Virus titres in tissues. The amount of virus in selected tissues including pancreas are shown. E2 replicated to a higher titre in all tissues tested, particularly in pancreas and heart, in both of which virus clearance was also delayed. (B) Viral antigen in pancreas on day 3 p.i. Viral antigen was detected by immunohistochemistry throughout the acinar tissue in both JVB- and E2-infected mice (b and c, respectively), but the levels in E2-infected animals were much higher. Only occasional islets containing viral antigen were seen with either virus strain (e and f). (a, d), Control tissues. Original magnifications: (a–c), ×200; (d–f), ×1000.
Fig. 5. Viral genome detected by in situ hybridization. The amount of viral genome in pancreatic tissues was determined by in situ hybridization using a digoxigenin-labelled RNA probe. Viral genome was only detected in JVB-infected tissues on days 1 and 3 p.i., while in E2-infected animals, virus persistence was detected on days 21 (f) and 35 (h) both in surviving islets and in tubular structures (arrows). Original magnifications: (a–h), × 200; (i, j), × 400.
Fig. 6. For legend see page 3062.
the remaining beta cells still retain some secretory function. TUNEL staining, a measure of cells undergoing apoptosis, also showed that the majority of cells within the E2-infected islets were viable on day 35, while we have found increasing numbers of TUNEL-positive cells up to day 95 (not shown).

**Fig. 6.** (A) Functional capacity of surviving E2-infected islets. Islets in E2-infected pancreatic tissues were stained for insulin, glucagon and pIAPP to determine whether they had retained some functional capacity. Although increasing numbers of dead cells were seen, the islets contained normal or increased numbers of glucagon-positive alpha cells (compare c with f, i and l). Very little insulin was detected (a, d, g and j), but a proportion of the cells were pIAPP-positive (d, e, h and k), identifying them as beta cells. Magnification × 200. (B) Total pancreatic insulin concentrations. The insulin content of the entire pancreas of SJL mice (n = 5) was measured by ELISA at various times p.i. with JVB or E2 and is shown as a percentage of the level in control mice sacrificed at the same time.

**Fig. 7.** Glucose homeostasis. (A) Glucose levels in blood obtained from the saphenous vein of JVB- or E2-infected mice was compared with control values between days 1 and 101 p.i. Mice infected with the E2 strain became hypoglycaemic on day 3 p.i. and their blood sugar levels remained low throughout the experiment. The JVB-infected mice showed a reproducible hyperglycaemic spike on day 10 but then reverted to normoglycaemia at later times. (B) Mice were supplemented with pancreatic enzymes, and glucose levels in heart blood at sacrifice were measured by the Trinder assay. The supplemented E2-infected mice were found to become hyperglycaemic around day 22 p.i. and stayed hyperglycaemic to day 56 p.i., while the JVB-infected mice became normoglycaemic by day 35 p.i.
Does JVB or E2 infection affect glucose homeostasis?

Following the acute stage of infection, both E2- and JVB-infected mice became active and resumed normal feeding. The E2 mice, however, started to lose weight and were significantly smaller by day 35 p.i. We measured non-fasting serum glucose levels in saphenous vein blood and found that E2-infected mice were extremely hypoglycaemic, despite the lack of insulin synthesis (Fig. 7A). To determine whether this was due to the lack of pancreatic exocrine function limiting digestion, mice were supplemented with pancreatic enzymes (including trypsin, chymotrypsin, lipase and amylase) from the time of infection. Blood glucose levels were measured, following heart bleed, at time of sacrifice (shown as a scatter plot in Fig. 7B). Even some of the unsupplemented E2-infected mice were found to be hyperglycaemic when blood was drawn directly from the heart instead of from the saphenous vein. In addition, the enzyme-supplemented mice became significantly hyperglycaemic after day 10 and up to day 56, the final time-point of the experiment. The surviving islets stained more strongly for insulin in the presence of supplementation, suggesting that the reduced insulin synthesis in E2-infected animals was at least in part due to a regulatory response to the low glucose levels. However, even after enzyme supplementation, total pancreatic insulin remained low and insufficient to maintain glucose homeostasis, so that hyperglycaemia resulted.

What is the role of the immune system?

Small numbers of mononuclear cells could be seen in the exocrine pancreas as early as 2 days p.i. with either JVB or E2. However, by day 5 we observed a marked difference between the viruses in the number of cells infiltrating the pancreas in each case. In JVB-infected animals, the exocrine tissue became packed with large numbers of CD45+ cells, the leukocyte common antigen that is expressed on all haematopoietic cells. Many fewer cells bearing this marker were detected in the case of E2 (Fig. 8, c and d). However, despite the large numbers of leukocytes present, we did not observe massive infiltration of islets suggestive of an
autoimmune reaction against islet antigens with either E2 or JVB. NK cells were the first lymphoid cells detected throughout the pancreas and within a proportion of islets on both day 3 and day 6 p.i. in agreement with the findings of others (Flodstrom et al., 2002; Vella & Festenstein, 1992). In addition, significant numbers of macrophages (MAC1+) were detected in the exocrine tissue between day 3 and day 10 p.i. However, the majority of the CD45+ cells in the acinar tissue were B and T cells in agreement with the findings of Ramsingh et al. (1997b). Their presence in large numbers in JVB-infected pancreata was associated with the regeneration of the exocrine tissue suggesting that they may play a role in the recovery process, perhaps by the production of cytokines and growth factors. Further analysis of these cells and their cytokine production is currently under way.

Are the strain differences between JVB and E2 qualitative or quantitative?

In order to determine whether injection of less E2 virus resulted in a disease course similar to that found for JVB strain, we injected $10^2$ and $10^3$ p.f.u. of E2 into mice and, in parallel, $10^6$ p.f.u. of JVB strain. The course of infection following inoculation with 100- to 1000-fold less E2 was altered in that the virus titres in the pancreas were lower on day 3 but persisted for longer (Fig. 9A). Associated with this, there was less damage in the pancreas at early times than we had seen previously, but by day 14, even with injection of $10^2$ p.f.u. of E2, the exocrine pancreas was severely damaged (Fig. 9B, d). Moreover, a 10-fold higher inoculum of JVB virus was still associated with recovery of the acinar tissue and neogenesis of islets as we had seen.

**Fig. 9.** Variation in virus inoculum. Virus titres (A) and histopathology (B) of pancreas of SJL mice infected with $10^2$ p.f.u. of E2 or $10^6$ of JVB. Even a very low inoculum caused destruction of the acinar tissue after E2 infection, while mice given a high inoculum of JVB ($10^6$ p.f.u.) recovered. Original magnification $\times 200$. 

(A) 

(B)
before, despite higher virus titres in the pancreas on day 3. Therefore, the virulence of E2 for the exocrine tissue is an intrinsic property of E2 and is not simply dose-related.

**DISCUSSION**

The E2 strain of CVB4 was isolated from the pancreas of a child who died following an aggressive virus disease associated with diabetic ketoacidosis (Yoon et al., 1979). The virus isolated from pancreas was identified as CVB4 and when inoculated into mice was shown to induce hyperglycaemia. Since that time it has been widely used as a diabetogenic strain of the CVBs, although several investigators have found that it induces hypoglycaemia not hyperglycaemia, raising questions as to its role in type 1 diabetes (Flodstrom et al., 2001; Horwitz et al., 1998). A comparative study of the pathogenesis of the E2 strain of CVB4 and the non-diabetogenic prototypic JVB strain is reported here, the results of which lead us to propose that this strain does indeed cause depletion of beta-cell mass, but predominantly through lack of islet neogenesis rather than beta-cell killing by either virus per se or the immune system. Our results showed that while E2 may have had a slightly greater propensity to infect islets, many survived the acute stage of E2 infection and it was the exocrine pancreas that suffered the most cytopathology. The remaining islets survived for prolonged periods, although by day 35 many contained a proportion of dead or dying cells. However, they still contained glucagon-positive alpha cells and also beta cells containing reduced insulin but normal levels of IAPP, a protein that is regulated in a similar way to insulin and secreted in the same granular structures. Over the next 2 months, these surviving islets contained increasing numbers of dead cells and in the absence of islet neogenesis, insulin levels became too low to maintain glucose homeostasis.

Glucose dysregulation was therefore induced by CVB4 but whether this manifested as high or low blood glucose levels depended on the availability of pancreatic digestive enzymes and in turn dietary glucose. When E2-infected animals were supplemented with enzymes normally supplied by the exocrine pancreas, they became reproducibly hyperglycaemic, while the unsupplemented mice were hypoglycaemic as dietary insufficiency results in glucose starvation of the tissues. Under these circumstances, the liver becomes the chief source of glucose through a combination of glycogenolysis and gluconeogenesis (Nordlie et al., 1999), but this process does not compensate for the lack of nutritional intake and the blood glucose levels remain low.

Many small islet clusters were seen in JVB-infected pancreata, indicative of islet neogenesis. The majority of these appeared to originate from tubular or ductal tissue that stained for the glucose transporter Glut-2, a marker for both beta cells and precursors (Fig. 3). However, the total pancreatic insulin content following infection with the non-diabetogenic JVB strain did not return to normal levels, at least for the 95 days of observation, suggesting that these animals may also be susceptible to further insult to the pancreas, either by a second virus infection, drugs or even diet. This may explain the conundrum of why the epidemiological evidence incriminates a number of CVB strains in inducing type 1 diabetes, while in experimental systems, few coxsackievirus isolates have been shown to infect islets in animal models. If the key to whether glucose dysregulation occurs lies in the degree to which the exocrine tissue is destroyed and islet neogenesis prevented, then a number of coxsackievirus strains (and other enteroviruses) could play a role (Hyoty et al., 1998). Moreover, successive infections with different viruses may cause accumulative damage, which could eventually lead to insulin insufficiency, an idea first promoted by A. L. Notkins and collaborators over 20 years ago (Toniolo et al., 1980).

In E2-infected pancreata in SJL mice, the role of the immune system appeared to involve clearing out the remnants of infection and enhancing tissue recovery. Widespread invasion of islets by lymphoreticular cells after infectious virus was no longer detectable, which would be suggestive of an autoimmune reaction, was not observed. On day 3 p.i., NK cells, involved in innate defence against viruses (Biron & Brossay, 2001), were the major cell type identified in both E2- and JVB-infected pancreata, as has been reported by others (Vella & Festenstein, 1992). By day 5, the infiltrate comprised largely CD45+ cells, but to our surprise the numbers of infiltrating cells were much higher in JVB-infected pancreata. These lymphocytic cells were dispersed throughout the acinar tissue and around islets, and their presence in juxtaposition to regenerating acinar tissue and islet neogenesis suggests that they may play a role in tissue recovery resulting in the pancreas in JVB-infected mice appearing normal by day 10 p.i. Immune-mediated killing of infected cells undoubtedly occurred but the overall result of lymphocytic infiltration was the restriction of further virus damage followed by tissue repair. In contrast, in the E2-infected animals far fewer infiltrating cells were seen, despite higher levels of virus replication and tissue damage. Perhaps the greater virulence of E2 leads to cytopathology before the infected cells can synthesize cytokines such as interferons to limit virus replication and chemokines to attract lymphocytes into the tissue. The particular importance of interferon-α in protecting islets from CVB4-induced damage was recently shown by N. Sarvetnick and colleagues (Flodstrom et al., 2002). The rapidity with which E2 infects and spreads throughout the exocrine pancreas may prevent the tissue from mounting an effective antiviral response in time.

The pathogenesis of E2 that we observed in 6-week-old SJL mice closely mimics the disease seen in the patient from whom this CVB4 variant was isolated (Yoon et al., 1979). A more fundamental question is whether our findings support the hypothesis that CVB infections are more
widely involved in the onset of type 1 diabetes, a disease long held to be due to autoimmune attack on beta cells by autoreactive T cells. Our results did not indicate that autoimmunity was a major component of the islet damage seen in E2-infected animals. However, recent studies have defined a group of diabetic patients who lack autoreactive antibodies or T cells indicating that autoimmunity is not a prerequisite of the disease. This idiopathic type 1B diabetes comprises between 5 and 20% of cases of insulin-dependent diabetes in different ethnic populations (Gavin, 2002). It is associated with younger children and abrupt onset with rapid loss of beta-cell capacity (Urakami et al., 2002). There is no evidence of autoimmunity, while a history of recent virus-like illness is common. In one study where pancreatic biopsy specimens were examined, T-cell infiltrates into the exocrine tissue were seen but no insulitis and high serum pancreatic enzyme concentrations suggested that damage to exocrine tissue had occurred (Imagawa et al., 2000). Others have also found high pancreatic enzyme activities in more than 25% of patients at the time of diagnosis (Semakula et al., 1996). Our findings with E2 fit well with this type of disease onset.

The fact that the pancreata of 6-week-old mice infected with the non-diabetogenic JVB strain did not regain normal insulin levels raises the possibility that any virus that causes exocrine damage may reduce the ability of the pancreas to reconstitute islet tissue and avert glucose dysregulation. It is possible that the pancreas has a finite capacity to regenerate and that successive insults (different virus infections, perhaps in conjunction with immune-mediated pathology, diet or alcohol) eventually result in an inability to replace damaged tissue. Diabetes would then ensue when insufficient islet neogenesis occurred to maintain glucose homeostasis. In this regard, coxsackievirus-induced diabetes may share similarities with HIV infections where AIDS results only when the capacity to replace CD4 cells is exhausted. Similarly, insulin insufficiency may only become apparent when the beta-cell precursors are no longer available to replenish cells lost due to successive virus infections or indeed to a single fulminant infection in the case of a virulent strain such as E2. This hypothesis invokes a role for all pancreatic viruses and not just rare variants with a particular tropism for beta cells, which fits much better with the epidemiological data reported (Roviainen et al., 1998; Hyoty et al., 1998).

While our study switches the focus of coxsackievirus-induced diabetes from the islet and beta-cell destruction to the exocrine tissue and islet neogenesis, other factors most likely play a role. CVB infections in humans are known to be associated with production of autoantibodies and autoreactive T cells that could accelerate islet destruction (Atkinson et al., 1994; Peakman et al., 2001), and molecular mimicry between CVB antigens VP1 and 2C and two of the major autoantigens identified in type 1 diabetes, IA-2 and GAD65, may play a role in this (Harkonen et al., 2002; Lonnrøt et al., 1996). In individuals with a genetic predisposition to autoimmune reactions, direct killing of beta cells by virus, while limited, may be sufficient to stimulate pre-existing autoreactive T cells that may participate in islet destruction (Horwitz et al., 2001). Related to this, bystander activation of immune cells drawn to the islets to eliminate virus could result in induction of cytokines such as TNFα, IL1 and interferon-γ, which are known to be capable of triggering beta-cell death, and this could also accelerate the onset of insulin insufficiency (Horwitz et al., 1998). The extent to which direct damage to the pancreas or islets, by virus or other environmental triggers, plays a role as opposed to immune-mediated damage by cytokines or self-reactive T cells probably varies between patients of differing genetic backgrounds. Thus, while we did not find evidence of insulitis in uninfected islets in the SJL mouse strain, autoimmune reactions may have a role in mice with different genetic backgrounds and have a role in CVB-induced disease in humans.

In conclusion, the results reported lead us to propose a novel mechanism for coxsackievirus-induced diabetes, which focuses on the prevention of islet replacement by neogenesis rather than beta-cell destruction by either virus itself or virus-induced autoimmune mechanisms. The remarkable regeneration of pancreas that occurs in JVB-infected mice lends optimism to the development of treatment strategies for type 1 diabetes based on limitation of acute virus-induced damage and treatment with growth factors and/or stem cells to encourage regeneration of both acinar and islet tissue.

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Coxsackievirus B4 and islet neogenesis


