

Coxsackievirus B3 infection and type 1 diabetes development in NOD mice: insulinitis determines susceptibility of pancreatic islets to virus infection

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Abstract

Group B coxsackieviruses (CVB) are believed to trigger some cases of human type 1 diabetes (T1D), although the mechanism by which this may occur has not been shown. We demonstrated previously that inoculation of young nonobese diabetic (NOD) mice with any of several different CVB strains reduced T1D incidence. We also observed no evidence of CVB replication within islets of young NOD mice, suggesting no role for CVB in T1D induction in the NOD mouse model. The failure to observe CVB replication within islets of young NOD mice has been proposed to be due to interferon expression by insulin-producing beta cells or lack of expression of the CVB receptor CAR. We found that CAR protein is detectable within islets of young and older NOD mice and that a CVB3 strain, which expresses murine IL-4, can replicate in islets. Mice inoculated with the IL-4 expressing CVB3 chimeric strain were better protected from T1D onset than were mock-infected control mice despite intraslet viral replication. Having demonstrated that CVB can replicate in healthy islets of young NOD mice when the intraslet environment is suitably altered, we asked whether islets in old prediabetic mice were resistant to CVB infection. Unlike young mice in which insulinitis is not yet apparent, older NOD mice demonstrate severe insulinitis in all islets. Inoculating older prediabetic mice with different pathogenic CVB strains caused accelerated T1D onset relative to control mice, a phenomenon that was preceded by detection of virus within islets. Together, the results suggest a model for resolving conflicting data regarding the role of CVB in human T1D etiology. © 2004 Elsevier Inc. All rights reserved.

Keywords: Coxsackievirus; Diabetes; Insulinitis

Introduction

The group B coxsackieviruses (six serotypes, CVB1-6) are human enteroviruses [*Picornaviridae*; Human Enterovirus B; (Oberste et al., 2004) which have a positive sense RNA genome that encodes 11 proteins within a single open reading frame (Tracy et al., 1997). The viral genome is

packaged in a naked capsid approximately 29 nm in diameter (Mucklebaur et al., 1995). The primary route of enteroviral infection is fecal-oral: the initial site of replication is believed to be within oral lymph nodes and the gut. Viremia occurs rapidly, spreading virus to secondary sites of infection which are often involved in disease states such as heart, pancreas, the central nervous system or skeletal muscle (Melnick, 1996). Group B coxsackieviruses are encountered worldwide and are established as common causes of diverse benign and serious human diseases (Melnick, 1996) including myocarditis [reviewed in (Kim et al., 2001, 2002; Martino et al., 1995)] and pancreatitis (Arnesjo et al., 1976; Gooby-Toedt et al., 1996; Imrie et al., 1977; Lal et al., 1988; Ramsingh, 1997;

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Ursing, 1973). The CVB are also believed to be able to trigger insulin-dependent type 1 diabetes (T1D) in humans, although its role in this disease has not been well established (Baekkeskov and Hansen, 1990; Craighead, 1975; Dippe et al., 1975; Foulis et al., 1990; Fuchtenbusch et al., 2001; Gamble, 1976; Hierholzer and Farris, 1974; von Herrath, 2000; Yoon et al., 1979).

The incidence of type 1 (juvenile or insulin-dependent) diabetes mellitus (T1D) is increasing worldwide (Onkama et al., 1999). An inability to regulate glucose in diabetic patients occurs due to autoimmune-mediated loss of insulin-producing beta cells in the pancreatic islets of Langerhans (Castano and Eisenbarth, 1990). Complex human genetic (Dorman and Bunker, 2000; Eisenbarth, 1986; Rotter et al., 1990; Wolf et al., 1983) plays a significant role in T1D onset but account for fewer than half of the cases (Barnett et al., 1981; Lo et al., 1991; Metcalfe et al., 2001; Redondo et al., 2001; Smith et al., 1998). Most cases of T1D are thus presumed to be a function of a combination of individual predisposing genetics and environmental factors, although the identities of putative environmental triggers remain unknown or largely surmised (Akerblom et al., 2002; Knip and Akerblom, 1999; von Herrath, 2000). Viruses, especially CVB, have been repeatedly suggested to act as triggers in the development of some cases of T1D (Jenson and Rpsenburg, 1984; Szopa et al., 1993; Ylipaasto et al., 2004; Yoon et al., 1979).

The nonobese diabetic (NOD) mouse is a small animal model of human T1D (Atkinson and Leiter, 1999; Kikutani and Makino, 1992; Makino et al., 1980). Young (4-week-old) NOD mice do not exhibit the autoimmune islet inflammation (insulinitis) which is responsible for the destruction of the beta cells later in life but insulinitis is readily apparent by 8–10 weeks of age. The developing insulinitis and concurrent beta cell killing eventually result in T1D onset starting to occur by 12–15 weeks of age, 70–100% of female NOD mice develop T1D by 30–40 weeks of age (Tisch et al., 1999; Tracy et al., 2002). In a previous report, it was demonstrated that inoculation of young NOD mice with any of numerous different strains of CVB3 or CVB4 resulted in significantly lowered incidences of T1D over 10 month (Tracy et al., 2002). Despite observations that CVB replicate to high titers in the murine pancreas (Tracy et al., 2000, 2002), we observed no evidence of CVB RNA in NOD mouse pancreatic islets, consistent with the observations of others (Mena et al., 2000; Vella et al., 1992; Vuorinen et al., 1989). These results argued against a mechanism, which invokes direct CVB-induced killing of pancreatic beta cells in young NOD mice as a mechanism for triggering T1D onset.

Two mechanisms have been proposed for why pancreatic islets in young NOD mice remain resistant to CVB infection. Intraislet production of type 1 interferons alpha/beta (IFN α / β) inhibits CVB4 replication in islets in vivo (Chehadeh et al., 2000; Flodstrom et al., 2002). These observations suggest a local, immune-mediated mechanism by which CVB could be denied productive replication within pan-

atic islets. The cells which are strongly associated with IFN production, are the insulin-producing beta cells. The other hypothetical mechanism by which CVB could be denied replication within islets is a lack of CAR protein expression, the receptor for the CVB (Bergelson et al., 1997; Carson et al., 1997; Tomko et al., 1997). Murine CAR mRNA was not detected in murine pancreatic islets by in situ hybridization analysis (Mena et al., 2000) although others have detected CAR protein expression using immunohistochemistry (Chehadeh et al., 2000). These hypotheses are not mutually exclusive.

In this report, we sought to reconcile contradictory data regarding the CVB etiology of human T1D by studying CVB infections in the NOD mouse host. To begin, we evaluated hypothetical mechanisms which have been proposed to account for failure to observe CVB replication in islets of young mice. Our results demonstrate that CAR can be detected and that CVB, if expressing the cytokine IL-4, can also be detected in islets after infection. These results show that it is possible for CVB to replicate in islets if the inraisle environment is specifically altered. Having shown that islets in young mice without insulinitis are resistant to productive CVB replication under normal circumstances, and reasoning that widespread insulinitis present in older NOD mice as they progress naturally toward T1D represents an alteration of the islet environment, we determined whether CVB could replicate in islets of old, prediabetic mice. We demonstrate that virulent CVB strains can be detected in the remaining healthy (non-infiltrated) regions of the islets shortly after infection and that this is linked to both to rapid T1D onset relative to control and a high titer of virus in the pancreas tissue. Together, the results provide a basis for resolving conflicting data regarding the impact of CVB infection upon human T1D development.

Results

Can CVB replicate within islets of young NOD mice?

Although CVB are hypothesized to be triggers of human T1D, the evidence for this in the related NOD mouse model is weak. For CVB to trigger T1D in NOD mice, we reasoned that the virus should be able to replicate within islets but this has not been observed in young NOD mice (Mena et al., 2000; Tracy et al., 2002). One hypothesis suggests that the lack of CAR expression in islets is a barrier to CVB replication (Mena et al., 2000), while another proposes that beta cell expression of interferons (IFN) suppresses intraislet CVB replication (Chehadeh et al., 2000; Flodstrom et al., 2002). To begin this work, we evaluated aspects of each hypothesis in an effort to understand the apparent blockade to CVB replication in young NOD which are still 2–4 months away from naturally developing T1D.

As CAR is the primary receptor protein for the CVB in mice and humans (Bergelson, 2003; Bergelson et al., 1998; Carson, 2001), the lack of CAR expression in islets would be a compelling argument for failing to observe intraislet CVB replication in normal mouse islets in vivo (Mena et al., 2000). Others, however, have reported the detection of CAR protein in islets using immunohistochemistry (Chehadeh et al., 2000). Further, in vivo cultured islets support CVB replication (Roivainen et al., 2000; Szopa et al., 1986; Toniolo et al., 1982; Vuorinen et al., 1992; Yoon et al., 1979), while cultivating human islets in culture in the presence of antibodies against CAR prevents CVB infection (Ylipaasto et al., 2004). We assayed NOD mouse pancreas tissue immunohistochemically for expression of the CAR protein with a commercially available antibody (K-20; Santa Cruz Biotechnology, Santa Cruz, CA) derived against a peptide sequence completely conserved between mouse and human CAR. To assess the specificity of the commercial CAR antibody, we used RD cells, which do not express detectable CAR, and a line of RD cells that have been engineered to reexpress human CAR (RD-hCAR). RD-hCAR cells have been demonstrated by Western blot analysis to express CAR (Cunningham et al., 2003). We compared results staining RD-hCAR cells to those obtained staining the parental RD cell line, which does not express measurable quantities of CAR. Using the K-20 antibody, positive staining was observed on RD-hCAR cells (Fig. 1B), while no staining was observed on RD cells (Fig. 1A).

The K-20 antibody also detected CAR in pancreas section from young (Fig. 1C) and older NOD mice (Fig.

1D). Insulin staining co-localized with CAR staining in mouse islets (Figs. 1D, E). CAR was not detected in sections that did not receive primary antibody (Fig. 1F). Assay of sections from an archival formalin-fixed, paraffin-embedded human pancreas sample using the K-20 antibody (Fig. 1G) and a previously characterized, human-specific CAR antibody, RmCB (Hsu et al., 1988) (Fig. 1H), revealed a similar localization in islets. No staining was detected in the absence of a primary antibody (Fig. 1I). The detection of CAR protein in islets of NOD mouse pancreatic tissue suggested that the primary block to productive CVB replication in young NOD mouse islets was not lack of CAR protein expression.

Interleukin 4 is a key cytokine [reviewed in (Paul, 1991)] with various effects, among which is the capacity to modulate IFN [(Gobl and Alm, 1992; Lee et al., 1995; Varano et al., 2000); reviewed in (Levings and Schrader, 1999)]. Interleukin-4 also suppresses T1D development in NOD mice (Gallichan et al., 1999; Katz et al., 1995; Mueller et al., 1996; Wang et al., 1998), an observation that has been ascribed to downregulation of autoimmune Th1 cells (Cameron et al., 1997; Fiorentino et al., 1989). Others have shown $\text{INF}\alpha/\beta$ inhibits CVB4 replication in NOD mouse islets in vivo (Chehadeh et al., 2000; Flodstrom et al., 2002), data which offer a different hypothetical mechanism to account for failure to observe CVB replication in islets of young NOD mice. We reasoned that a CVB3 strain that expresses biologically active murine IL-4 [CVB3-mIL4; (Chapman et al., 2000a)] might sufficiently alter the intraislet microenvironment to permit virus replication. Young (4

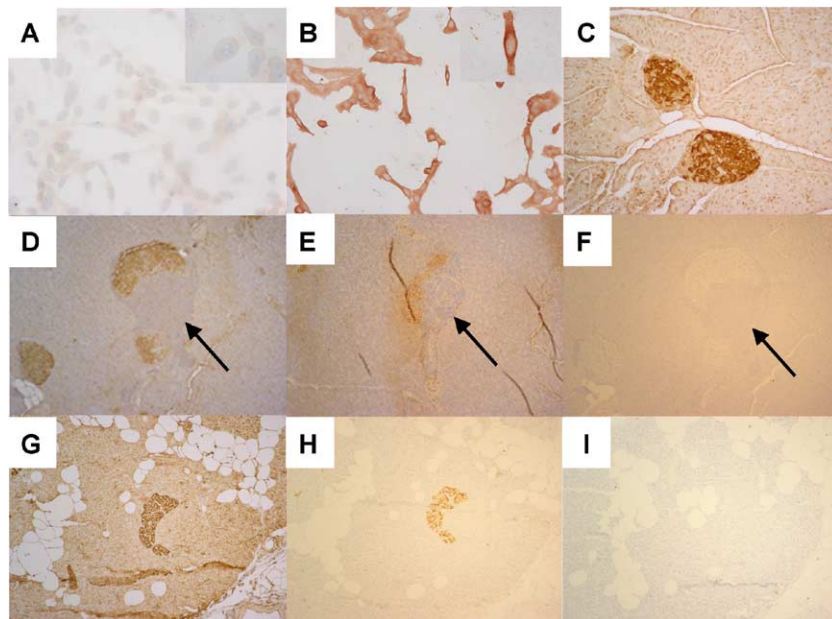


Fig. 1. Detection of CAR protein in NOD mouse and human pancreatic islets of Langerhans. Human RD cells and RD cells expressing human CAR (RD-hCAR) were stained with an antibody against CAR [K-20, Santa Cruz Biotechnology; panels A, B, respectively]. (C) CAR is detected in islets of young (4 weeks old) NOD mice using the K-20 antibody. Sections from a 14-week-old NOD mouse were stained with an antibody against insulin (D), an antibody against CAR (K-20) (E), or no primary antibody (F). Arrows follow the same islet in serial sections (D–F). Sections from a formalin-fixed, paraffin-embedded, archived human pancreas were stained with the K-20 CAR antibody (G), the human-specific RmCB CAR antibody (H), or no primary antibody (I). Panels A, B, original magnification, 40 \times (insert 120 \times); panels C–I, 100 \times .

weeks old) NOD mice were inoculated with CVB3-mIL4 or the control parental CVB3 strain. Productive replication of CVB3-mIL4 and the parental virus in NOD mouse pancreata was confirmed by measuring infectious virus titer in total pancreas tissue as a function of days post-inoculation (p.i.) (Fig. 2A). Titers of both viruses peaked shortly after inoculation, then declined, confirming and extending previous findings for other CVB strains in murine pancreas tissue (Tracy et al., 2000). The parental strain CVB3/0 replicated approximately 1–2 logs higher titers than CVB3-mIL4 on day 4 and infectious titers for both viruses were undetectable by day 8 p.i. consistent with avirulent CVB strain replication (Tracy et al., 2000). Pancreas sections from mice killed 4 days p.i. (mock-infected mice, Figs. 2B–D; CVB3-mIL4 infected mice, Figs. 2E–G) were probed for CVB protein. Identity of islets was confirmed by staining with insulin (Fig. 2C) or glucagon (Fig. 2F); the same islets are shown in hematoxylin and eosin-stained serial sections (Figs. 2B and E, respectively). Cells that stained well for viral protein were readily observed in islets of CVB3-mIL4-inoculated mice (Fig. 2G; the same islet is shown at high magnification in Fig. 2H). This staining was appreciably greater than background staining with the same antibody against enteroviral capsid proteins observed in tissue from mock-infected mice (Fig. 2D), suggesting that detectable CVB3-mIL4 replication occurred in islets. These immunohistochemical results were further tested using in situ hybridization (Figs. 2I–K). Pancreas from mock-infected mice was negative for detection of viral RNA (Fig. 2I; islets shown with arrows). Viral RNA of the control parental virus CVB3/0 (which does not produce mIL-4) was not detected in islets but was apparent in acinar tissue (Fig. 2J). Viral RNA was detected only in islets of mice previously inoculated with CVB3-mIL-4 (Fig. 2K). These results demonstrated that unlike wild-type CVB strains, a CVB3 strain which expresses biologically active murine IL-4 can replicate within islets of young NOD mice.

Injection of mice with the double-stranded RNA analog polyinosinic:polycytosinic acid (poly I:C) strongly stimulates the innate immune system and induces production of innate interferons (Biron et al., 1986; Brehm and Kirchner, 1986; Korngold et al., 1983; Padalko et al., 2004; Pyo et al., 2004). We reasoned that poly I:C inoculation before CVB3-mIL4

inoculation might also suppress CVB3-mIL4 replication in pancreas despite the expression of IL-4. To test, this we inoculated young mice with poly I:C and on the following day, we inoculated mice with CVB3-mIL4. Mice were killed at 2 and 4 days post-inoculation with virus. Infectious virus titer was reduced by 2–3 logs in pancreas tissues of mice treated with poly I:C versus control mice that were injected only with saline (Fig. 3A). Pancreas sections were stained for

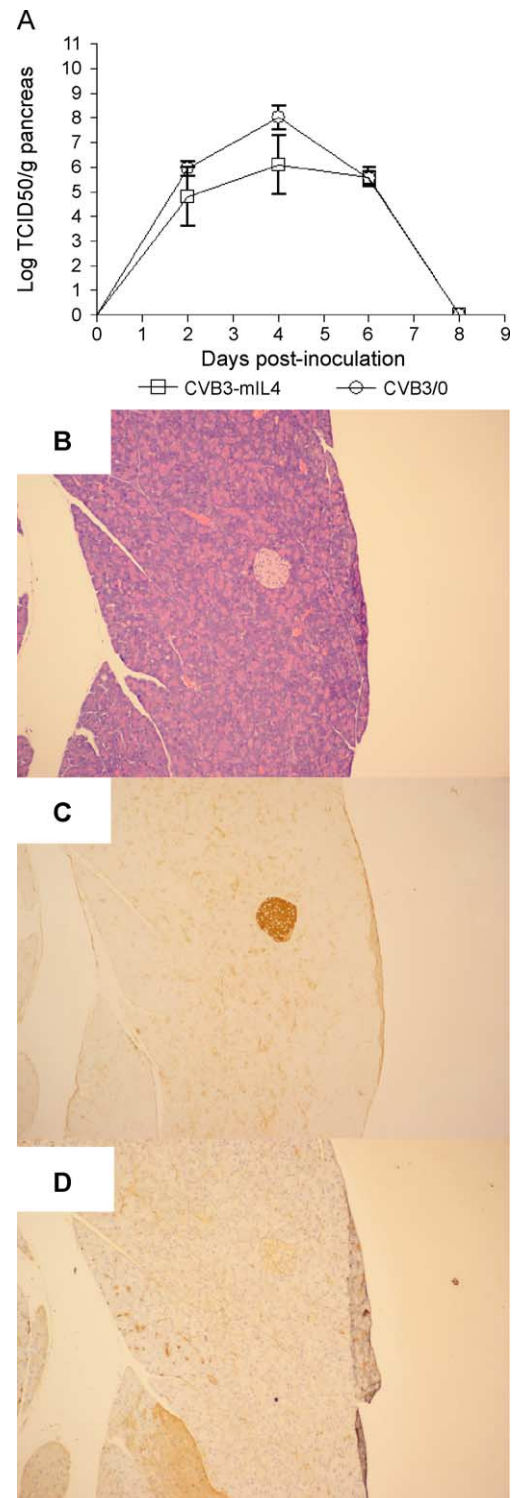


Fig. 2. CVB3-mIL4 replicates in NOD mouse pancreas and pancreatic islets. Titering infectious virus in pancreata of mice previously inoculated with CVB3-mIL4 or CVB3/0 demonstrate that both viruses replicate in NOD mouse pancreas tissue (A). Serial pancreas sections from mock-infected (control) NOD mice (B–D) and CVB3-mIL4-inoculated mice (E–G) were stained with hematoxylin and eosin (B, E), an antibody against insulin (C), glucagon (F), or an antibody against enteroviral capsid proteins (D, G). Panel H shows the same islet as in panel G but at 400 \times magnification; arrows indicate cells staining positive for virus protein. In situ hybridization with a DIG-labeled riboprobe against the CVB3 positive strand RNA was used to probe pancreata of mice inoculated with saline (controls) (I), CVB3/0 (J) or CVB3-mIL4 (K). Arrows in panels I–K indicate islets. Original magnification panels B–G, 100 \times ; G, 400 \times ; panels I–K, 200 \times .

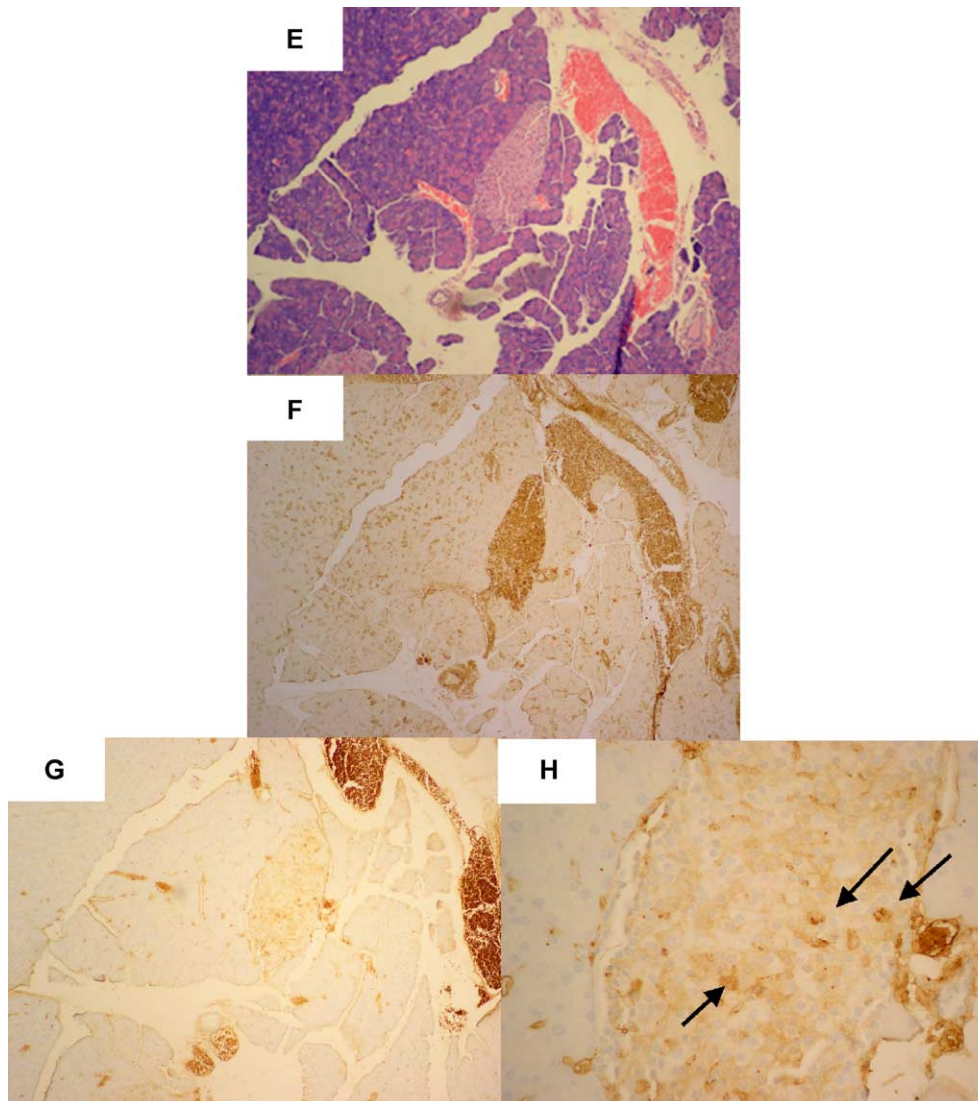


Fig. 2 (continued).

insulin (Figs. 3B, D, F) and CVB3 protein (Figs. 3C, E, G). Virus protein staining in islets co-localized with insulin staining. Islets in pancreata of mice which had been inoculated with poly I:C before CVB3-mIL4 inoculation showed suppressed staining for viral protein in islets (Fig. 3G) relative to control, CVB3-mIL4-inoculated mice (Fig. 3E). Taken together, these results demonstrate that CVB replication can occur or be suppressed in islets of normal, young NOD mice, depending upon the state of the intraislet microenvironment.

Despite replication of CVB3-mIL4 in islets, fewer mice develop T1D than controls

Our observation that recombinant CVB3-mIL4 replicated in islets of young NOD mice permitted testing of the hypothesis that intraislet CVB replication may trigger T1D onset. Others have shown that a strain of CVB4 induces T1D in beta cell expressing suppressor of cytokine signal-

ing-1 (SOCS-1) transgenic NOD mice (Flodstrom et al., 2002), results that have been used to support the hypothesis that some cases of T1D may be induced by CVB infection of islets. Groups of NOD mice were inoculated at 4 and 6 weeks of age with CVB3-mIL4 or parental control CVB3/0, then were maintained to 39 weeks of age. Onset of T1D was assessed weekly by assaying urine glucose; mice were considered diabetic when urine glucose levels were ≥ 2 g/dL urine (Tracy et al., 2002). Ninety percent (9/10) of mock-infected control mice developed T1D before the end of the experiment (Fig. 4), while only 3/10 (30%) mice inoculated with CVB3-mIL4 developed T1D during this time; 7 mice remained healthy. Mice inoculated with the control virus strain, CVB3/0, fared less well than CVB3-mIL4-inoculated mice; 6/10 mice (60%) of these mice developed T1D, although initial onset of T1D was delayed by 7 weeks relative to control mice. These results demonstrate that despite intraislet replication of CVB3-mIL4, significantly fewer mice developed T1D as a consequence of CVB3-

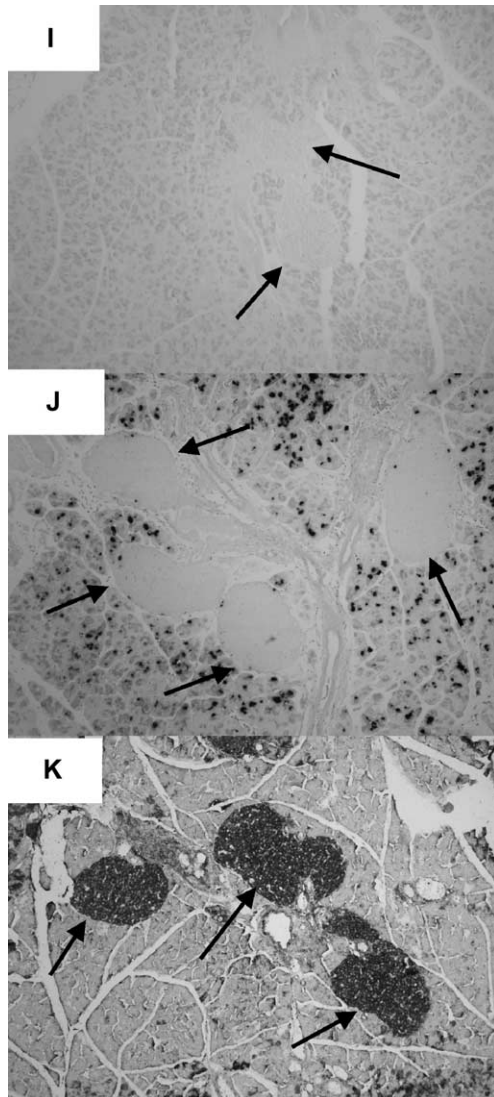


Fig. 2 (continued).

mIL4 inoculation than control mice. The results suggest that inraislelet replication of virus must not necessarily be deleterious to the health of the host.

CVB3 can rapidly accelerate T1D onset in a majority of old, immediately prediabetic NOD mice

The role of CVB infection as a triggering event for the onset of human T1D or in murine systems remains unclear (Coleman et al., 1974; Craighead, 1975; Dippe et al., 1975; Fuchtenbusch et al., 2001; Gamble, 1976; Hierholzer and Farris, 1974; Nairn et al., 1999; Oldstone et al., 1990; Ramsingh et al., 1997; Takei et al., 1992; Tracy et al., 2002; Wagenknecht et al., 1991). While inoculation of 4-week-old NOD mice with CVB prevents T1D onset in the majority of mice relative to no treatment (Tracy et al., 2002), there is no observable insulinitis at this age. This soon changes, by 8 weeks of age, insulinitis is consistently detected in pancreatic islets of NOD mice. Notably, inoculation of mice at 8 weeks

of age (in contrast to 4-week-old mice) with CVB has been observed to cause accelerated T1D onset in some mice relative to mock-infected control mice (Serreze et al., 2000; Tracy et al., 2002). Accelerated, CVB-triggered T1D onset in 8-week-old mice is related to the extent of insulinitis at the time of inoculation: accelerated T1D onset is due to an expanding population of autoimmune lymphocytes (Serreze et al., 2000). However, if CVB infection triggers accelerated T1D onset in NOD mice only when insulinitis has reached a critical threshold, then few mice at 8 weeks of age meet this

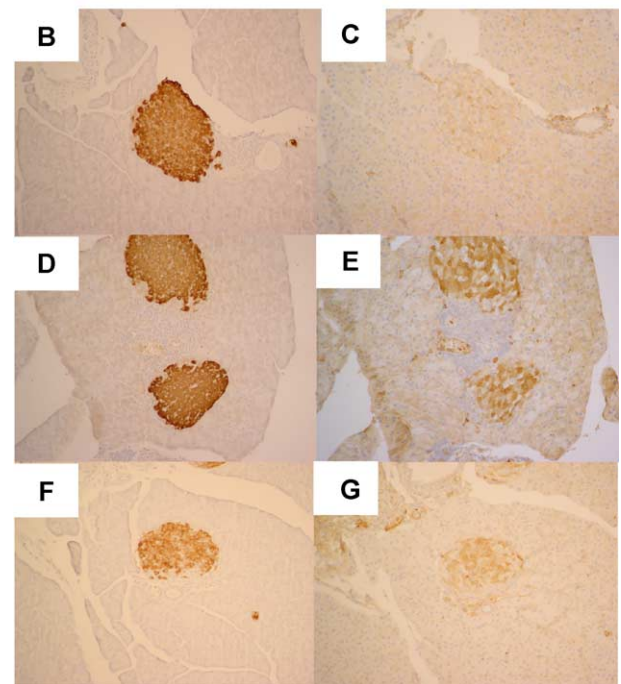
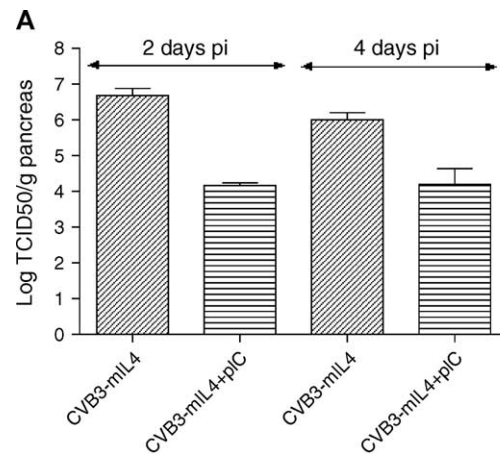


Fig. 3. Inoculation with poly I:C suppresses CVB3-mIL4 replication in pancreas. Mice (groups of 3) were inoculated with poly I:C or with saline, then 24 h later inoculated with CVB3-mIL4. (A) Titers of CVB3-mIL4 in mouse pancreata 2 and 4 days post-inoculation. (B–G) Formalin-fixed, paraffin-embedded sections from pancreata of mice 2 days post-inoculation with CVB3-mIL4 were probed immunohistochemically for insulin (panels B, D, F) and virus (C, E, G) as indicated in the figure. Panels B, C, control (saline inoculated) mice; panels D, E, saline and CVB3-mIL4 infected control mice; panels F, G, poly I:C treated and CVB3-mIL4-inoculated mice. Original magnification, 200 \times .

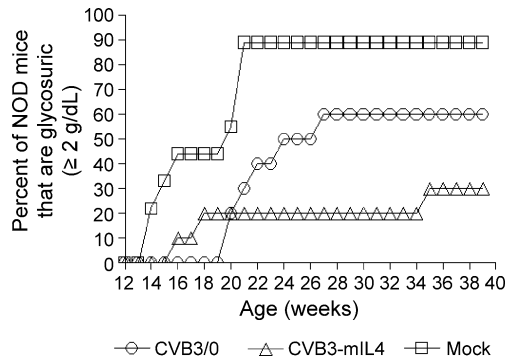


Fig. 4. Inoculation of young NOD mice with CVB3-mIL4 suppresses T1D incidence. Groups of 10 NOD mice were inoculated with the CVB3 strains shown in the figure; mock-infected mice were inoculated with saline. T1D onset was ascertained by measuring urine glucose content. Surviving mice were killed at 39 weeks of age.

criterion (Serreze et al., 2000). However, at 12–15 weeks of age, with widespread insulinitis and T1D beginning to occur, every NOD mouse can be considered to be immediately prediabetic. We reasoned that older, immediately prediabetic mice would be at risk for accelerated T1D onset following CVB inoculation based on the hypothesis that the extent of lymphocytic infiltration in islets is linked to accelerated T1D onset. There would also be significantly fewer intact beta cells in the prediabetic mice relative to healthy, younger mice. Widespread insulinitis and decreased beta cell populations might constitute a significant islet microenvironment alteration, analogous to that obtained in young SOCS-1 transgenic mice (Flodstrom et al., 2002) or in young mice inoculated with CVB3-mIL4.

To determine the effect of CVB replication upon T1D onset and incidence in older NOD mice, we inoculated NOD mice (15 weeks of age) with CVB3/28, a virulent strain, or CVB3/GA, an avirulent strain (Tracy et al., 2000, 2002). Diabetes developed in 7/10 mice (70%) within 2 weeks of inoculation with CVB3/28 (Fig. 5A), in contrast to long-term protection afforded the majority of mice when inoculated at 4 weeks of age (Tracy et al., 2002). The T1D incidence in mock-infected mice did not reach 70% for another 9 weeks. Inoculation with CVB3/GA, however, delayed T1D onset by 5 weeks relative to mock-infected (control) mice but thereafter 70% of mice developed T1D by 30 weeks of age. Stained pancreas sections revealed typical CVB3/28-induced widespread pancreatitis while CVB3/GA induced no pancreatitis (data not shown), consistent with previous results (Tracy et al., 2002). These results demonstrated that a CVB infection can dramatically impact T1D development in a population of immediately prediabetic, older NOD mice. Further, the outcome of the infection was significantly different, either adverse or temporarily beneficial, as an apparent function of the viral virulence phenotype.

A second experiment tested the hypothesis that the CVB3 virulence phenotype can affect timing of initial T1D onset in older, prediabetic mice following inoculation. NOD

mice (17 weeks of age) were inoculated with CVB3/AS, a virulent strain (Tracy et al., 2000, 2002). CVB3/CO, a strain which induces variable pancreatitis (Tracy et al., 2000) or the avirulent strains, CVB3/GA or CVB3/49. Virulent CVB3/AS inoculation greatly accelerated T1D development relative to mock-infected control mice; 70% of mice (7/10) were diabetic within 3 weeks of inoculation (Fig. 5B) versus less than 30% of control mice, consistent with and confirming previous results using CVB3/28 (Fig. 5A). CVB3/GA and CVB3/49 each slowed the rate of T1D onset relative to mock-infected control mice; both groups developed T1D incidences of 40% by the time the experiment was ended. CVB3/CO induced accelerated T1D in two mice relative to the control group but thereafter, no significant difference was observed relative to control mice in the rate of T1D development; the final T1D incidence was 70% in this group (Fig. 5B). These results demonstrated that the outcome of inoculating older, immediately prediabetic NOD mice with different CVB3 strains is dependent upon the viral virulence phenotype: virulent CVB3 strains significantly accelerate T1D onset in a majority of mice, whereas avirulent CVB3 strains initially delay T1D onset relative to mock-infected control mice. These results differ sharply from those obtained using 4-week-old NOD mice in

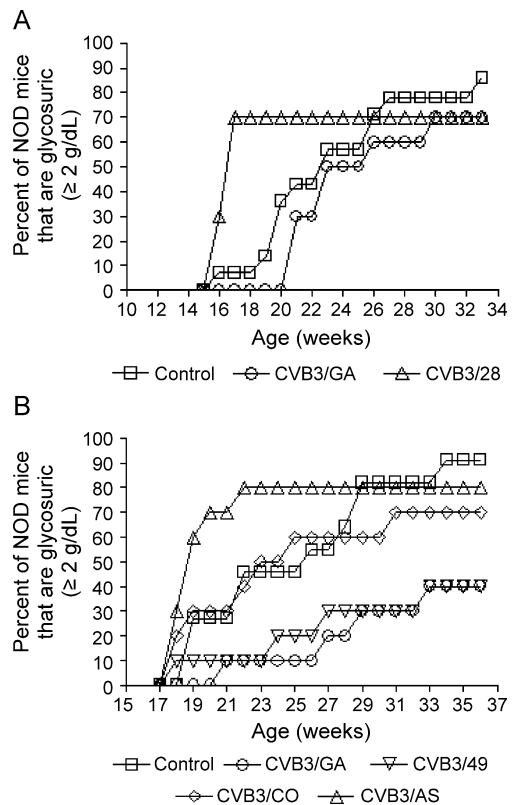


Fig. 5. Impact on T1D development of inoculating older, prediabetic mice with different CVB3 strains. Groups of 10 NOD mice were inoculated at 15 weeks (A) or 17 weeks (B) with strains of CVB3 that differed by virulence phenotype. Control mice were mock infected with saline. T1D onset ascertained by measuring urine glucose content. Surviving mice were killed at 33 weeks (A) or 36 weeks of age (B).

which insulinitis is not apparent inoculated with the same CVB3 strains (Tracy et al., 2002): inoculation of young mice with any CVB strain significantly reduced the change of T1D development, with virulent virus strains being the most protective.

Virulent CVB3 is detected in pancreatic islets of immediately prediabetic NOD mice

Because inoculation with virulent CVB3 strains greatly accelerated T1D onset in older and immediately prediabetic mice, we reasoned that virus replication within cells in the islets may be linked to the rapid onset of T1D. To test whether CVB were detectable in islets of old mice following inoculation, we assayed pancreas section of 17-week-old NOD mice killed 4 days after inoculation with different CVB strains for evidence of virus in islets. CVB protein was detected by immunohistochemistry in islets of mice inoculated with virulent strains indicating viral replication, in marked contrast to the results in young mice inoculated with either of two avirulent strains was indistinguishable from control mice. Fig. 6 displays typical results using sections from pancreata of mice inoculated with CVB3/28 (Figs. 6A–D), CVB3/CO (Figs. 6E–H), CVB3/49 (Figs. 6I, J), or from mock-infected control mice (Figs. 6K, L). Insulin staining identified islets as well as remaining functional islet tissue (Figs. 6B, F, I, K) in the presence of advanced insulinitis which is shown in hematoxylin and eosin-stained sections (Figs. 6A, E). Panel I demonstrates a highly inflamed islet; minimal insulin staining was observed.

Staining for viral protein was positive in islets (thin arrows) and in acinar tissues (large arrows) of mice previously inoculated with virulent CVB3/28 (Fig. 6D). Detection of virus protein in islets (Fig. 6H) of mice inoculated with less virulent CVB3/CO was less clearly defined than that observed with virulent strains, but trended higher than either islets from mice inoculated with avirulent strains like CVB3/49 (Fig. 6J) or mock-infected control mice (Fig. 6L). In addition, pancreatic acinar tissue in CVB3/CO-inoculated mice was usually positive for viral protein detection (Fig. 6H, large arrow), whereas acinar tissue from mice inoculated with virulent strains was consistently positive for the detection of viral protein (Fig. 6D). These results showed that replication of virulent CVB3 strains occurs in the remaining healthy areas of islets in older prediabetic mice before the rapid T1D onset which occurs within 2–3 weeks of virus inoculation.

Detection of CVB3 in islets of prediabetic mice, which is related to virulence phenotype of the virus, did not explain why less virulent or avirulent strains were not as readily detected in islets. Having previously established that virulent CVB3 strains replicate to higher titers than avirulent strains in pancreas tissue of young NOD and other strains of mice (Tracy et al., 2000, 2002), we determined whether CVB3 strains similarly generate different titers as a function of virulence phenotype in older NOD mice. Infectious titers in pancreata of 15-week-old mice previously inoculated with one of 5 different strains of CVB3 were determined 4 days post-inoculation, a time when virus titer peaks in the pancreas (Tracy et al., 2000; Fig. 2A). Highly virulent strains

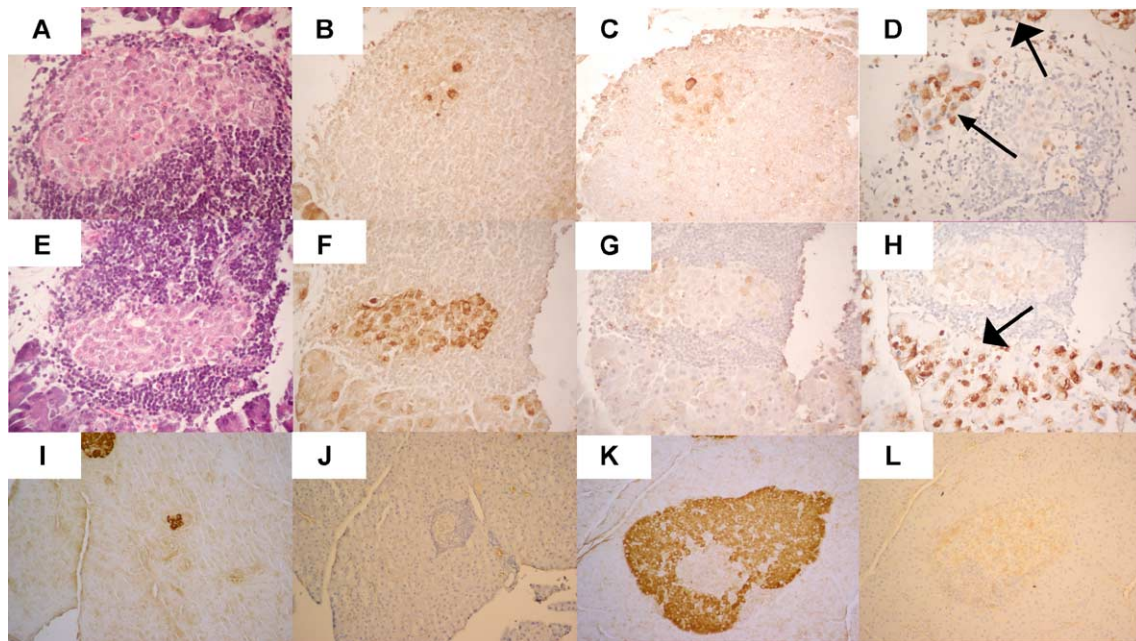


Fig. 6. CVB3 can be detected within islets of older, prediabetic NOD mice. Mice were killed 4 days post-inoculation with CVB3/28 (panels A–D), CVB3/CO (panels E–H), CVB3/49 (panels I, J) or saline (mock-infected; panels K, L). Serial pancreas sections were stained with hematoxylin and eosin (panels A, E) an antibody against insulin (panels B, F, I, K), an antibody against CAR (K-20; panels C, G), or an antibody against enterovirus capsid protein (D, H, J, L). Large arrows indicate acinar tissue staining for viral proteins (panels D, H); small arrows indicates positive staining for virus protein in islet (panel D). Original magnification, panels A–H, $\times 400$; panels I–L, $\times 200$.

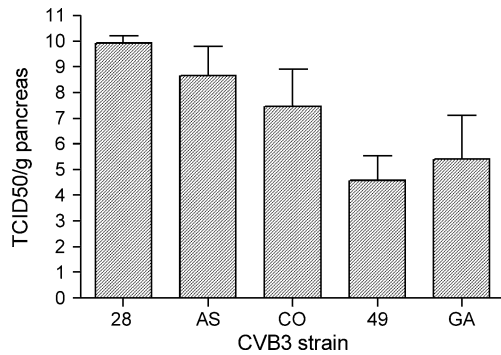


Fig. 7. Infectious titers of different CVB3 strains in pancreata of older, prediabetic mice. Mice (groups of 3) were inoculated at 25 weeks of age with one of five strains of CVB3 shown in the figure. Mice were killed 4 days later and infectious titers in pancreas tissue determined. The mean titers are shown with bars denoting standard deviation.

(CVB3/AS, CVB3/28) replicated to titers that were 3–5 logs higher than the avirulent strains, CVB3/GA and CVB3/49 (Fig. 7). Moderately virulent CVB3/CO replicated to 1–2 logs higher in titer than the avirulent strains. This variation in titer was similar to that observed earlier in younger mice, providing a rationale to explain why only virulent strains induced rapid T1D onset in the majority of older mice.

Discussion

Human T1D etiology is a complex problem and a poorly understood combination of individual genetic factors, putative environmental triggers [reviewed in (Atkinson and Maclaren, 1994; Bach, 1994; Baekkeskov and Hansen, 1990; Barnett et al., 1981; Bertrera et al., 1999; Blom et al., 1989; 1991; Chowdhury et al., 1999; von Herrath, 2000)]. The NOD mouse permits the study of T1D in a small animal model which although not a perfect model of human T1D, recapitulates many aspects of human T1D (Atkinson and Leiter, 1999). Experimental exposure of NOD mice to diverse agents, either infectious or chemical, can variably suppress T1D incidence (Atkinson and Leiter, 1999); this list also includes several murine viruses. Previous results (Tracy et al., 2002) were consistent with these observations: inoculation of young NOD mice with any of several phenotypically diverse and genetically distinct CVB3 or CVB4 strains lowered T1D incidences relative to mock-infected mice. However, our observations were unique in that of all the viral agents studied in the NOD mouse model, only the CVB are commonly encountered human viruses worldwide and are implicated as causes, not inhibitors, of T1D induction in humans. Despite the protective effect by CVB demonstrated in this initial paper, we did not address reports which strongly suggest that CVB infections are associated with some cases of human T1D onset [for example, (Andreoletti et al., 1997; Champsaur et al., 1980; Chehadeh et al., 2000; Clements et al., 1995; Hyoty et al., 1998; Ylipaasto et al., 2004)]. We explored this further by

developing a model in older mice to test the hypothesis that CVB can trigger T1D.

We began by assuming that CVB-induced destruction or impairment of the insulin-producing pancreatic beta cells was key to the hypothesis that CVB can induce T1D. Previous results demonstrated a remarkable CVB-induced protein of young NOD mice from T1D onset and no evidence for productive CVB3 replication in pancreatic islets of young mice despite high viral titers in pancreata within 1–2 days post-inoculation (Tracy et al., 2002). This argued against a deleterious role for the viruses in promoting T1D onset in the T1D model using young NOD mice. From the work of others, it appeared likely that CVB are unable naturally to infect islets in young NOD mice due to one or more factors, described either as limited or no CAR expression (Mena et al., 2000) and/or the production of IFN α/β by pancreatic islet beta cells (Chehadeh et al., 2000; Flodstrom et al., 2002).

We detected CAR protein murine islets with a commercially available CAR antibody, the specificity which was demonstrated by the detection of RD cells engineered to express human CAR (Cunningham et al., 2003). CAR was also detected in human islets using this antibody and a previously characterized human CAR-specific antibody (Hsu et al., 1988). These results confirmed reports which demonstrate that CVB can replicate in cultured islets and can be inhibited by the presence of anti-CAR antibody (Roivainen et al., 2000; Szopa et al., 1986; Toniolo et al., 1982; Vuorinen et al., 1992; Yoon et al., 1979). We cannot explain the disparity between these and others' results (Chehadeh et al., 2000) and those reported previously (Mena et al., 2000) in which CAR mRNA in murine islets was not detected using in situ hybridization, although it cannot be excluded that CAR mRNA may be transcribed in islets at levels below the limits of the in situ hybridization system employed.

Another hypothesis to account for the inability of CVB to replicate in islets of young mice which has been proposed by two different groups is that intraislet IFN expression inhibits CVB replication in mouse islets (Chehadeh et al., 2000; Flodstrom et al., 2002). Interleukin-4 is an anti-inflammatory cytokine with various effects upon the immune system [(Gobl and Alm, 1992; Lee et al., 1995; Varano et al., 2000); reviewed in (Levings and Schrader, 1999)]. Interleukin-4 additionally suppresses T1D development in NOD mice (Gallichan et al., 1999; Katz et al., 1995; Mueller et al., 1996; Wang et al., 1998), most likely due to downregulating pathogenic autoimmune Th1 cells (Cameron et al., 1997; Fiorentino et al., 1989). Using a CVB3 strain that expresses biologically active murine IL-4 (Chapman et al., 2000a), we observed CVB3-mIL4 in islets of young NOD mice shortly after inoculation, mice parental CVB3 was not detected in islets. We also showed that CVB3-mIL4 titers fall in the pancreata of young mice treated before infection with the potent inducer of innate immune system, poly I:C (Biron et al., 1986; Brehm and

Kirchner, 1986; Korngold et al., 1983; Padalko et al., 2004b; Pyo et al., 2004). Thus, the simple expression of murine IL-4 by CVB3 facilitated productive CVB replication in islets; this occurred without resorting to approaches such as generating transgenic mice (Flodstrom et al., 2002), which has the potential of inducing other unknown effects due either to the genetic manipulation and/or the global loss of these IFN (Gerlai, 1996; Steinman, 1997; Wolfer et al., 2002). Further work is required to clarify the mechanism by which CVB3-expressed IL-4 can replicate within islets.

The foregoing results showed CAR expression occurred within islets of young NOD mice, data that were mechanistically confirmed by the demonstration that CVB3-mIL4 replicated in mouse islets. These data showed that as long as the intraislet environment was suitably altered, replication of CVB in young NOD mice could occur. We used this finding to ask what impact CVB3-mIL4 replication within islets of young mice would have upon T1D onset. We observed that fewer of these NOD mice developed T1D compared to control groups, contrary to a simple etiologic hypothesis of CVB-induced T1D that invokes CVB-induced loss of islet beta cells. This finding suggests that CVB infections of pancreatic islets may occur in some islets of young mice, is rapidly cleared from the mouse by the innate and early adaptive immune responses before significant damage occurs, thus failing to trigger accelerated T1D. This is supported by results, which demonstrate that pancreatic viral titers peak at 4 days p.i. and are no longer detectable by day 8, results that are similar to those which show that avirulent CVB3 strains are more rapidly cleared than virulent strains (Tracy et al., 2000). Rapid clearance of CVB3-mIL4 from the pancreas as a whole, coupled with lower titers of the recombinant virus, would help to minimize islet damage. It has also been demonstrated that induction of a Th2 response in NOD mice by IL-4 protects mice from T1D (Cameron et al., 1997; Gallichan et al., 1999); we speculate that a similar mechanism, previously demonstrated following inoculation of CVB3-mIL4 in a different strain of mouse (Chapman et al., 2000a), may at least partially account for the lower T1D incidence in CVB3-mIL4-inoculated NOD mice.

The rapidity with which T1D induction occurred in older, immediately prediabetic NOD mice following inoculation with virulent CVB3 suggests significant damage occurs as a function of the intraislet virus infection. Immunohistochemical staining revealed that CVB replicates only in uninvolved sections of islets, the same regions in which insulin is expressed. Similar accelerated onset results have been observed in a minority of 8-week-old NOD mice inoculated with CVB (Serreze et al., 2000; Tracy et al., 2002). Those observations indicate that CVB infections trigger rapid T1D onset when a sufficient population of autoimmune lymphocytes has been achieved (Serreze et al., 2000). Because CVB triggers T1D only in a few mice at 8 weeks of age, it may be assumed that insulinitis in those few diabetic mice was

significant, much as is observed later at 12–15 weeks of age. Thus, the CVB-induced rapid T1D onset, which can occur sporadically in 8-week-old mice, occurs in nearly every mouse at about 15–17 weeks of age following with a pathogenic CVB strain. In contrast to results in 4-week-old mice with little or no insulinitis, and in which the inoculation of any CVB strain provided protection from T1D onset (Tracy et al., 2002), the impact of CVB infections in older prediabetic NOD mice with widespread insulinitis can be understood as manifesting one of three outcomes: immediately deleterious due to infection by highly virulent strains, temporarily beneficial when infected by avirulent strains, or without noticeable impact relative to the control (mock-infected) mice when infected by pancreovirulent strains.

Results in this report demonstrate that CVB can replicate in islets of NOD mice when the intraislet environment has been altered to be different from that found in healthy young NOD mice. Others had shown that this can be attained in transgenic mice (Flodstrom et al., 2002) or by cultivating islets *in vitro* in the presence of anti-IFN alpha neutralizing antibody (Chehadeh et al., 2000). Our results are consistent with those reports which hypothesize that intraislet and/or beta cell IFN production is a barrier to CVB replication in islets of young NOD mice (Chehadeh et al., 2000; Flodstrom et al., 2002). By extrapolating our results to humans, our findings offer a plausible explanation for reports of sudden T1D onset during or following human CVB circulation [for example, (Andreoletti et al., 1997; Helfand et al., 1995; Nairn et al., 1999)]. We theorize that two criteria, defined here in the NOD mouse model, would have been met in such cases of sudden T1D onset: (1) the infecting CVB strain would have been a pathogenic one, similar to the strains CVB3/AS and CVB3/28 used in this report [and as described elsewhere; (Lee et al., 1997; Tracy et al., 2000)] and (2) the affected individuals would have been immediately prediabetic with extensive insulinitis present before the virus infection. Although the latter condition is effectively impossible to verify in humans due to ethical and clinical difficulties, it is possible to test whether a CVB strain is pathogenic. If the specific CVB strain were not available, one might less rigorously test the hypothesis by assaying those strains which circulated in the local population at the time of T1D onset. Our results also suggest an explanation for why the great majority of CVB infections are not associated with sudden onset T1D. Infection of prediabetic NOD mice with less virulent strains either had no impact on the rate with which T1D developed in the mice relative to controls, or T1D onset was delayed well past the time of the acute infection. In either case, were a similar condition to obtain in humans, it would be extremely difficult to link subclinical infections with subsequent T1D onset. The rates of T1D onset in older NOD observed inoculation with avirulent or less virulent CVB strains suggest that an etiologic scenario of a hit-and-run infection (Ramsingh et al., 1997) followed by CVB-initiated T1D onset at a much later time may be unlikely.

The results described here better define the complex interplay between NOD mouse host immunity and CVB genetics which can result either in what appears to be lifelong protection from the T1D onset. We believe that defining both the CVB-induced protective mechanism in mice and the molecular CVB genetics which mediate its efficiency will be crucial to better understanding the roles of virus infection in T1D etiology as modeled in NOD mice. Although the NOD mouse T1D model is not a perfect reflection of the human disease and results obtained using it must be carefully weighed, the manner in which these two factors interact in NOD mice may help to reconcile often contradictory reports regarding a role for CVB infections in human T1D etiology while simultaneously suggesting an approach for a vaccine strategy. These results may also hint at an outline of a general mechanism which could apply to the etiology of other autoimmune diseases in which viral infections have been implicated as a factor in their development such as multiple sclerosis (Soldan and Jacobson, 2001; Theil et al., 2001).

Methods

Viruses and cells

All strains of CVB3 and CVB4 used in these experiments, their propagation in and titrating on HeLa cell monolayers, have been described previously (Chapman et al., 2000a, 2000b; Lee et al., 1997; Tracy et al., 2002). The strain CVB3/46 expresses biologically active murine IL-4 and has been described (Chapman et al., 2000a); it is termed CVB3-mIL4 in this report for ease of reference. RNA preparations from the CVB3-mIL4 stock used here were assayed by direct RT-PCR-mediated sequence analysis before use to verify the presence of the murine IL-4 encoding insert (data not shown). CVB3/49 (Chapman et al., 2000b) is a chimeric strain in which the 5' NTR of CVB3 was replaced with that from poliovirus type 1 (Semler et al., 1986); this strain is avirulent in mice, causing neither pancreatitis nor myocarditis. RD (human rhabdomyosarcoma) cells were obtained from the ATCC (Manassas, VA). The creation of RD cells that express human CAR (Rh-hCAR) have been described (Cunningham et al., 2003).

Mice

Nonobese diabetic (NOD) female mice were purchased from Taconic (Germantown, PA) at 4 weeks of age and aged as necessary. Mice were housed five per cage for as described previously (Tracy et al., 2002). Mice were inoculated intraperitoneally (i.p.) with 0.1 mL containing 5×10^5 TCID₅₀ of virus, diluted in sterile 100 mM NaCl. Poly I:C treatment of mice was by inoculation of 0.1 mL saline containing 100 µg poly I:C (Sigma, St. Louis MO)

which had been sterilized by twice passaging through 0.2 micron syringe tip filters; control mice received saline alone. Twenty-four hours later, mice were inoculated with 5×10^5 TCID₅₀ CVB3-mIL4 in saline. To monitor T1D onset, mice were assayed weekly for glucose in urine using commercial dipsticks (Diasix; Bayer, Elkhart, IN) as described (Tracy et al., 2002) starting at 8 weeks of age. Mice with glucose at 2 g/dL urine were considered diabetic. Pancreata were excised after death. Pancreas was frozen under dry ice for later titer determination or fixed in buffered formalin before embedding in paraffin for sectioning.

Infectious virus titers in pancreas

Infectious titers in pancreatic tissues were determined on HeLa cell monolayers (Tracy et al., 2002). Briefly, weighed, frozen pancreata were homogenized in complete tissue culture medium. After freezing and thawing, the centrifugally cleared supernatants were assayed for the presence of infectious virus. Titers were expressed as TCID₅₀/g pancreas tissue.

In situ hybridization

Detection of CVB3 RNA by in situ hybridization was carried out as described, (Tracy et al., 2002). Briefly, a digoxigenin (DIG) labeled riboprobe complementary to the CVB3 positive strand 5' non-translated region was synthesized in a runoff T7 DNA polymerase reaction. Hybridization was carried out on deparaffinized sections of murine pancreas. Sections were lightly counterstained with nuclear fast red.

Immunohistochemistry

Immunohistochemical detection of insulin, glucagon, coxsackievirus adenovirus receptor (CAR), and enteroviral protein was carried out essentially as described (Tracy et al., 2002) with modifications. Pancreas sections were cut at 6-µm thickness from formalin-fixed, paraffin-embedded tissue. For detection of CAR on RD cells, cells [RD or RD-hCAR; (Cunningham et al., 2003)] were plated on chamber slides the day before fixing in cold acetone for 30 s, followed by air drying. For detection of CAR or CVB protein, pancreas sections were deparaffinized, hydrated through an ethanol series, immersed in 10 mM sodium citrate buffer (pH 6), and steamed in a vegetable steamer (Black and Decker, Towson, MD) for 30 min. Slides were cooled to room temperature and washed in PBS (phosphate-buffered saline). For detection of CAR on RD and RD-hCAR cultures, slides were immersed in 0.2% hydrogen peroxide in methanol for 5 min at room temperature following binding of primary antibody. Enteroviral protein was detected using a mouse monoclonal antibody (1:200; Dako, Carpinteria, CA), insulin with a guinea pig antibody to pig insulin (1:200; Sigma), and glucagon with a rabbit

antibody (1:200; Chemicon, Temecula, CA). The following biotinylated secondary antibodies were used: sheep anti-mouse F'ab2 fragment (Chemicon), goat anti-guinea pig IgG (Vector Labs, Burlingame CA), goat anti-rabbit IgG (Vector), and rabbit anti-goat IgG (Vector). CAR was detected with a goat polyclonal antibody (K-20; 1:200; Santa Cruz Biotechnology) or on human section using the antibody RmcB (Hsu et al., 1988). According to Santa Cruz Biotechnology, which did not divulge the peptide sequence against which the K-20 antibody was raised, the peptide sequence is within a region that is conserved between human and mouse CAR sequences. The extracellular protein primary structure of murine and human CAR differ by 10% and are more than 95% conserved in the intracellular domains (Bergelson et al., 1998). Following washes in PBS, slides were incubated with ABC Elite (Vector) and developed with the DAB substrate kit (Vector). Slides were lightly counterstained with hematoxylin, rinsed in saturated lithium carbonate in water for 10 s at room temperature, and rinsed in water. Slides were dehydrated and coverslipped with Permount.

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