



# Virus Infections in Type 1 Diabetes

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The precise etiology of type 1 diabetes (T1D) is still unknown, but viruses have long been suggested as a potential environmental trigger for the disease. However, despite decades of research, the body of evidence supporting a relationship between viral infections and initiation or acceleration of islet autoimmunity remains largely circumstantial. The most robust association with viruses and T1D involves enterovirus species, of which some strains have the ability to induce or accelerate disease in animal models. Several hypotheses have been formulated to mechanistically explain how viruses may affect islet autoimmunity and  $\beta$ -cell decay. The recent observation that certain viral infections, when encountered at the right time and infectious dose, can prevent autoimmune diabetes illustrates that potential relationships may be more complex than previously thought. Here, we provide a concise summary of data obtained in mouse models and humans, and identify future avenues toward a better characterization of the association between viruses and T1D.

## WHY AN ENVIRONMENTAL FACTOR FOR T1D?

Although there is a well-documented genetic basis for T1D, its rising incidence in developed countries has to be attributed to environmental changes (Bodansky et al. 1992; Gillespie et al. 2004). Population genetics simply do not change enough between generations to account for the dramatic surge in T1D prevalence as observed in, for example, Finland (Harjutsalo et al. 2008). Moreover, varying disease penetrance rates and the differing ages of disease onset in monozygotic twins are suggestive of nongenetic variables in T1D pathogenesis (Redondo et al. 2008). It has been postulated that the largest increase in disease frequency is occurring in the

most developed countries, following a north to south gradient that is reminiscent of the distribution seen in other autoimmune diseases (Borchers et al. 2010). Indeed, the “hygiene hypothesis” has frequently been referenced in this context to explain high ratios of autoimmune disease by the relative lack of exposure to infectious agents (Bach 2002). This theory, as well as the relationship between hygiene standards and T1D incidence is, however, far from absolute as exemplified by the second-highest worldwide incidence in Sardinia, Italy or the substantially lower incidences found in the countries surrounding Finland, which has the world’s highest rate. This article will provide an overview of the data that support the implication of viruses as environmental triggers of islet autoimmunity in T1D.

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## HISTORY OF VIRAL ASSOCIATIONS WITH T1D

### Suspects Released without Charge

It has long been acknowledged in the medical literature that T1D onset within populations follows a seasonal pattern; a finding which initially led to the formulation of a viral etiology (Adams 1926). Ever since, a variety of viruses have come under scrutiny as potential inducers of T1D. In the majority of cases, however, associations are weak, irreproducible, or were in some instances convincingly disproved. Examples of initially promising data include reports on T1D association with cytomegalovirus (CMV) (Pak et al. 1988), parvovirus (Guberski et al. 1991; Kasuga et al. 1996), encephalomyocarditis virus (Craighead and McLane 1968), and retroviruses (Conrad et al. 1997), all of which were challenged or are awaiting scientific replication.

Rotaviruses, responsible for a significant share of childhood gastroenteritis, have also been subjected to investigation for their relationship to T1D. Interest was sparked by the demonstration of sequence analogies between T-cell epitopes within the islet antigens GAD and IA-2 and rotavirus protein, suggesting potential cross-reactivity mechanisms (Honeyman et al. 1998). An association between rotavirus infection and islet autoantibody positivity in at-risk children was reported (Honeyman et al. 2000), but later challenged by studies in the Finnish population (Blomqvist et al. 2002; Makela et al. 2006). Therefore, it can be concluded that the role of rotavirus in the etiology of T1D is unconfirmed.

Congenital rubella infection and subsequent onset of diabetes after birth constitutes an interesting paradigm (Gale 2008). Congenital rubella syndrome encompasses an array of physical and behavioral abnormalities. Of note, diabetes development is associated with the presence of the DR3-DQ2 T1D susceptibility haplotype (Menser et al. 1974). It is thought that the virus causes diabetes by disturbing the normal development of  $\beta$ -cell mass, rather than inducing islet autoimmunity (Patterson et al. 1981). Since the introduction of an effi-

cient vaccine in 1969, the virus has been largely eliminated in developed countries, and thus is not expected to be a factor in the current increase of T1D incidence. Beyond this, the mere notion of whether this form of diabetes has any relationship to T1D also remains in doubt (Gale 2008).

Mumps infection has also been implicated in some instances, including a recent report documenting a case of “fulminant” T1D (Goto et al. 2008). In analogy to rubella, however, efficacious vaccination programs have not been able to curb the rising T1D incidence levels (Honeyman 2005).

### Enteroviruses and T1D: A Long History

The most robustly documented correlation between a virus and T1D has been with enteroviruses, a viral single-stranded RNA (ssRNA) genus belonging to the picornaviruses. A recent meta-analysis by Yeung and coworkers established that there is a clinically significant association between enterovirus infection, detected with molecular methods, and T1D (Yeung et al. 2011). Early reports suggesting a link between coxsackievirus, a member of the enterovirus genus, and T1D showed higher neutralizing antibody titers in serum from recent-onset patients as compared to nondiabetic subjects (Gamble et al. 1969), and were later confirmed using conventional polymerase chain reaction (PCR) testing (Clements et al. 1995). Some studies simultaneously probed for antibodies against other viruses and found that the most significant association was with coxsackievirus (Bannatvala et al. 1985).

Cross-sectional studies have focused predominantly on recent-onset individuals with T1D, although enterovirus was also identified as a risk factor in prediabetic children (Sadeharju et al. 2001) and pregnant women (Dahlquist et al. 1995; Hyoty et al. 1995; Elfving et al. 2008). There is still a lack of large prospective studies that establish a clear temporal relation between enterovirus infection and the development of islet autoimmunity. That said, two recent studies offer support for a causative role for enteroviruses in T1D. Studying blood

samples collected by the Diabetes and Autoimmunity Study in the Young (DAISY) consortium, Stene et al. found that the rate of progression from islet autoimmunity (detection of islet autoantibodies) to T1D was significantly increased following detection of enteroviral RNA in serum (Stene et al. 2010). Oikarinen and colleagues further showed that detection of enterovirus RNA is associated with increased risk for the primary development of islet autoimmunity, with a peak infection frequency during the 6 mo window that precedes the appearance of islet autoantibodies (Oikarinen et al. 2011). In combination, these reports suggest that enteroviruses may be involved in both the initiation of islet autoimmunity as well as progression to overt hyperglycemia and thus, act at multiple stages of disease development.

The timing of enteroviral infection as related to T1D onset is, in a sense, an altogether unresolved issue. Data from the nucleotide oligomerization domain (NOD) mouse seem to favor a scenario in which insulinitis serves as a prerequisite for coxsackievirus to be diabetogenic (see next section) (Horwitz et al. 2001; Drescher et al. 2004). Susceptible individuals may thus suffer from subclinical insulinitis for years before a viral challenge eventually culminates in hyperglycemia.

The notion that any potential association is not absolute and depends, to a considerable degree, on genetic susceptibility, or perhaps acts in concert with other environmental factors, is supported by several studies noting no such correlation (Fuchtenbusch et al. 2001; Graves et al. 2003). A 1971 study followed the diabetes incidence rate after a well-documented epidemic of coxsackievirus B4 (CVB4) infection in the remote Pribilof Islands (Alaska, USA). Five years later, the incidence of diabetes in the infected versus noninfected persons was found to be unaffected (Dippe et al. 1975). CVB4 isolates do, reportedly, have the intrinsic capacity to infect  $\beta$  cells and cause insulinitis and diabetes in susceptible mouse strains (Yoon et al. 1978) following their direct isolation from a child after their onset of T1D (Yoon et al. 1979). Despite the virus' perceived islet tropism, host susceptibility or additional environmental factors are thus

required for diabetogenicity. That said, some of these facets, including the identification of multiple strains (isolated from human pancreata) having the ability to induce diabetes remain rare in reports, and require more in the way of additional study.

## ANIMAL MODELS AND MECHANISMS BY WHICH VIRUSES COULD IMPACT T1D

### Animal Models for Studies of the Role for Viruses in T1D

Despite their shortcomings, animal models remain indispensable tools to map pathological mechanisms. Although the use of rodents in T1D research is discussed in a distinct article of this collection, we will summarize here which viruses have shown the ability to induce or alter experimental diabetogenic responses *in vivo*.

#### Mice

One of the oldest known and most unequivocal relationships between viral infection and diabetes development was revealed after inoculation of mice with encephalomyocarditis virus (EMC; picornavirus, ssRNA) (Craighead and McLane 1968). Diabetes induction usually occurs 3–4 d after infection and critically depends on the virus variant used (Onodera et al. 1978b), dosing (Baek and Yoon 1991), and the genetic background of the host (Onodera et al. 1978b). The virus induces diabetes after specifically infecting pancreatic  $\beta$  cells followed by direct cytolysis (high dose) or the recruitment of macrophages (low dose) (Baek and Yoon 1991; Yoon and Jun 2006). Parallels have been drawn between this model and the acute subform of fulminant diabetes that is found in the Japanese population based on its rapid and aggressive onset, exocrine tissue damage, and lack of autoantibodies (Shimada and Maruyama 2004). A recent study exploited this model to show that the viral sensor molecules MDA5 and TLR3, which are both involved in IFN-I responses to viral infection, are required to prevent diabetes in mice infected with EMCV (McCartney et al. 2011).

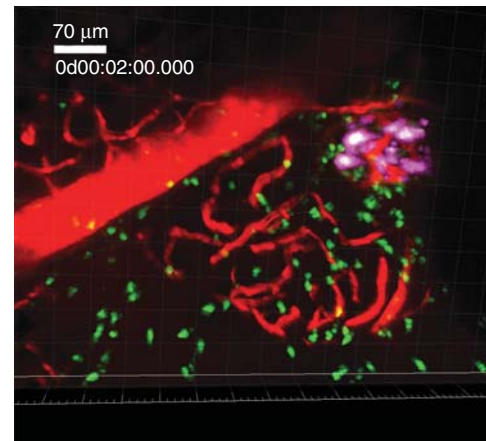


A more ambiguous role is reserved for coxsackie B viruses (CVB; picornavirus, ssRNA) in the NOD mouse. Interest was sparked by the isolation of a virus resembling CVB4 from a 10-yr-old boy, which famously triggered insulinitis and hyperglycemia in mice (Yoon et al. 1979). Detailed analysis in the NOD mouse revealed that coxsackieviruses provoke diabetes only when a preexisting mass of insulinitis has accumulated (Serreze et al. 2000). When administered earlier, however, inoculation has a strong preventive outcome (Tracy et al. 2002; Filippi et al. 2009). This model is thus highly suitable to study the combined effect of genetically determined immunological abnormalities and an environmental factor (i.e., viral infection).

A final example of a widely studied viral mouse model differs from the previous two by the fact that the host has been genetically altered to express a viral antigen from the lymphocytic choriomeningitis virus (LCMV; arenavirus; ssRNA) on its  $\beta$  cells (Ohashi et al. 1991; Oldstone et al. 1991). This is thus a pure mimicry model, in which antiviral T cells redirect to the  $\beta$  cells after viral clearance and, depending on expression of viral antigen in the thymus, cause rapid or slow onset of clinical hyperglycemia (von Herrath et al. 1994). Our laboratory recently developed an advanced two-photon imaging approach to allow for imaging of cytotoxic T lymphocyte (CTL) effector kinetics in vivo in the pancreas using this model (Fig. 1) (Coppieters et al. 2010). Although mechanisms of cross-reactivity at play in the rat insulin promoter (RIP)-LCMV model more than likely diverge from the etiology of T1D, we propose that the behavior of activated CTL in the target tissue may still serve as a suitable model of their kinetics in T1D.

### Rats

Although less commonly used as an animal model, valuable information on virus-induced diabetes has been obtained from studies in rats. A well-documented diabetogenic virus is the Kilham rat virus (KRV; parvovirus; ssDNA) that causes diabetes in the diabetes-resistant



**Figure 1.** Antiviral, diabetogenic CTL in the RIP-LCMV.GP diabetes model as visualized by intravital two-photon imaging in the pancreas. The technique is described in Coppieters et al. (2010). Briefly, purified and labeled P14 TCR-transgenic CTL is transferred to RIP-LCMV.GP hosts, followed by infection with LCMV. As such, the kinetics of the diabetogenic CTL response can be dynamically visualized in the pancreas. (Magenta) Insulin-producing  $\beta$  cells, (green) CTL, (red) vasculature after dye injection.

BioBreeding (DR-BB) rat (Guberski et al. 1991). In contrast with EMCV, KRV does not infect  $\beta$  cells but rather promotes diabetes by induction of autoimmunity against the  $\beta$  cells. Recruitment of macrophages and perturbation of regulatory and autoreactive T-cell species have been proposed as possible mechanisms that contribute to  $\beta$ -cell decay in this model (Mordes et al. 2004). More recently, infection with KRV, but also rat cytomegalovirus (RCMV), was found to result in autoimmune diabetes in LEW\*1WR1 rats, a strain that normally experiences spontaneous onset in only 2% of cases (Tirabassi et al. 2010). Other viruses such as H-1, vaccinia, and CVB4, however, did not induce diabetes, indicating that diabetogenicity is virus-specific.

Table 1 lists other, less characterized, observations in mice and rats that link viral infection with diabetes development. In the following subsections we will list some of the hypotheses that have been put forward based primarily on research in the animal models outlined above (Fig. 2).

**Table 1.** Additional examples of viruses that can promote or prevent autoimmune diabetes in mice or rats

Virus	Classification	Host species— Diabetogenicity	Reference
Ljungan virus	Picornaviridae (ssRNA)	Viral particles detected in wild-trapped bank voles and BB rats.	Niklasson et al. 2003, 2007
MHV-68	Herperviridae (dsDNA)	Delays diabetes in the NOD in an age-dependent manner.	Smith et al. 2007
Rotavirus	Reoviridae (dsRNA)	Delays or accelerates diabetes in the NOD in an age-dependent manner.	Graham et al. 2007, 2008
Reovirus	Reoviridae (dsRNA)	Induces diabetes and infects $\beta$ cells. Delays diabetes in the NOD mouse.	Onodera et al. 1978a; Wetzel et al. 2006
Retrovirus	Retroviridae (ssRNA)	Presence of endogenous retrovirus correlates with diabetes susceptibility and progression.	Suenaga and Yoon 1988; Gaskins et al. 1992
Mengovirus	Picornaviridae (ssRNA)	Causes diabetes in addition to encephalitis owing to islet necrosis.	Yoon et al. 1984
Mouse hepatitis virus	Coronaviridae (ssRNA)	Reduces incidence in the NOD mouse.	Wilberz et al. 1991
Lactate dehydrogenase virus	Arteriviridae (ssRNA)	Suppresses diabetes development in the NOD mouse.	Takei et al. 1992

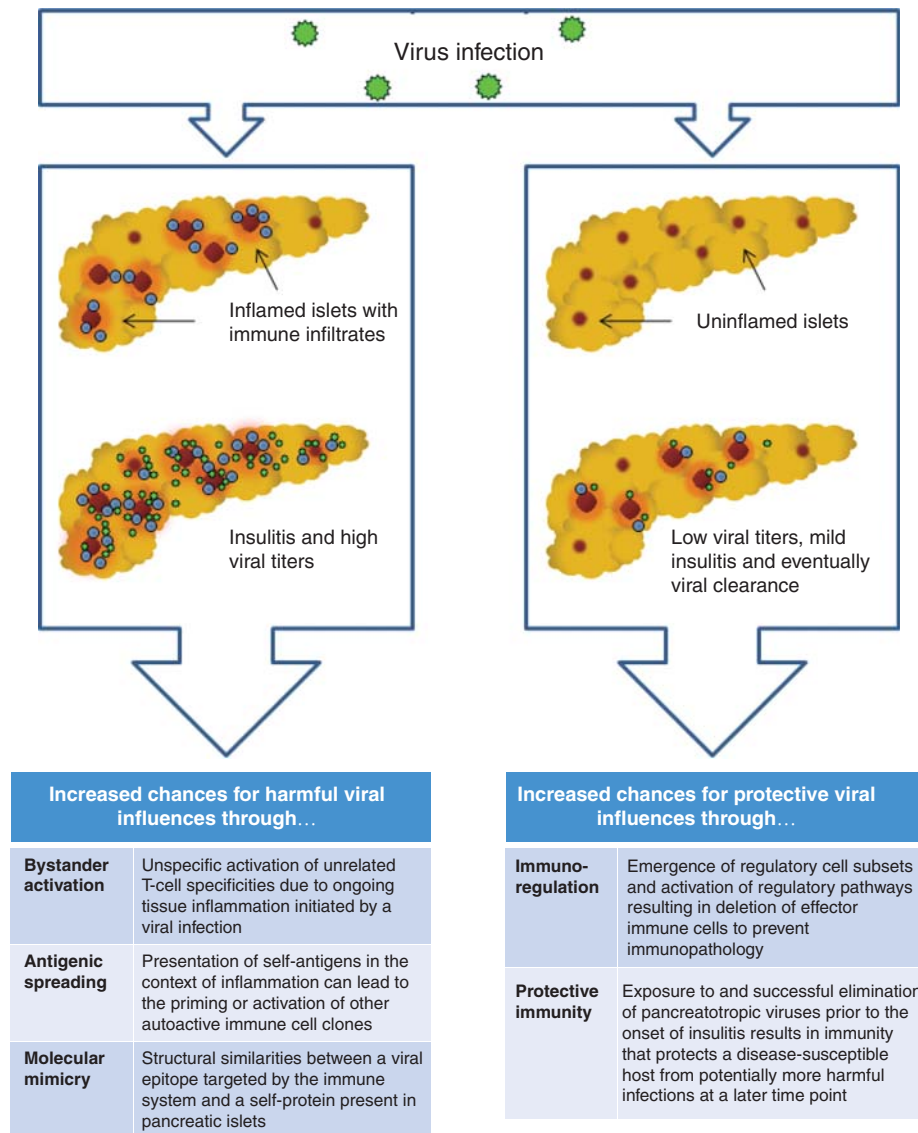


### Evidence for Islet-Specific Infection

The possibility of a viral infection specifically affecting pancreatic endocrine cells constitutes a straightforward explanation for the selective demise of  $\beta$  cells, either through lysis induced by cytopathic viruses or immune-mediated destruction of infected  $\beta$  cells. The example of EMCV-induced diabetes highlights the potential of some viruses to specifically infect pancreatic islets and cause  $\beta$ -cell decay. Coxsackievirus, in contrast, displays pancreas tropism rather than a preference for  $\beta$  cells, and it has been reported that it exclusively affects acinar cells while sparing the islet cells (Mena et al. 2000). Although some evidence exists that CVB can indeed infect islets in vivo (Drescher et al. 2004), it is uncertain whether the virus can persist and whether islet-specific infection is required for diabetogenicity. The detection of viral particles in the human T1D pancreas will be discussed separately.

### Molecular Mimicry

The demonstration of remarkable sequence similarities between the 2C protein from coxsackievirus and a GAD65 (glutamate decarboxylase) epitope, a major target antigen in T1D, gave rise to the idea of viral mimicry in T1D (Kaufman et al. 1992). Although molecular mimicry is undoubtedly the reason for rheumatic heart disease following infection with *Streptococcus pyogenes* (Guilherme et al. 1995), in T1D, such type of connection proved difficult to establish. It was shown that CVB infection of young NOD mice failed to activate or expand the autoreactive precursors that are specific for those  $\beta$ -cell epitopes that share structural similarities with viral epitopes (Horwitz et al. 1998). Analogously, autoreactive human T-cell clones specific for the GAD65 epitope did not proliferate following stimulation with the viral epitope (Schloot et al. 2001). Studies in the mouse RIP-LCMV system suggest that complete



**Figure 2.** Potential immune mechanisms leading to islet inflammation or tolerance following viral infection.

sequence identity is required to initiate diabetes as single amino acid changes in the viral epitope on  $\beta$  cells protected these mice from diabetes (Sevilla et al. 2000). Collectively, the current body of evidence favoring a simple event of cross-reactivity between viral and self-antigen is weak (Richter et al. 1994; Horwitz et al. 1998; Schloot et al. 2001). Nevertheless, molecular mimicry has been shown to accelerate disease progression under conditions of virus-induced

pancreatic inflammation, suggesting that sequence homologies may not be the initiating trigger, but are able to codetermine the pace by which disease develops (Christen et al. 2004).

### The Concept of “Bystander” Activation

Bystander T-cell activation is defined as the activation of a T cell through a mechanism that is independent of specific T-cell receptor (TCR)



stimulation. Alternative mechanisms encompass activation through soluble factors or membrane-bound molecules that bind to receptors other than the TCR. In a strict sense, mechanisms such as “epitope spreading” or “molecular mimicry” are not included, because these involve specific recognition of a presented peptide by the TCR. Thus, bystander T-cell activation circumvents the requirement for specific TCR stimulation.

In spontaneous diabetes models, antigen specificity appears to be required for migration of autoreactive T-cell species to the pancreatic islet (Lennon et al. 2009). Recognition of islet antigens was shown to occur early at the vascular level, ensuring that only antigen-specific T cells can extravasate and access the islets (Savinov et al. 2003). In the context of CVB infection, circulating naive islet-specific T cells became activated, either through release of sequestered antigen or by “true” non-TCR bystander activation, and rapidly triggered diabetes development (Horwitz et al. 1998). However, the role for non-TCR-dependent activation of naïve T cells during viral infection appears limited (Ehl et al. 1997; Zarozinski and Welsh 1997).

In contrast to “bystander activation” of naïve T cells, the induction of “bystander damage” via, e.g., cytokine release may be the more relevant scenario during virus-induced diabetes (Seewaldt et al. 2000). Here, the  $\beta$  cells are the bystanders and are killed in a non-TCR-dependent fashion through the release of soluble factors by antiviral effectors.

### The Fertile Field Hypothesis

The fertile field hypothesis combines the aforementioned concepts and postulates that virus-induced inflammation preconditions (“fertilizes”) the pancreas milieu for autoimmunity, which ultimately results in T1D only in susceptible individuals (von Herrath et al. 2003). Following the establishment of localized inflammation by the virus, autoreactive T cells are generated by molecular mimicry or bystander activation, or a combination of both. The subsequent demise of  $\beta$  cells and consequent presentation of  $\beta$ -cell antigens in the draining lymph

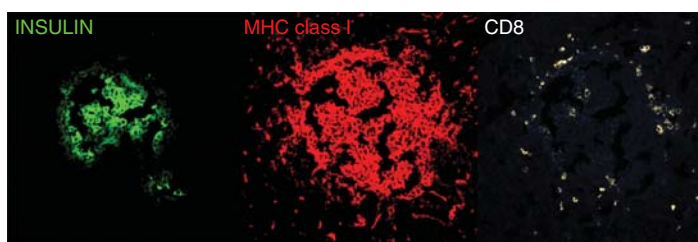
nodes would then lead to epitope and antigenic spreading. This would explain the emergence of a broad autoreactive T-cell repertoire over time in most T1D individuals. Mouse models of virally induced T1D and multiple sclerosis (MS) also clearly show the occurrence of secondary autoreactivity against molecules that are not initially targeted by the antiviral effectors that drive the early stages (Miller et al. 1997; Coon et al. 1999; Holz et al. 2000). Contradicting the fertile field hypothesis are studies that emphasize preexisting insulinitis as a crucial prerequisite for a viral infection to cause T1D (see above). Thus, it remains to be conclusively determined whether viral infections are required to produce disease-promoting conditions in autoimmunity-prone individuals or, conversely, if preexisting autoimmunity determines the outcome of a pancreatropic viral infection.

### EVIDENCE FOR VIRAL INFECTION OF THE PANCREAS

The target organ in T1D, the pancreas, is unfortunately extremely difficult to study because of its inaccessible anatomical location (Coppeters and von Herrath 2009). Owing to greatly improved clinical management of T1D, pancreas samples from recently diagnosed T1D patients today only rarely become available. Programs such as the Network for Pancreatic Organ Donors (nPOD) ([www.jdrfnpod.org](http://www.jdrfnpod.org)), aimed at the nationwide procurement of tissue relevant to T1D research, respond to this unmet need and will also offer samples from non-diabetic, islet-antibody positive individuals. At present, the important question as to whether viruses, and in particular, enteroviruses, can directly affect pancreatic islets has been addressed by a relatively small set of studies.

### Indirect Evidence: The “Viral Signature”

Foulis and coworkers were the first to systematically document the hyperexpression of HLA Class I and interferon- $\alpha$  within islets of recently diagnosed diabetic children (Fig. 3) (Foulis et al. 1987). Normal islets are completely devoid of these markers. The presence of these markers



**Figure 3.** Aberrant MHC class I hyperexpression in islets from a 12-yr-old boy with one year of T1D duration (nPOD case 6052). Frozen pancreas section was stained for immunofluorescence and analyzed by confocal microscopy. Note the abundance of CD8<sup>+</sup> cellular infiltration. (For details see Gianani et al. 2010.)

is commonly referred to as a “viral signature,” as the up-regulated expression of HLA/MHC class I molecules is typically driven by type I IFNs following viral infection. The islet-specific MHC class I expression would render the cells suitable targets to CD8 T cells that are known to be an integral part of insulinitic lesions. Indeed, in a mouse model of type T1D, only  $\beta$  cells that have been unmasked by MHC class I expression were attacked by activated, autoreactive T cells, demonstrating the necessity of MHC class I up-regulation for immune-mediated  $\beta$ -cell destruction (von Herrath et al. 1994). In this context it is interesting to note that defined polymorphisms in the *IFIH1* gene may result in lower levels of type I interferons (IFNs) in response to viral infections and may confer protection from autoimmune diabetes (Nejentsev et al. 2009).

CVB-induced hyperexpression of the interferon-inducible chemokine CXCL-10 by pancreatic islet cells was also proposed as an early molecular marker of infection (Berg et al. 2006). Its localized production was found to coincide with islet-specific enteroviral infection in fulminant T1D, but also conventional T1D (Tanaka et al. 2009; Roep et al. 2010). Some data from animal models suggests that virus-induced CXCL-10 is essential in the recruitment of CXCR-3<sup>+</sup> autoreactive T cells to the islets, although recent studies in our laboratory found that CXCL10 is in fact dispensable (Christen et al. 2003) (KT Coppieters and MG von Herrath, unpubl.). Collectively, although the indispensable role of CXCL10 has yet to be confirmed, these data suggest that viral infections

have the potential to establish a molecular “signature” that aids in the recruitment of diabetogenic T cells to pancreatic islets.

### Direct Evidence of Viral Infection of the Pancreas in T1D

Whereas initial efforts to directly detect viral sequences in the islets from patients with MHC class I up-regulation were unsuccessful (Foulis et al. 1990, 1997), the same samples were recently revisited using a more modern methodology (Richardson et al. 2009). Using immunohistochemical detection, enteroviral particles were found in islets from 44 out of 72 recent-onset patients versus three out of 50 controls. It is worth noticing that positive detection was also achieved in 10 out of 25 type 2 diabetes patients. Another group probed 65 pancreata from T1D patients for enteroviral RNA by in situ hybridization and found enterovirus-positive islet cells in four cases, compared to none in nondiabetics (Ylipaasto et al. 2004). Likewise, Dotta and co-workers found immunohistochemical traces of enteroviral protein in three out of six pancreatic islets from recent-onset patients and corroborated these data by sequencing (Dotta et al. 2007). Of interest, islets from these patients showed an unusual, NK cell-dominated form of insulinitis. In contrast to the above analyses performed after diagnosis, a recent case report sampled pancreatic tissue from an autoantibody-positive, nondiabetic child (Oikarinen et al. 2008b). It was found that the pancreatic islets had no signs of insulinitis, in line with data from In't Veld et al. in a larger cohort (In't





Veld et al. 2007). However, enterovirus was detected by immunohistochemistry specifically in the islets but not by in situ hybridization. Awaiting confirmation from larger studies, it is possible that this isolated case exposes the very early stages of T1D development, showing the establishment of local viral infection even before onset of subclinical insulinitis.

Anatomical sites close to the pancreas may serve as a reservoir for viral infections in T1D. Oikarinen et al. subjected intestinal biopsies from 12 T1D patients to in situ hybridization and immunohistochemistry for enterovirus (Oikarinen et al. 2008a). Detection was achieved in 50% of T1D patients as compared to none in nondiabetic subjects. Based on these findings, the authors postulated that the gut—which showed normal histopathological composition in all cases—could represent a chronic infectious site with close anatomical ties to the pancreatic milieu. Alternatively, immune recognition could occur at the intestinal level followed by subsequent homing of activated lymphocytes to the pancreas.

It seems rather unlikely that the majority of T1D cases are attributable to direct lysis of infected  $\beta$  cells. Such a scenario would result in a more acute onset of disease, which is not the disease phenotype that is commonly observed in patients, although such cases have been described in the literature (Imagawa et al. 2000). Although evidence of enteroviral infection has been shown in these fulminant diabetes cases, the pathogenesis observed in such patients lacks the development of islet autoreactivity (e.g., islet autoantibodies), a fundamental feature of conventional T1D (Tanaka et al. 2009).

## DIFFICULTIES IN IDENTIFYING A ROLE FOR VIRUSES IN T1D AND THERAPEUTIC IMPLICATIONS

### Temporal Divergence of Viral Insult and Clinical Onset

Despite decades of investigation, no evidence exists for the involvement of a particular viral strain with T1D. The search for a correlation with certain viral agents can be expected to be

complex for a variety of reasons. In both mouse models and T1D patients, development of clinical hyperglycemia is thought to represent the final stage of the autoimmune process. Therefore, it can be assumed that the event that initiates the loss of tolerance against islet antigens likely precedes the onset of diabetes by several months or years. This temporal discrepancy poses considerable restraints in studying the role of viral infection in T1D development, as the vast majority of patients are traditionally sampled after diagnosis. Taking into account that inciting viral agents may use a “hit-and-run” strategy, or act by repeated, sequential infection, analyses around clinical onset may at least in some occasions miss out on the culprits.

Alternatively, viral infection may only serve as an accelerating factor that, superimposed onto advanced insulinitis, leads to rapid culmination into hyperglycemia. The latter scenario would suggest that detection of viral particles around onset is an achievable goal in determining a causal relationship.

### Multiple Viruses May Provoke Disease in Similar Fashion

The fact that no absolute association has been identified with certain viral strains or even viral genera or families indicates that, if T1D is indeed caused by viruses, multiple infectious strains may result in the same disease phenotype. Historically, samples from T1D patients have been probed by a “one test-one pathogen” approach that unavoidably introduces experimental bias. Whether the assay is based on detection of virus-specific antibodies or nucleic acid sequences, such strategies are costly, inefficient, and time-consuming and generally make poor usage of the limited sample volumes that are available from T1D patients. To cast the net more widely in the evaluation of a viral etiology, emerging nucleic acid technologies to detect pathogens on a broad-spectrum basis should be applied on blood and pancreas samples from T1D patients at various stages pre- and postdiagnosis. Indeed high-density microarrays, such as the Virochip pan-viral microarray and deep sequencing, can test for



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thousands of potential pathogens simultaneously (Wang et al. 2002, 2003).

### Alignment with the Hygiene Hypothesis

Based on our present knowledge, enteroviruses would appear associated with at least a fraction of T1D cases. But if enteroviruses are indeed a major contributor to T1D pathogenesis, how can we explain the increase in T1D incidence in countries where exposure to enteroviruses has been dropping (e.g., Finland) (Viskari et al. 2005)? In other words, is the theory that T1D can be caused by a viral infection compatible with the hygiene hypothesis? Based on the findings in the NOD mouse, one could argue that the lack of exposure to enteroviruses in developed countries results in a reduced frequency of individuals with protective immunity through early childhood infections. When genetically driven islet inflammation occurs in these unprotected individuals, they would be more susceptible to an enteroviral infection that has the potential to initiate overt autoreactivity and  $\beta$ -cell damage.

### Immunization Strategies: Why Not Now?

So why don't we initiate population-wide vaccination programs to more thoroughly and directly evaluate the role of enterovirus in T1D? Theoretically, virologists deem the development of enterovirus vaccines relatively straightforward and achievable (S Tracy, pers. comm.). The main limitation at present is that the enterovirus genus of the Picornavirida family consists of five virus species. These virus species in turn contain many different strains and serologically distinct viruses (Fauquet 2005). Any one or a combination of these viruses could be the virus detected by, for example, the anti-VP1 antibody that is commonly used in immunohistochemical analysis. Finnish groups are currently attempting to delineate which enteroviral strains are most prevalent in T1D patients to clarify serotypes that should be immunized against (Roivainen 2006).

A disturbing observation related to the idea of prophylactic vaccination is the finding that

CVB infection protects against diabetes development in the young NOD mouse. This protection is orchestrated via at least two distinct suppressive immune mechanisms, the up-regulation of the inhibitory PD-1/PD-L1 pathway and increasing numbers of circulating T cells with regulatory capacities (Filippi et al. 2009). Such data illustrate the dual role of viral infections in autoimmunity, and portray T1D development as a balancing act between immune "education" by viruses (see "hygiene" hypothesis) and the induction of aberrant immunity in response to these agents. Moreover, they suggest that the protective effect of viral infections is a proactive mechanism that involves the emergence of regulatory mechanisms and thus, exceeds the achievement of sterile immunity which would be the ultimate goal in vaccination programs.

As a final note, the introduction of childhood immunizations programs and the growing prevalence of T1D in developed countries have also provided rationale for assessing a possible correlation between the two entities. Multiple large-scale studies found no support for any causal relation between childhood vaccination and T1D (Blom et al. 1991; EURODIAB Substudy 2 Study Group 2000; DeStefano et al. 2001; Hviid et al. 2004). As there appears to be no significant association between vaccination and T1D, the risk-benefit ratio as of today balances strongly in favor of continued protection efforts by means of immunization.

### CONCLUDING REMARKS

The available data set on the role of viral infections in T1D development and progression allows us to conclude with a reasonable degree of confidence, that at least a fraction of patients at some point suffer some type of viral insult. Viruses belonging to the enterovirus genus have the capacity to initiate and/or accelerate islet autoimmunity, but cannot fully explain the etiology as a sole environmental trigger. What is needed is a comprehensive, microarray-based approach using patient samples at various stages pre- and postdiagnosis of disease. Emerging PCR-based technologies should be

used to offer a more definitive and unbiased evaluation of the potential role of viruses in T1D pathophysiology. Results from large longitudinal follow-up studies such as DAISY and The Environmental Determinants of Diabetes in the Young (TEDDY) study are expected to contribute significantly in the near future. In-depth knowledge on which viruses act when, and at which anatomical level, could enable us to design rational vaccination approaches in susceptible individuals for T1D prevention.

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