Viral diabetes: Virus diabetogenicity and host susceptibility

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Received: September 17, 2015
Published online: October 19, 2015

Diabetes mellitus (DM) is on the rise worldwide, and is associated with improvement in socioeconomic conditions, increasing wealth, high caloric and fat intake, and reduced physical activity. Accumulating evidence also suggests a causal/trIGGERING link with environmental factors, such as toxins and viruses. A variety of viral infections have been associated with the development of diabetes. There might be no ‘diabetes virus’, but there might be a variety of ‘diabetogenic viruses’ that contribute to diabetes in people who are particularly susceptible due to genetic or physiologic conditions. Increasing evidence is showing that human enteroviruses (EVs) are prominent among possible causal candidates for type 1 diabetes mellitus (T1D). However, evidence for diabetogenicity of viruses is still missing. For a pathogen, causality should fulfill the etiologic criteria known as ‘modified Koch’s postulates’. Recently, we presented evidence that mutations of Tyk2 gene are responsible for diabetes susceptibility of mice infected with the diabetogenic D strain of encephalomyocarditis virus (EMC-D). Observations have been extended to identify the human polymorphism of TYK2 gene associated with increased risk for virus-induced diabetes in humans. An update is given on the possible diabetogenic role of EVs with reference to experimental work in animals and to human studies (viral serology, pancreas histopathology, virus detection in blood and in tissues of diabetic patients). For the identification of a diabetogenic virus, the development of a sensitive experimental model is imperative. In vivo assay systems should include an animal model appropriately simulating a susceptible human and carrying susceptibility genes or factors. This work summarizes current knowledge on virus-induced diabetes together with evidence from experimental models, susceptibility genes in mice, candidate diabetogenic viruses and susceptibility genes in humans. Perspectives on the identification of diabetogenic viruses - which may possibly lead to innovative strategies for the cure or prevention of diabetes - are also presented.

Keywords: Diabetes; Susceptibility; Genetics; Immunity; Innate immunity; Virus; Picornavirus; Enterovirus; Coxsackievirus; Encephalomyocarditis virus


Introduction

Diabetes mellitus is on the rise worldwide and is associated with improved socioeconomic conditions, increasing wealth, higher caloric and fat intake, lower physical activity. This occurs irrespective of races and countries, in Europe, Nordic countries, the Middle-East, the Asia-Pacific region, and the United States. Diabetics are estimated at 346 million worldwide ¹¹. The increasing number of patients with diabetes represent a big challenge to the society both for the increasing medical costs and for lowered quality of life especially due to diabetes complications. By themselves, social and intrinsic factors seem unable to explain the explosive diabetes epidemic that
Table 1. Viruses associated with diabetes in humans and experimental animals

<table>
<thead>
<tr>
<th></th>
<th>Humans</th>
<th></th>
<th>DNA viruses</th>
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<th>Experimental Animals</th>
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<tbody>
<tr>
<td>RNA viruses</td>
<td>Coxsackie B viruses</td>
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<td>Hepatitis A virus</td>
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<td>Coxsackie B viruses</td>
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<td>Rubella virus</td>
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<td>Mumps virus</td>
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<td>Rotavirus</td>
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<td>Retrovirus</td>
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<td>Kilham rat virus</td>
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<td>DNA viruses</td>
<td>Cytomegalovirus</td>
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<tr>
<td></td>
<td>Epstein-Barr virus</td>
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<tr>
<td></td>
<td>Human Herpesvirus 6</td>
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we are seeing today. Accumulating evidence suggests the association of many candidate viruses as possible environmental factors that contribute to the increasing number of diabetic patients [2-4] (Table 1). Consistently, clinical observations in Japan suggest that viral infection is associated with about 20% of T1D cases and up to 70% of cases of fulminant diabetes [5].

Virus-induced diabetes is a more complex disease than previously thought and is ascribed to diverse mechanisms that may lead to damage of pancreatic beta-cells. The mechanisms include beta-cell necrosis (virolysis), apoptosis of beta-cells, local inflammatory responses (Figure 1). Apparently, viral infection of islets is linked to lower than normal resistance to virus infection [3]. In addition, impaired regeneration of beta-cells together with a tendency to trigger autoimmunity against beta-cells appear to be involved in determining the outcome of virus-induced damage to insulin-producing cells [6]. The precise mechanisms of pancreatic beta-cell damage associated with viral infection remain, however, not determined. Host factors controlling virus-induced diabetes have not been fully elucidated. In human cases, evidence strongly suggests the pathogenic contribution of enteroviruses (EVs), which belong to the family Picornaviridae [7-11]. Since resistance to picornavirus infection is dependent on innate immunity [12], pathways regulating innate immune responses are candidates for susceptibility to virus-induced diabetes [13]. These include the
interferon system, interferon production and interferon receptor-mediated signaling pathways, pattern recognition receptors (PRR) directed against pathogen-associated molecular patterns (PAMPs). Among these, toll-like receptors (TLRs), intracellular helicases such as retinoic acid-inducible gene I (RIG-I), interferon induced with the helicase C domain 1; melanocyte differentiation antigen (MDA)-5 (MDA-5/IFIH1) [13]. Interferon-regulatory factors and interferon receptor-associated downstream molecules (e.g., JAK1, TYK2, STAT1 and STAT2) are also important (Figure 2) [13]. Recently, we found that the Tyk2 gene may serve as a susceptibility factor in virus-induced diabetes both in mice and humans [14, 15]. A recent study on influenza virus indicates that T cells can target infection sites only upon instructions from cells of the innate immune system and that neutrophils are the first-responder cells [16]. Neutrophils appear to form long membrane strands as they migrate and leave membrane particles with the CXLC12 chemokine behind. T cells are attracted along the chemokine trail, infiltrate virus-infected tissues and initiate the adaptive response.

With regard to a diabetogenic virus of mice (the encephalomyocarditis virus, EMC), it has been shown that a single point mutation can contribute to diabetogenicity [17], indicating that virus evolution can contribute to determining diabetogenicity and possibly increase the risk of diabetes.

In this work, current knowledge on virus-induced diabetes is summarized. Evidence from experimental models, susceptibility genes in mice, susceptibility genes in humans, and candidate diabetogenic enteroviruses are reviewed. Finally, perspectives on the identification of diabetogenic viruses - which may possibly lead to innovative strategies for the cure and prevention of diabetes - are presented.

1. Experimental Virus-Induced Diabetes

1.1) Diabetogenicity of encephalomyocarditis virus

In experimental animals, virus-induced diabetes has been reported as a consequence of infection with EMC and mengovirus (both members of the picornavirus group), cytomegalovirus, and Kilham rat virus (a parvovirus) [18, 19]. In infectious diseases, clinical outcomes are dependent on many factors, including stochastic elements, pathogenicity of the infecting agent, host resistance factors (immunologic resistance, age, sex, physiologic conditions). EMC virus provides an excellent animal model for studying virus-induced diabetes [19], and pathogenic mechanisms have been investigated extensively [20].

Using the plaque purification method, Yoon, McClintock, Onodera, and Notkins isolated the highly diabetogenic D clone of EMC virus (EMC-D) and its non-diabetogenic B
variant (EMC-B) \[21\]. Interestingly, the differential diabetogenic potential of the two clones of EMC viruses is dependent on genetic variation \[22\]. Although EMC-D and EMC-B virus could not be distinguished by either neutralization assay or competitive radioimmunoassay, nucleotide sequence analysis of the two clones showed only 14 nucleotide differences between them \[22\]. Further investigations using several mutant viruses generated from stocks of both EMC-D and EMC-B found that only one amino acid (alanine, the amino acid 776 of the viral polyprotein), is apparently critical for diabetogenicity \[23\]. Moreover, reverse genetic analysis showed that a “G” nucleotide at position 3155 (Ala[GCC]-776 on the polyprotein) is unique to all diabetogenic variants, whereas an “A” nucleotide at the same position (Thr[ACC]-776) is conserved among non-diabetogenic variants \[23\]. Recombinant chimeric EMC strains containing Thr, Ser, Pro, Asp or Val at position 152 of the major capsid protein VP1 (amino acid position 776 in the polyprotein) bound poorly to beta-cells, while EMC strains containing Ala at position 152 bound efficiently to beta-cells. The results indicate that the diabetogenic effect of EMC does depend on virus affinity for beta-cells \[24\]. These studies pointed out that diabetogenicity may be ascribed to a single mutation in a receptor-binding site of the viral capsid.

1.2) Host factors

Since EMC-D-induced diabetes develops within 4 days after infection, and infection of T- or B-lymphocyte-deficient mice failed to make the animals more susceptible to virus-induced diabetes, it is thought that innate immunity (the IFN system, macrophages, early inflammatory responses) is likely responsible for the outcome of infection \[22\]. Recent advances in the field of innate immunity have elucidated the significance of PRRs directed against PAMPs and the role of IFN receptor (IFNR)-associated molecules (Figure 2).

Since innate immunity plays a significant role in the protection against experimental EMC-induced diabetes, intact functionality of the IFN system may be important for diabetes resistance. McCartney and others reported that MDA5 and TLR3 are both required to activate IFN-dependent anti-viral responses and preventing EMC-D-induced diabetes in mice \[26\]. In agreement with this concept, it should be borne in mind that EMC-D infection does produce diabetes in a limited number of mouse strains (e.g., DBA, SJL, SWR, NIH Swiss mice), the A/J and BALB/c mice being moderately susceptible, and the C57BL/6, CBA, AKR, C3H/He strains being totally resistant (Figure 3) \[27-30\].

It was reported that a single autosomal recessive gene inherited in a Mendelian fashion, controls susceptibility to EMC-D \[30\]. However, susceptibility genes for EMC-induced diabetes in highly susceptible mouse strains have not been fully identified in spite of investigations started 40 years ago. Development of EMC-induced diabetes is influenced by the mouse strain and the infectious dose, but also by host factors. Among those, sex, infiltration of macrophages, production of cytokines/chemokines, chemical mediators, genetic background of the host \[17, 19, 20, 27-30\]. Recently, it has been reported that the Tyk2 gene (an IFN receptor signaling pathway molecule) is responsible for susceptibility to EMC-D-induced diabetes \[14\]. Highly susceptible strains such as SJL and SWR mice have been shown to carry a mutated Tyk2 gene associated with decreased promoter activity. This apparently leads to reduced expression of Tyk2 gene in
pancreatic beta-cells and reduced IFN-dependent antiviral response \[^{14}\]. Since the reported Tyk2 gene mutation is absent in highly susceptible DBA/2 mice, genes other than Tyk2 are expected to play a role in susceptibility of this mouse strain. Taken together, the results indicate that multiple diabetes susceptibility genes do play critical roles in mice.

2. Virus-Induced Diabetes in Humans

2.1) Viral infections possibly linked to diabetes

Clinical and experimental observations have long considered viral infections as a possible cause of T1D. Different viruses, including group B coxsackieviruses, cytomegalovirus, varicella-zoster and rubella virus, have been detected in the pancreatic islets of patients with severe fatal viral infections \[^{31}\], suggesting that systemic viral infection could lead to pancreatic beta-cell damage.

A few case reports indicated the association of mumps with the development of diabetes \[^{32}\]. It has also been shown that subjects with congenital rubella are at increased risk for the later development of diabetes (including T2DM) and slowly progressive or latent diabetes \[^{33}\]. In some cases, the rubella virus has been isolated at autopsy from the pancreas of subjects with congenital rubella syndrome \[^{34}\]. There are also data on the possible association of rotavirus infection with autoimmune diabetes (possibly due to molecular mimicry of rotavirus VP7 protein and the IA2 islet antigen) \[^{35}\].

With regard to cases of fulminant diabetes (mostly reported from Japan and other Asian countries), about 70% of patients appeared to be affected by a flu-like syndrome at the time of clinical onset. A variety of viruses, including EBV, HHV6, influenza B, rubella, EVs have been implicated in this rapid form of diabetes \[^{36}\].

About four decades ago, the increased prevalence of antibody positivity to group B coxsackieviruses (particularly CV-B4) and the detection of EV-specific IgM at the time of clinical onset suggested an association of CVBs with T1D \[^{37,38}\]. Recently, a large Finnish survey of EV-neutralizing antibodies in European populations indicated CV-B1 as strongly associated with T1D and with the induction of islet-specific autoimmunity \[^{39,40}\]. Based on experiments showing that EMC-induced diabetes in mice could be prevented by a viral vaccine \[^{41}\], the serologic studies made in Finland led to the proposal of an antidiabetic EV vaccine for humans \[^{42,43}\].

Pathologic studies initiated over thirty years ago \[^{31}\], demonstrated EV proteins and/or genome in pancreatic islets, particularly in beta cells \[^{44,45}\]. Indirect signs of an ongoing viral infection in the pancreas of diabetic individuals have been also found: expression in beta cells of IFN-related genes, hyperexpression of MHC class I molecules, detection of double-stranded RNA (indicating the replication of RNA viruses) \[^{46,47}\].

### Table 2. Taxonomy of Human Enteroviruses within the family Picornaviridae

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species (No. serotypes)</th>
<th>Serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picornaviridae</td>
<td>Enteroivirus</td>
<td>Human enterovirus A (21) CV-A2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16: Enterovirus-A71 (EV-A71), 76, 89, 90, 91, 92, 114, 119, 120, 121</td>
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<td>Human enterovirus B (59) CV-B1, 2, 3, 4, 5 (incl. SVDV), 6; CV-A9; Echovirus-1 (E-1, including E-8), 2, 3, 4, 5, 6, 7, 9 (including CV-A23), 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 25, 26, 27, 29, 30, 31, 32, 33; Enterovirus B69 (EV-B69), 73, 74, 75, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 93, 97, 98, 100, 101, 106, 107, 111</td>
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<tr>
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<td></td>
<td>Human enterovirus C (23) CV-A1, 11, 13, 17, 19, 20, 21, 22, 24; Enterovirus C95 (EV-C95), 96, 99, 102, 104, 105, 109, 113, 116, 117, 118 Poliovirus-1 (PV-1), 2, 3</td>
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<td>Human enterovirus D (4) Enterovirus D68 (EV-D68; including human rhinovirus-87), 70, 94, 111</td>
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<td></td>
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<td>Human enteroviruses not fitting into current species (2) EV-122, EV-123</td>
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</table>

Abbreviations: CV-A: coxsackieviruses group A; EV: numbered (not named) Enteroviruses; CV-B: coxsackieviruses group B; SVDV: swine vesicular disease virus; E: Echoviruses; PV: Polioviruses. Data from: http://www.picornaviridae.com/ (September 2015).
produce little if any beta-cell damage, diabetes developed if infected with Coxsackie B3 and B5, which ordinarily virus, diabetes developed. Furthermore, when mice were streptozotocin (a beta cell toxin), then infected with EMC-D diabetes were first treated with sub-diabetogenic doses of

2.2) Taxonomy of enteroviruses in the picornavirus group

As summarized in Table 2, the taxonomy of human EVs includes four species (A, B, C, D) that comprise 109 virus types (not considering human rhinoviruses). Thus, detection and identification of so many different viral genomes is a formidable task for virologists. A mix of different EV types circulates every year, and select EV types can be highly prevalent in different years. Non-polio EVs are second only to the "common cold" viruses (the rhinoviruses) as the most frequent viral infectious agents of humans. EVs cause an estimated 10-15 million symptomatic infections a year in the United States (www.cdc.gov/non-polio-enterovirus). The same is occurring in Europe and other geographic areas. Thus, at least 300 million new EV infections do occur each year worldwide.

EVs are associated with a variety of acute presentations (Table 3): aseptic meningitis and encephalitis (CVA viruses), myocarditis and pericarditis (CVB viruses, echoviruses), neonatal infection (CVB viruses, echoviruses), hand-foot-and-mouth disease (EV-71 and CVA viruses), pleurodynia (CVB viruses, echoviruses), pancreatitis (CVB viruses), undifferentiated febrile illness. Some EV types have unique disease associations: polioviruses (poliomyelitis), CV-A24 and EV-D70 (acute hemorrhagic conjunctivitis), EV-A71 (hand-foot-and-mouth disease, neurogenic pulmonary edema), EV-D68 (respiratory syndrome and polio-like paralysis) [47,48].

2.3) Coxsackieviruses group B: studies in animals

When strains of mice normally resistant to EMC-induced diabetes were first treated with sub-diabetogenic doses of streptozotocin (a beta cell toxin), then infected with EMC-D virus, diabetes developed. Furthermore, when mice were infected with Coxsackie B3 and B5, which ordinarily produce little if any beta-cell damage, diabetes developed if the mice were first treated with sub-diabetogenic doses of streptozotocin. These findings indicated that T1D may result from beta-cell damage induced by cumulative insults from chemicals and viruses [49]. The capacity of prototype strains of group B coxsackieviruses to produce diabetes in diabetes-prone mice (e.g., SIL, SWR strains) was studied before and after passage in various cell types [50]. CVBs that had been passaged in monkey kidney cells or in primary mouse embryo fibroblasts induced exocrine pancreatitis (particularly CV-B1), but failed to produce abnormal glucose tolerance tests. In contrast, CVB types 1-6 serially passaged in the pancreata of mice or in beta-cell cultures produced abnormal glucose tolerance tests. Immunofluorescence and histologic studies revealed that passage of CVBs in cultured beta-cells changed the tropism of these viruses from the acinar pancreas to the islets of Langerhans. However, expression of viral antigens in islet cells was minimal and glucose abnormalities were transient. It was concluded that the six CVB serotypes have the potential for infecting and damaging pancreatic beta-cells in mice. Subsequent studies performed in non-human primates showed that Cynomolagus, Rhesus, and Cebus monkeys failed to show glucose tolerance or insulin secretion abnormalities after infection with either EMC-D or CV-B4. However, Patas monkeys infected with CV-B4 or treated first with a subdiabetogenic dose of streptozotocin and then infected sequentially with CV-B4 and CV-B3 showed transient elevation of glucose tolerance tests, depressed insulin secretion, and glucose in the urine. The experiments support the concept that CVB infections may alter glucose homeostasis not only in rodents but also in higher primates, and possibly in humans [51].

The mechanisms of CV-B4-induced diabetes were investigated in different strains of mice. Upon infection, mice with diabetes-prone MHC alleles had no acceleration of diabetes, but mice with a T cell receptor transgene specific for an islet autoantigen rapidly developed diabetes [52]. The results show that diabetes induced by CV-B4 is the result of local infection leading to inflammation, tissue damage, and

Table 3. Enterovirus infections: typical acute clinical manifestations

<table>
<thead>
<tr>
<th>Virus</th>
<th>Acute clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coxsackieviruses group A</td>
<td>Undifferentiated febrile illness, aseptic meningitis, encephalitis, hand-foot-and-mouth disease</td>
</tr>
<tr>
<td>Coxsackieviruses group B</td>
<td>Undifferentiated febrile illness, myocarditis and pericarditis, neonatal infection, pleurodynia, pancreatitis</td>
</tr>
<tr>
<td>Echoviruses</td>
<td>Undifferentiated febrile illness, myocarditis and pericarditis, neonatal infection, pleurodynia</td>
</tr>
<tr>
<td>Polioviruses</td>
<td>Undifferentiated febrile disease, diarrhea, paralytic poliomyelitis</td>
</tr>
<tr>
<td>Coxsackievirus A24 and Enterovirus D70</td>
<td>Acute hemorrhagic conjunctivitis</td>
</tr>
<tr>
<td>Enterovirus A71</td>
<td>Hand-foot-and-mouth disease, encephalitis, neurogenic pulmonary edema</td>
</tr>
<tr>
<td>Enterovirus D68</td>
<td>Respiratory syndrome, polio-like paralysis (suspected*)</td>
</tr>
</tbody>
</table>

*47,48.
the release of sequestered islet antigen. The events appear to lead to re-stimulation of resting autoreactive T cells and indicate that sensitization to islet antigens is probably an indirect consequence of viral infection.

Paradoxically, when non-obese diabetic (NOD) mice were infected with different CVB types at a young age, the later incidence of spontaneous diabetes was significantly reduced [53]. The phenomenon was analyzed in detail using different strains of CV-B3. However, the mechanisms by which infection protects the host from the later development of diabetes remain unclear. Further studies examined the possibility of a persistent/chronic CV-B3 infection of pancreas in NOD mice. It emerged that (as in the heart) naturally occurring deletions at the 5′terminus of the virus genome are accumulating in the pancreas where the virus can persist for long times [54]. The terminally-deleted variants of CV-B3 are characterized by reduced viral replication and suppression of cytopathic effects in cell culture. The results of these experiments closely mimic the histopathologic findings in human T1D cases. In humans, EV antigens and genomes can be found in islets for years after the clinical onset. Further studies in NOD mice compared the effects of CV-B4 infection in wild type mice carrying functional toll like receptor 3 (TLR3) to those produced in TLR3 knockout NOD mice [55]. Mice not expressing TLR3 were strongly protected from CV-B4-induced diabetes and virus-associated insulitis was less severe than in wild type animals. The results show that TLR3 recognition of double-stranded RNA is critical for virus-induced diabetes.

An inverse correlation between the incidence of T1D and the prevalence of EV infections has been observed in human populations. The finding could be explained by an extension of the “poliovirus hypothesis” (or “hygiene hypothesis”) suggesting that the absence of maternal antiviral antibodies in the offspring does increase the risk for diabetes [56]. Experimental proof was provided by experiments in NOD and Soes1-transgenic NOD mice [56]. As expected, CVB-infected mice produced neutralizing antibodies. Compared to non-infected females, the offspring of CVB-immune females were clearly protected from diabetes. It was concluded that the absence of maternal protection does increase the risk for diabetes in the offspring.

Atrophy of lymphoid organs has been reported in mice that were infected with CV-B3 a few days after birth. Lymphoid atrophy was not produced by CVBs other than CV-B3. In thymus, the main lesion was depletion of cortical cell, whereas pathology of lymph nodes and spleen was not selective [57]. CV-B3-infected mice were also characterized by reduced B- and T-cell responses in the absence of documented virus replication in lymphoid cells [58]. In addition to the direct and indirect effects of CVBs in the pancreas, infection of thymus epithelial cells has been documented in mice infected with CV-B4. It has been proposed that infection of epithelial thymus cells may impair immune tolerance and favor the development of islet-specific autoimmunity [59]. Experiments suggest that autoimmunity to insulin is favored by the decreased expression of insulin and insulin-like growth factor 2 in infected thymus epithelial cells.

Recently, novel bioinformatic tools have been used to designing nucleic acid probes capable of detecting and identifying EVs at the type level. The probes have been tested in infected mouse pancreata and found capable of detecting a vast array of EV types suspected to play a role in human diabetes [60]. In histopathologic studies, these tools will help identify the EV types that infect islet cells in diabetic subjects.

2.4) Enteroviruses as prominent candidate agents for TID in humans

Extensive studies from Europe, the Nordic countries and the U.S. indicate EVs as prominent candidates among viral agents. EV antigens could be detected not only in islets of T1D diabetics, but also in subjects diagnosed with T2DM [45, 61]. The latter finding could be due to difficulties in the exact identification of all multifaceted forms of diabetes, or to the fact that mixed forms of diabetes (type-2 and type-1, for instance) may occur in nature.

Case reports, histopathologic studies, molecular and serologic investigations plausibly point to EV infections as prominently associated with T1D cases, both at the time of clinical presentation and in long-lasting disease [62, 63]. Scientists have been able to isolate viruses from patients with type 1 diabetes in just a few cases [9, 64-68]. Overt hyperglycemia, in fact, is the final consequence of a prolonged pathologic process that - if linked to virus-induced damage - may involve slowly-replicating agents that challenge current methods of virus isolation in vitro.

A meta-analysis of molecular studies aimed at virus detection in newly-diagnosed cases of T1D lends support to the idea that EVs are present at varying but remarkable frequencies in the early phase of the disease [69]. EVs have been detected also in intestinal epithelial cells of diabetic subjects [70], although not confirmed [71]. It remains unclear whether these correlations are merely coincidental with a “failing” immune system in early diabetics or have causal implications.

All studies point to the fact that the type of virus-host
interaction seen in T1D is not an acute one, but a long-lasting infection during which little virus is produced and the consequences on the host are profoundly different from those of acute symptomatic disease that is clinically diagnosed [72]. To establish persistent infection, a virus must be able to reduce its cytopathic effects (i.e., its ability to kill or damage infected cells), maintain its genome within host cells over time, avoid elimination by the host immune system. Though EVs are cytolytic to their host cells (mainly due to the shutdown of host transcriptional and translational machinery resulting in substantial inhibition of host cell metabolism) evidence suggests that persistent EV infection may be associated with the post-polio syndrome, myasthenia gravis, chronic myocarditis, type 1 diabetes and, possibly, autoimmune thyroiditis [73-77].

Several models of EV persistence have been reported: monkey kidney cells, human cells (e.g., HEp2 line, cells of neural and endothelial origin, astrocytes, a neuroblastoma line) [79]. Cell cultures persistently infected by EVs show peculiar characteristics: a) only a small percentage of cells do express viral antigens, b) viral titers (i.e., the amount of infectious viral particles released by infected cells in the medium) are low (≤103 plaque-forming units/ml), c) infected cells produce cytokines of pathogenic relevance. In persistently infected hosts, virus spread to uninfected cells is not only achieved by a lytic mechanism, but also by the release of host cell-derived microvesicles, the formation of cell protrusions, or intercellular bridges [79]. These modes of spread let the virus escape immune defenses and continue to infect cells adjacent to those already infected.

The high genetic variability of these agents (due to their high mutation rate and to recombination events) does contribute to the selection of non-cytolytic variants. Studies of clinical cases and of EV-infected mice have shown that: 1) EVs may produce persistent heart infection and neurologic disease [80,81]; 2) persistent infection can be established in specialized cells such as motor neurons, astrocytes, insulin-producing beta cells, cardiomyocytes, vascular endothelial cells, thyroid and thymus cells, macrophages/dendritic cells [82, 83]; 3) EV genome mutations as well as deletions in the 5’ terminus appear to hinder virus replication and contribute to virus persistence [76].

2.5) Additional evidence for an association of enteroviruses with T1D

The Diabetes Virus Detection study (DiViD) has been the first to examine fresh pancreatic tissue at the diagnosis of T1D for the presence of viruses [84]. Pancreatic tissue was obtained 3-9 weeks after onset of type 1 diabetes from 6 adult Norwegian patients (age 24-35 years). The presence of EV capsid protein 1 (VP1) was investigated by immunohistochemistry, the presence of EV genome was analyzed from frozen whole pancreatic tissue using PCR and sequencing. Non-diabetic pancreas donors served as controls. VP1 was detected in the islets of 6/6 T1D patients (vs. 2/9 controls). Enterovirus specific RNA sequences were detected in 4 of 6 cases (vs. 0/6 controls). Results have been confirmed in different laboratories (Tampere, Uppsala, Varese). Less than 2% of the islets contained VP1 positive cells and the amount of EV RNA was low. EVs of different species have been detected. The results provide evidence for the presence of EV in pancreatic islets being consistent with the possibility that a low grade EV infection contribute to triggering/causing T1D in humans.

In a collaborative study of tissue samples provided by the Network of Pancreatic Organ Donors with Diabetes [85], the proponent isolated live enteroviruses from spleens of 9/12 long-standing T1D cases (vs. 2/13 non-diabetic controls). Immunostaining for VP1 identified small numbers of intensely positive cells in 9 T1D cases (5 being virus-positive), in one autoantibody-positive case, and in two non-diabetic controls (one of which was virus-positive). Virus isolation and VP1 staining were concordant in >70% of cases [45]. Notably, Infectious virus was detected in long-standing cases up to 32 years from diagnosis. The finding shows that EVs may persist in at least some patients and raise the question of whether chronic infection may hinder metabolic control and contribute to diabetes complications.

EVs have been detected in peripheral blood leukocytes of over 75% pediatric patients at the time of clinical onset [66,85]. At that time, EV infection has been documented to spreads out to family members [86]. Follow up, of virus-positive diabetic children showed that EVs can persist for over 3 years in one third of diabetic children/adolescents. Detection of EVs in blood is linked to the development of T1D [70].

Taken together, the data demonstrate that in humans low level EV infection involves pancreatic islet cells (especially beta-cells), intestinal epithelial cells, lymphoid cells of spleen and lymph nodes, peripheral blood leukocytes [87]. Thus, EV persistence in diabetics seems to be associated with a systemic infection affecting not only pancreatic islets, but also the intestine and the immune system. An interesting question is whether EVs can also persist in vascular endothelial cells, since microangiopathy plays a crucial role in diabetic complications [88]. Notably, persistent EV infection of human vascular endothelial cells is followed by cell activation, enhanced expression of adhesion molecules, production of cytokines of pathogenic significance [89-91].
Finally, exocrine insufficiency of pancreas is a clear feature of T1D, but it is generally considered clinically irrelevant compared to beta cell loss. Early pathology observations [92] and recent studies have documented pancreatic atrophy in patients recently diagnosed with T1D [93] and in pre-diabetic individuals positive for T1D-associated autoantibodies [94]. Recently, inflammatory infiltrates in the exocrine pancreas of T1D patients have been detected [95]. It seems thus possible that acute pancreatitis (possibly due to a viral infection) takes place in the early stages of the disease and that, in some cases, the process becomes chronic. In the long term, this could lead to attraction of immune cells into the organ and to sensitization against beta cell antigens in predisposed individuals [9, 76]. Thus, further investigations should be addressed to the hypothesis of T1D as a combined endocrine-exocrine disease in which the loss of beta cells is the clinically prominent feature.

3) Determinants of Diabetes Susceptibility in Humans

In human cases the situation is highly different from that in experimental animals where mice have been infected with a virus and it is possible to prove that infection is causing diabetes. However, in humans the accumulation of circumstantial evidence to identify the possible etiologic role of viral infections and the virus-induced susceptibility genes is important [97]. It was reported that polymorphisms of the IFIH1 gene, which is an intracellular pathogen recognition receptor for picornavirus including EVs, operating as an inducer of interferon production, is associated with risk or resistance for the T1D, serving possible virus-induced susceptibility gene in humans [98, 99]. Interestingly, a human genome-wide study suggested the T1D susceptibility region to be in chromosome 19p13, where the TYK2 gene is located (19p13.2) [100], and recent fine mapping research could identify TYK2 as a candidate gene for T1D [101]. In addition, natural Tyk2 gene mutations are responsible for susceptibility to experimental EMC-D virus-induced diabetes [14]. The observations suggest that the human TYK2 gene may be associated with the risk for T1D and also may confer a link with susceptibility to virus-induced diabetes in humans. Accordingly, it was found that polymorphisms of TYK2 gene at the promoter region and exons and that a TYK2 promoter haplotype with multiple genetic polymorphisms, which are in complete linkage disequilibrium, named TYK2 promoter variant, presenting decreased promoter activity, is associated with an increased risk of not only T1D (odds ratio (OR), 2.4; P=0.01), but also T2D (OR, 2.1; P=0.03) [15]. The risk is high in patients with T1D associated with flu-like syndrome at diabetes onset (OR, 3.6; P=0.005), and also those without anti-glutamic acid decarboxylase autoantibody (OR, 3.3; P=0.002) [15] (Figure 4). Moreover, there was increased TYK2 promoter variant rate at statistical significance only in non-obese T2D with less than 26 BMI (OR, 2.4; P=0.002), but not obese T2D with more than 26 BMI (OR, 0.8; P=1.0) [15], suggesting that obesity is not likely involved in the increased risk associated with TYK2 promoter variant in T2D. These observations suggested that human TYK2 gene might be associated with virus-induced diabetes not only in type 1 but also Type 2 diabetes, indicating that virus infection may serve as one of risk factor for T2D as well as causal factor for T1D (Figure 5). These observations are consistent with the previous experimental evidence that cumulative environmental insults may lead to the development of diabetes [39]. Since the outcome of virus-induced diabetes is influenced by many factors including viral diabetogenicity and host susceptibility, the discovery of other human risk genes associated with virus-induced diabetes in addition to IFIH1 and TYK2 genes is both possible and feasible.

4) Conclusions and Perspectives

To identify the causal pathogen in inducing an infectious disease, historically and ideally, ‘Koch’s postulate’ should be fulfilled [102]. Although this paradigm cannot be applied to all microbial diseases, it is of great help to show that an isolated pathogen can cause the disease in experimental animals, to prove the causality of the pathogen. There were only a few reports to prove the diabetogenicity of the clinically isolated viruses from patients with diabetes [64, 103], and no reproducible findings have been published. Mouse models simulative of virus-induced human diabetes, should be developed as an in vivo assay system to evaluate the diabetogenic potential viral agents that have been or will be.
detected in humans. Mouse strains endowed with high susceptibility to picornavirus-induced diabetes may represent an important tool to evaluate the diabetogenicity of candidate human viruses.

**Conflicting Interests**

None to declare

**Acknowledgements**

The authors thank all collaborators, Dr. Yoshiaki Nose and international scientists for their help and contributions to the field of Viral Diabetes. Financial support to SN from Type 1 Diabetes Research Fund of Japan and the generous support to AT of the Juvenile Diabetes Research Foundation (JDRF-nPOD-V grant 25-2012-770) is gratefully acknowledged.

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