Role of coxsackievirus B4 in the pathogenesis of type 1 diabetes

H. Jaïdane a, b, D. Hober a, *

a Laboratoire de virologie/UPRES EA3610, faculté de médecine, université Lille-2, CHRU de Lille, centre de biologie pathologie et parc Eurasanté, 59037 Lille, France
b Laboratoire de virologie LR99ES27, unité de pathogénèse et virulence virales, faculté de pharmacie de Monastir, avenue Avicenne 5000 Monastir, Tunisia

Received 12 March 2008; received in revised form 23 May 2008; accepted 23 May 2008
Available online 31 October 2008

Abstract

Environmental factors, especially viruses, are thought to play an important role in the initiation or acceleration of the pathogenesis of type 1 diabetes (T1D). Data from retrospective and prospective epidemiological studies strongly suggest that enteroviruses, such as coxsackievirus B4 (CV-B4), may be associated with the development of T1D. It has also been shown that enterovirus infections are significantly more prevalent in at-risk individuals such as the siblings of diabetic patients, when they develop anti-β-cell autoantibodies or T1D, and in recently diagnosed diabetic patients, compared with control subjects. The isolation of CV-B4 from the pancreas of diabetic patients supports the hypothesis of a relationship between the virus and the disease. Furthermore, studies performed in vitro and in vivo in animal models have increased our knowledge of the role of CV-B4 in T1D by helping to clarify the pathogenic mechanisms of the infection that can lead to β-cell destruction, including direct virus-induced β-cell lysis, molecular mimicry, ‘bystander activation’ and viral persistence. The role of enteroviruses as the sole agents in T1D, and a causal link between these agents and T1D, have not yet been established, although arguments that support such a role for these viruses in the pathogenesis of the disease cannot be ignored.

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Résumé

Rôle des coxsackievirus B4 dans la pathogenèse du diabète de type 1. Des facteurs environnementaux, et notamment des virus, sont supposés jouer un rôle important dans l’initiation ou l’accélération du développement du diabète de type 1 (DT1). Les résultats des études épidémiologiques rétrospectives et prospectives suggèrent fortement que des entérovirus, et notamment le coxsackievirus B4 (CV-B4), puissent être associés au développement du DT1. Il a été démontré que les infections à entérovirus étaient significativement plus fréquentes chez les sujets à risque tels que les frères et sœurs de patients diabétiques, lorsqu’ils ont développé des auto-anticorps dirigés contre les cellules β et/ou un DT1, et chez les patients diabétiques récemment diagnostiqués, que chez les sujets non diabétiques. L’isolement de CV-B4 à partir du pancréas de patients diabétiques a renforcé l’hypothèse d’un lien entre le virus et la maladie. Par ailleurs, des études réalisées in vitro et in vivo chez l’animal ont abouti à une meilleure connaissance du rôle de CV-B4 dans le DT1. Ces études ont permis de découvrir des mécanismes pathogéniques de l’infection susceptibles d’aboutir à la destruction des cellules β, tels que la lyse viro-induite de ces cellules, le mimétisme moléculaire, ‘bystander activation’ et la persistance virale. La responsabilité des entérovirus en tant qu’unicus agents du DT1 et un lien de causalité entre ces agents et le DT1 n’ont pas été établis, cependant un faisceau d’arguments suggère que le rôle de ces virus dans la pathogenèse de la maladie ne peut être exclu.

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Keywords: Coxsackievirus B4; Type 1 diabetes; Pathogenesis; Review
Mots clés : Coxsackievirus B4 ; Diabète de type 1 ; Physiopathologie ; Revue

1. Introduction

Type 1 diabetes (T1D), initially termed ‘insulin-dependent diabetes mellitus’ (IDDM), is an organ-specific autoimmune disease characterized by a defect in insulin production as a result of selective and massive destruction of islet β cells (80–90%) or of their functional impairment. The progression of the
autoimmune process is generally slow and may take several years before the onset of the disease.

The subclinical phase is characterized by the circulation of autoantibodies targeting islet β-cell autoantigens, including islet-cell autoantibodies (ICA) directed against islet cells, insulin autoantibodies (IAA) against insulin, GADA (GAD65 antibodies) against glutamate decarboxylase and IA-2A (IA-2 antibodies) against the IA-2 molecule related to tyrosine phosphatase [1].

Starting generally at a young age, T1D is also referred to as 'juvenile diabetes'. Indeed, it affects children and young adults in particular, and generally occurs before the age of 40 years, with incidence peaks at 2, 4–6 and 10–14 years [2].

T1D represents 5–10% of all cases of diabetes, a disease with frequently serious and severe consequences for the patient (such as ketosis, ketoacidosis, microangiopathy, cerebral vascular accidents, retinopathies, cataract, glaucoma, hypertension, ischaemic cardiopathy, glomerulosclerosis, autonomous neuropathy, peripheral vasculopathy and gangrene). Polymuria, polyphagia, polydypsia, weight loss, weakness and recurrent infections are its principal signs and symptoms. The annual incidence of T1D varies widely from one country to another (from less than 1 per 100,000 inhabitants in Asia to approximately 14 per 100,000 in US and more than 30 per 100,000 in Scandinavia), but is, however, in steady increase across the globe (approximately 3% per year between 1960 and 2000), especially among children aged less than five years [3].

Prevention of T1D remains a challenge partly because its exact cause is still unknown. In fact, there is no one factor implicated as the unique cause; T1D appears to be the result of the interactions of a multitude of factors.

In this review, we first describe the primary processes involved in the pathogenesis of T1D and then focus on a putative interaction of a multitude of factors.

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2. Factors involved in the pathogenesis of T1D

2.1. Perturbation of thymus function and autoimmunity

As with other autoimmune diseases, T1D can result from the failure of central and/or peripheral tolerance. In this section, we consider only those phenomena involving the thymus, and not the abnormalities of regulatory T lymphocytes (TREG) in the periphery [4].

2.1.1. Alteration of factors affecting positive selection

Some abnormalities of positive selection are strictly related to the major histocompatibility complex (MHC) of an individual, especially in the case of T1D [4]. In subjects expressing the haplotype DQ8, for example, thymus epithelial cells allow the selection of an excessive number of autoreactive T lymphocytes with a strong affinity for autoantigens [5].

2.1.2. Defect of negative selection

A defect in the negative selection of autoreactive thymocytes may be the result of the following conditions:

- a low level of thymus expression of autoantigens. The majority of autoantigens, whether ubiquitous or organ-specific, are expressed in the thymus as, for example, GAD and proteins of the insulin family involved in T1D to be presented to thymocytes [6]. Mechanisms that decrease the expression of these autoantigens disturb the negative selection of autoreactive T lymphocytes and, thus, predispose to the development of autoimmunity. In humans, susceptibility to T1D is associated with a polymorphism in the 5′ region of the insulin gene, which influences the rate of expression of peptides derived from insulin by antigen-presenting cells in the thymus. The protective allele is associated with a high level of thymus expression of insulin and the susceptibility allele, with a low level [6]. Non-obese diabetic (NOD) mice, which do not express proinsulin 2 in either the pancreas or thymus, rapidly develop diabetes [7]. Likewise, bio-breeding diabetes-prone (BBDP) rats do not express type 2 insulin-like growth factor (IGF2) in the thymus [8]. It has also been reported that intrathymus transplantation of pancreatic islet cells reduces autoimmunity against β cells and prevents diabetes development in NOD/Lt mice [9]. This last observation suggests that the thymus may play a role in acquired tolerance, making it a potential candidate for therapy in autoimmune diseases;
- a defect of peptide presentation may be due to the weak affinity of the T-cell receptor (TCR) for unstable MHC–peptide complexes and/or a defect of antigen-processing by proteases of thymus antigen-presenting cells [4];
- there may be a disturbance in the architecture of the thymus stroma as seen in NOD mice, where medullary epithelial cells are present in the cortex [5];
- a defect of apoptotic factors may play a role as negative selection is based on the apoptosis of autoreactive T lymphocytes [4].

2.2. Genetic determinism

A large body of evidence indicates that genetic factors influence both susceptibility and resistance to T1D. Siblings of diabetic patients have a 6% risk of developing the disease versus only 0.3% in the general population. Furthermore, approximately 10% of the children diagnosed with T1D have a family member who is also affected by the disease at the time of diagnosis [10].

Several chromosomal regions have been correlated to the disease, suggesting that it is a polygenic disorder. The most important and most studied genes are those coding for human leukocyte antigen (HLA) molecules, especially the DQ and DR loci. The alleles HLA-DQ and HLA-DR, with serine, alanine or valine in position 57 of the β chain, appear to fix peptides with a negative charge in position 9, which would facilitate a cytotoxic Th1 response and so increase the risk of T1D. On the other hand, the risk is decreased by the presence of aspartic acid in
activate endogenous retrovirus genes that then behave like ‘dis-

immune system. Thus, environmental factors may provide or

against expressed retroviral antigens previously unknown to the

individuals could be the result of cross-reactivity or immunity

viruses. The development of diabetes in genetically predisposed

birth, and common viral infections and/or the sex-hormone

so that their genes can be either inherited or acquired after

ple) [19]. Retroviruses can integrate with the human genome

becomes a target of autoimmunity in T1D.

responsiveness to specific epitopes and help to explain why IA-2

expression in the pancreas rather than in the lym-

IA-2 [18]. Alternative splicing causes differential IA-2 mRNA

ability of mounting an autoimmune response to autoantigen

there is evidence that alternative splicing can affect the prob-

sion in a tissue-specific manner and predispose to T1D. Indeed,

mechanisms, such as alternative splicing, can affect gene expres-

fectibility could be due to modulation of insulin transcription

VNTR class I alleles appear to be predisposing and class

III alleles appear to be protective, and are associated with low

and high levels, respectively, of insulin transcripts in the thymus

and peripheral lymphoid tissues where, as mentioned above,

production of self-antigens may be crucial for the shaping and

maintaining of a self-tolerant T-cell repertoire [16].

At least 20 other susceptibility loci have been described in the

literature, but many of them have recently been found to be

‘false positives’ and have been replaced by identified genes.

Besides inherited alleles, other mechanisms that regulate
gene expression include epigenetic modifications (such as DNA

methylation) and parent-of-origin effects (imprinting of either

the maternal or paternal allele) that can influence susceptibil-

ity by modifying the transmission and transcription of inherited

gen genes [17].

There is also evidence that other, non-mendelian regulatory

mechanisms, such as alternative splicing, can affect gene expres-

sion in a tissue-specific manner and predispose to T1D. Indeed,

there is evidence that alternative splicing can affect the prob-

ability of mounting an autoimmune response to autoantigen

IA-2 [18]. Alternative splicing causes differential IA-2 mRNA

and protein expression in the pancreas rather than in the lym-

phoid organs. Such differences may have an effect on immune

responsiveness to specific epitopes and help to explain why IA-2

becomes a target of autoimmunity in T1D.

It is also an intriguing possibility that additional genetic fac-

tors, or their expression, may be acquired after birth perhaps

through environmental exposure (via viruses or diet, for exam-

ple) [19]. Retroviruses can integrate with the human genome

so that their genes can be either inherited or acquired after

birth, and common viral infections and/or the sex-hormone

changes associated with puberty may activate quiescent retro-

viruses. The development of diabetes in genetically predisposed

individuals could be the result of cross-reactivity or immunity

against expressed retroviral antigens previously unknown to the

immune system. Thus, environmental factors may provide or

activate endogenous retrovirus genes that then behave like ‘dis-

ease genes’ [19]. It has been reported that a human endogenous

retrovirus, termed IDDMK1,2 22, is expressed and released from

leukocytes in T1D patients, suggesting a role for this endogenous

retrovirus in T1D pathogenesis [20].

Thus, a variety of genetic mechanisms may influence the

autoimmune responses leading to β-cell destruction.

2.3. Environmental factors

It has been noted that less than 5% of genetically predisposed

subjects develop the disease, and the concordance rate is only

30–50% between monozygotic twins [21]. Furthermore, it is
clear that the alarming increase of the incidence of T1D over the

past few decades cannot be the result of an increase in genetic

susceptibility within the population.

This has led to the hypothesis that the process could be trig-

gered in genetically predisposed subjects by ‘environmental’
factors such as drugs, toxins [22], nutrients such as cow’s

milk [23] and, more recently, viruses [24] such as rubella virus,
mumps virus, cytomegalovirus, retroviruses, rotaviruses and

especially enteroviruses. The involvement of viruses is notably

supported by the variable incidence of T1D from one coun-

try to another and from one season to another, as well as by

observations regarding the relationship between immigration

and disease development [2,25].

3. CV-B4 and T1D

Enteroviruses belong to the Picornaviridae family, which

includes the Rhinovirus, Hepatovirus, Parechovirus, Car-

diovirus, Kobuvirus, Aphthovirus, Erbovirus and Teschovirus

genera. Human enteroviruses are traditionally classified, on

the basis of serological criteria, into 64 serotypes distributed

across five subgenera: poliovirus (PV); coxsackievirus A (CV-

A); coxsackievirus B (CV-B); echovirus (E-V); and unclassified

enteroviruses. A new classification of human enteroviruses was

proposed by the International Committee on Taxonomy of

Viruses (ICTV), based on sequence homologies, and included

only five species: PV; and human enterovirus (HEV)-A, -B, -C

and -D [26]. According to this new classification, CV-B belongs
to the HEV-B species, and the Enterovirus genus encompasses
89 serotypes at this time.

Enteroviruses are small (around 25–30 nm in diameter)

non-enveloped viruses that have cubic symmetry (icosahedral
capsid) and single-stranded, linear, non-fragmented positive

RNA (infectious and serving as a messenger RNA) capped by

the VPg protein (genomic viral protein) in 5′. The enterovirus
genome of about 7500 bases comprises a unique large, open

reading frame (representing more than 80% of the RNA) cod-
ing for a massive polyprotein of 2200 amino acids (aa) that

undergoes successive cleavages, leading to the production of 11

mature proteins, of which four are structural (VP1, VP2, VP3

and VP4 form the capsid and are coded for in the P1 region) and

seven are functional (of which VPg, the RNA polymerase and

proteases are coded for in the P2 and P3 regions) [27], flanked

by two non-coding regions in 5′ and 3′ that are essential for

translation initiation and viral RNA synthesis.
Enteroviruses have a ubiquitous distribution and are mainly transmitted by the faecal–oral route via the ingestion of water or food soiled by contaminated faeces. Of these human pathogens, CV-B constitute one of the most clinically significant groups, especially in countries where PV infections are controlled by vaccination programs. Enterovirus infections have been associated with acute manifestations such as meningitis, encephalitis and pericarditis, as well as with chronic diseases such as meningoccephalitis in agammaglobulinaemic patients, post-poliomyelitis syndrome, amyotrophic lateral sclerosis, chronic myocarditis, dilated cardiomyopathy and T1D.

The possible involvement of enteroviruses in T1D was raised for the first time in 1969 by Gamble and Taylor, who observed that anti-CV antibodies were found more frequently in patients with T1D than in control subjects [28]. Later, in 1979, Yoon et al. described the isolation of a virus from the pancreas of a 10-year-old boy who had died of diabetic ketoacidosis. This virus, recognized by an antisera directed against a diabetogenic variant derived from the prototype CV-B4 Van Barscholten (CV-B4 JVB) strain, adapted to the mouse through successive passages on β cells, suggested that this human isolate was CV-B4 [29]. It was similar to the CV-B4 E2 variant and purified, as with variants E1 and E3, by Hartig et al. in 1983 [30] by successive cloning on plates of the CV-B4 Edwards strain, isolated in 1956 by Kibrick and Benirschke from the myocardial tissue of a newborn child who had died following a generalized infection with focal necrosis and inflammation of the pancreas [31]. The strain isolated by Yoon et al.—called ‘CV-B4 E2’—when transferred to susceptible mice such as SJL/J, led to hyperglycaemia with inflammation of the Langerhans islets and β-cell necrosis [29].

A similar pattern of results was obtained with a strain of CV-B5 isolated from the stools of a diabetic patient [32]. In another study conducted with SJL/J mice, it was found that CV-B4 E2 provoked hyperglycaemia six weeks post-infection, with the appearance of anti-GAD antibodies in 90% of the animals, suggesting that this virus was able to initiate or accelerate autoimmunity against β cells [33].

A temporal relationship between the peaks of enterovirus infections (late summer/early fall) and of diabetes onset (late fall/early winter) was also noted in several studies [34,35].

The hygiene theory postulates that the increase in the incidence of T1D could be related to changes in the epidemiology of enterovirus infections [36]. Indeed, it has been observed that, in a given population, there is an inverse correlation between the incidence of T1D and the frequency of such infections. Interestingly, the epidemiology of enterovirus infections changed just over a hundred years ago. At that time, there was a rapid decrease in poliovirus transmission as a consequence of improvements in sanitation. However, on the other hand, the risk of paralytic complications was higher.

Better hygiene in the developed countries resulted in a decrease of enterovirus infections in the general population. This meant that such infections occurred when subjects were no longer protected by maternal antibodies. Also, because of the reduced exposure to enteroviruses in general, maternal antibodies were directed against only a few serotypes. If the initial infection struck when the child was protected by maternal anti-bodies able to inhibit the spreading of viruses within the body, the child’s immune system was then able to elaborate factors to limit the pathogenic effects of enteroviruses in case of an infection arising after the disappearance of the passively transmitted, protective maternal antibodies. This is consistent with the idea developed by Rolf Zinkernagel, who described how the antibody repertoire of the mother can influence the susceptibility of her child to infectious agents and autoimmune diseases, using poliovirus and CV-B as examples [37].

Since the first suspected implication of enteroviruses in the physiopathology of T1D, about 40 years ago, investigations have been conducted to test the hypothesis.

### 3.1. Association between CV-B4 and T1D

Numerous epidemiological investigations have been conducted to better define the possible relationship between enterovirus infections and T1D. The main findings from both retrospective and prospective studies are given below.

#### 3.1.1. Lessons from retrospective studies

Anti-enterovirus antibodies are found more frequently in recently diagnosed diabetic patients than in healthy controls matched for age and location [38–40]. Studies carried out in several countries showed that the enterovirus genome is also detected more frequently in the peripheral blood of recently diagnosed diabetics than in healthy controls [40–43].

The most frequently implicated enteroviruses were CV-B, especially CV-B4 [39,40,43,44]. It was also found that T1D patients harbour CV-B4 RNA homologous to that of CV-B4 E2 and CV-B4 VD2921 (an isolate similar to CV-B4 E2) in their peripheral blood mononuclear cells (PBMC) [42].

A study conducted by the present authors showed that interferon-alpha (IFN-α), a marker of viral infection, is found at high levels in plasma in association with CV-B infections in 75% of T1D patients at various stages of the disease, but not in controls [44]. In that study, the simultaneous presence of IFN-α, enterovirus RNA (in particular, CV-B4 RNA) and anti-enterovirus antibodies suggested that acute or persistent enterovirus infections were associated with T1D in the majority of cases.

Recently, enterovirus RNA was detected by in situ hybridization (ISH) in autopsy pancreas samples from T1D patients, but not in those from controls [45]. In addition, researchers detected enteroviruses by electron microscopy and immunohistochemical staining of the capsid protein VP1 in β cells from three out of six diabetic organ donors, but from none of 26 control donors [46]. In that study, the enterovirus isolated from the endocrine pancreatic tissue of one of the diabetic donors turned out to be CV-B4.

#### 3.1.2. Lessons from prospective studies

Prospective studies based on the follow-up of children who were not diabetic until the onset of the disease have the advantage of revealing information on the pathogenic processes that arise during the preclinical phase of the disease. However, such large-scale studies are time-consuming and expensive. For this reason,
investigators have focused their attention on the appearance of early immunological markers—in particular, the autoantibodies directed against islet antigens (ICA, IAA, GADA and IA-2A)—as the endpoints of their studies. This approach allows the number of cohorts and the duration of the follow-up to be reduced, while allowing analyses of the temporal relationship between viral infections and the initiation of autoimmune processes corresponding to the appearance of autoantibodies [41]. Here, we report the results of three Finnish prospective studies.

The Childhood Diabetes in Finland Study (DiMe) was the first prospective series to include the siblings of diabetic children. Blood samples were collected from these subjects every six months [47–49]. The diagnosis of enterovirus infection was based on a significant increase of the titre of anti-enterovirus antibodies in serum, as determined by radioimmunoassay (RIA) and enzyme-immunoassay (EIA) methods. This study found that enterovirus infections were more frequent in the children who progressed to diabetes than in the others. Moreover, it was observed that the enterovirus infections were clustered in the period immediately preceding the appearance of autoantibodies. These serological data were later confirmed by the more frequent detection of enterovirus RNA in the serum of prediabetic children compared with the controls [50].

The Finnish Diabetes Prediction and Prevention (DIPP) trial was a follow-up study of genetically predisposed newborn children (according to their allelic HLA-DQ profile) [51]. Several methods for diagnosing enterovirus infections were used (including EIA and reverse-transcription polymerase chain reaction [RT-PCR]), and the intervals between the samplings were shorter than in the DiMe study (every 3–6 months for serum and every month for faeces) to maximize the results of the study. Thus, it was shown that enterovirus infections were more frequent in children who became positive for autoantibodies than in the controls. These infections were also clustered in the period immediately preceding the appearance of autoantibodies directed against β cells [52,53]. A relationship between viral infection and autoimmunity was found with none of the other tested viruses except for enteroviruses.

Finally, the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) was a study in which the presence of autoantibodies was determined in two-year-old children who were genetically predisposed to develop T1D, and who were fed up to the age of 6–8 months with a hydrolyzed casein formula. A relationship between enterovirus infections (diagnosed by seroneutralization, EIA and RT-PCR) and the induction of autoimmunity against β cells was observed [54].

In these prospective studies, the controls were the newborn siblings of T1D patients, or genetically predisposed newborns, who were matched for age, location, gender and HLA haplotype (in the studies of genetically predisposed newborns), but who did not become positive for autoantibodies (anti-β cells).

Studies focusing on the role of exposure to enteroviruses during intrauterine life showed that IgM and IgG directed against CV-B are found more frequently in mothers of diabetic children than in other mothers, suggesting that the pathogenic process may be related to maternal infection during pregnancy [55,56].

Nevertheless, in contrast to the above-cited studies, other studies have reported no association between enterovirus infections and T1D [25]. It is, however, important to remember that these studies were based on traditional serological assays looking at a limited range of enteroviruses.

Overall, it appears that a relationship between enterovirus infections and T1D may be suspected; however, further clinical studies based on larger series need to be performed to confirm these theories.

A multicentre, multinational, epidemiological study—The Environmental Determinants of Diabetes in the Young (TEDDY) study—is in progress to identify the infectious agents, dietary factors and other environmental factors associated with an increased risk of autoimmunity and T1D [57]. TEDDY is an observational cohort study in which newborns younger than four months and with the high-risk HLA alleles in the general population, or with first-degree relatives who have T1D, will be enrolled. Recruitment will take place over a five-year period in six clinical centres across the US and Europe, and the participating children will be followed-up to the age of 15 years.

### 3.2. CV-B4 and the pathogenesis of T1D

#### 3.2.1. Lessons from in vitro investigations

The infection of cultured cells with CV-B4 has made a considerable contribution to the investigation of the hypothetical role of enteroviruses in T1D.

**3.2.1.1. CV-B4 infection of pancreatic cells.** The diabetogenic strain CV-B4 E2 and the strain CV-B4 VD2921, as well as the prototypes of CV-B2, -B3, -B4 and -B5 serotypes, can infect and damage human β cells in vitro [45,58–60]. It has also been demonstrated that clinical isolates of CV-B4 circulating in the general population have a predilection for murine β cells [61].

In cell cultures, the infection of islets by some viruses—in particular, CV-B4—activates the expression of MHC molecules, which can facilitate the autoimmune response against β cells [62]. Coxsackievirus B (especially CV-B5 in the reported studies) and CV-B-induced cytokines (such as interleukin [IL]-1β and IFN-γ) both stimulate the expression of various chemokines, IL-15 and ICAM-1 (intercellular adhesion molecule), which can contribute to the activation of mononuclear cells in islets that participate in the early pathogenic processes [63].

A persistent infection by CV-B4 (E2 and JVB) and CV-B3 of human pancreatic islet β cells was achieved by the present authors, but with no observable cytopathogenic effects up to the conclusion of the islet cultures (30 days post-infection) [59].

The predilection of enteroviruses, especially CV-B4, for pancreatic cells in diabetic patients was confirmed by the detection of viral RNA in this organ and/or the isolation of CV-B4, as reported recently by several teams of researchers [45,46].

**3.2.1.2. Thymus infection with CV-B4.** In the thymus, it is proposed that a disruption of central tolerance, through CV-B4 infection, can participate in the pathogenesis of autoimmune disease and, in particular, T1D. The present authors in collaboration...
with V Geenen, Liège, have demonstrated that CV-B4 E2 and CV-B4 JVB can replicate and persist in human thymus epithelial cells in vitro with an increased production of IL-6, leukaemia inhibitory factor (LIF) and granulocyte–macrophage colony-stimulating factor (GM-CSF) [64]. Furthermore, the infection of fragments of human fetal thymus with CV-B4 E2 can perturb T-lymphocyte maturation. The infection mainly involves immature CD4+ and CD8+ thymocytes, and leads to increased expression of MHC class I molecules by these cells and epithelial cells, as well as to severe thymocyte depletion [65]. Thus, CV-B4 is able to infect epithelial cells and immature thymocytes, and may potentially disturb thymus selection.

In our laboratory, it has recently been shown, in outbred Swiss albino mice, that CV-B4 E2 inoculated by the oral route (the usual route of contamination in humans) was able to reach the thymus through systemic infection, with detection of the viral genome by RT-PCR up to 70 days post-infection [66]. It was later found that CV-B4 E2 and CV-B4 JVB replicate in murine thymus cells in primary cultures [67]. Moreover, murine fetal thymus organ cultures (FTOC), when infected by CV-B4 E2, had disturbed T-lymphocyte maturation and differentiation processes [68]. It has also been observed that a thymus epithelial cell line derived from newborn mice could be persistently infected, which reduced the rate of IFG2 transcripts, a gene of a protein potentially involved in the establishment of fragments of human fetal thymus with CV-B4 E2 can persistently infected, which reduced the rate of IGF2 transcripts, which suggests that these antibodies have the ability to facilitate or potentiate the infectivity of the virus in circulating monocytes [76]. Interactions among the virus, non-neutralizing antibodies and specific receptors allowed CV-B4 to infect the PBMC via an antibody-dependent mechanism and to induce the synthesis of IFN-α by these cells [74–76]. The inhibition of IFN-α through the addition of neutralizing antibodies to cultures, notably CD14+ monocytes, revealed the presence of infectious virus (evidenced by culture on Hep-2 cells) in the supernatant fluid of infected PBMC.

We also demonstrated that the pre-incubation of human plasma with the viral protein VP4, obtained from CV-B4 E2 heated to 56 °C, inhibited the plasma-dependent enhancement of CV-B4-induced production of IFN-α by PBMC in vitro [77]. In addition, the IFN-α concentrations in culture supernatants from these experiments were positively correlated to plasma anti-VP4 antibodies, which were more frequently detected by enzyme-linked immunosorbent assay (ELISA) at higher rates in diabetic patients than in control subjects. These data show that the protein VP4 was the target of antibodies involved in the increase of CV-B4-E2-induced synthesis of IFN-α by PBMC. Recently, our laboratory found that the VP4 capsid protein and anti-VP4 antibodies isolated from plasma are both involved in the enhancement of infection of PBMC with CV-B4 E2 [78]. The target amino-acid sequence of VP4 involved in the plasma-dependent enhancement of CV-B4-E2-induced production of IFN-α by PBMC has been determined, and it has been observed that the levels and prevalence of anti-VP4 and anti-VP4 peptide antibodies are higher in patients with T1D than in non-T1D subjects [79]. VP4 is buried in the capsid at cold temperatures, according to X-ray crystallography studies, although the binding of antibodies in plasma to VP4 argues that, at physiological temperatures, the viral conformation is different. It may be that a part of this protein is continuously exhibited by the viral particle or that the CV-B4 E2 capsid undergoes discontinuous conformational changes enabling the exposure of VP4 sequences [79].

The antibody-dependent increase of CV-B4 infection of circulating blood cells may play a role in the dissemination of virus within the organism and, as a consequence, in the pathogenesis of disease induced by the virus. Further work is required to determine whether it has such a role in the viral pathogenesis of T1D.
3.2.2. Lessons from in vivo investigations

Experiments in animals have shown that several enterovirus strains are pancreotropic. Enteroviruses generally infect the exocrine pancreas, but some strains preferentially infect islets. The molecular basis of this pancreotropism is unknown, but it may be explained in part by the variable expression of viral receptors in various tissues, as well as by other cellular elements regulating viral replication in the different cell types [41], such as the specific interaction of intracellular proteins with viral RNA [80]. An interaction between CV-B and trace elements in the pancreas has been described and is thought to play a role in triggering T1D. Indeed, CV-B3 leads to an increase in iron levels, and in transcripts of the divalent metal transporter-1 (DMT-1) and zinc transporter-5 (ZnT-5), together with a decrease in zinc levels and in transcripts of metallothionein-1 (MT-1) in the pancreas of BALB/c mice [81]. According to that study’s authors, such variations may reflect the early-stage development of pancreatitis and preclinical diabetic disease.

In addition, some strains initially non-diabetogenic, such as CV-B4 JVB or CV-B4 P15, but adapted beforehand to the murine pancreatic tissue, are able to cause diabetes in mice [82,83]. In fact, the six serotypes of CV-B can become diabetogenic after serial passages on the murine pancreas in vivo, and in vitro on cultured murine β cells. This change in phenotype may be, according to an investigation using CV-B5, linked to genetic modifications of capsid proteins (5 aa substitutions, including three at the level of capsid proteins) [84].

Several mechanisms may be involved in β-cell destruction, which can be modulated by the genetic background of the host as well as by the diabetogenicity and predilection of the viral strain [24,82]. We propose here a synthesis of the main mechanisms likely to play a role in β-cell destruction, based on the results of experimental investigations in vivo in animals, and supported by data and observations collected from studies carried out in humans (Fig. 1).

3.2.2.1. Beta-cell infection. Our hypothesis is that there is direct virus-induced β-cell lysis following infection (Fig. 1A). In CD-1 mice, CV-B4 E2 infects islet cells and induces autoimmuneity against β cells [85]. Insulitis and anti-islet autoantibodies are the result of the persistence of viral RNA in islets for several months, which appears to be critical for the development of diabetes in these animals. Infection of SJL/J mice with a CV-B4 JVB strain, adapted beforehand by successive passages on diabetes in these animals. Infection of SJL/J mice with a CV-

3.2.2.2. Molecular mimicry. The idea of molecular mimicry is based on the partial sequence homology between a viral antigen and a β-cell protein that constitutes a target autoantigen in T1D pathology. The immune response directed against the viral antigen can then lead, by cross-reactivity, to the production of cytotoxic T lymphocytes (Tc) [86] and/or antibodies that are able to attack pancreatic tissue and, thus, participate in the pathogenesis of T1D (Fig. 1B). This hypothesis has been investigated using serum and/or T cells from animals immunized against synthetic peptides of β-cell autoantigens, as well as serum and/or T cells from diabetic patients or individuals with CV-B infection.

The most well-known model involves viral protease 2C and GAD65 sharing a common amino-acid sequence known as ‘PEVREK’ [87]. Although the phenomenon of cross-reactivity has been described [88], this is not always found with either Tc lymphocytes [89] or antibodies [90]. Thus, the role of this homology in the pathogenesis of T1D remains to be proven. Cross-reactivity could be restricted to some MHC alleles such as HLA-DR1 (which does not a priori predispose to T1D), or HLA-DR3 in humans or I-A^d in mice (both predisposing to T1D), for effective presentation of the immunogenic epitope [87,91,92]. However, in CV-B4-infected mice with susceptible MHC alleles in which the P2C/GAD65 molecular mimicry hypothesis has been tested, there was no viral acceleration of diabetes [92].

There is homology between the capsid protein VP1 or the precursor VP0 and the tyrosine phosphatase IAR/IA-2 as well, with evidence of a humoral and cellular immune response directed against the C-terminal part of this phosphatase. The region has an identical sequence of 5 aa, followed by an analogy of 5 aa, with the preserved enterovirus VP1 motif (PALTAVETGA/HT) that is a strongly immunogenic epitope for either B and T cells in the immune responses induced by enterovirus infections. The results obtained in human sera collected during an enterovirus infection indicate that such infection can occasionally lead to a humoral response that reacts with IA-2/IAR [93].

Homology has also been found between the heat-shock protein HSP60, an autoantigen present in β cells, and regions of enterovirus capsid proteins—one in VP1 and the other in the precursor VP0. In the latter example, the molecular mimicry is associated with antibody-mediated cross-reactivity [93].

3.2.2.3. ‘Bystander activation’ or ‘innocent-bystander killing’. The infection can induce inflammation of the endocrine pancreas with production of nitric-oxide radicals, cytokines and other toxins, thereby destroying β cells indirectly [25]. β cells can be destroyed by preexisting autoreactive Tc lymphocytes as well. Indeed, mice that do not develop diabetes spontaneously, but overexpress a TCR transgene specific to an islet autoantigen, go on to develop diabetes 2–4 weeks after CV-B4 inoculation [94]. In this model, CV-B4 infection induces, after inflammation of the pancreas, activation of preexisting autoreactive T cells through a ‘bystander’ effect. Another study showed that, in NOD mice, CV-B4 can accelerate the spontaneous development of diabetes through this bystander effect, but only in the presence of a sufficient number of preexisting autoreactive T cells [95].
Fig. 1. Schematic representations of the mechanisms most likely to play a role in virus-induced β-cell destruction: A direct β-cell infection and lysis; B indirect autoimmune β-cell destruction through molecular mimicry; C indirect β-cell destruction through "bystander activation" or "innocent bystander killing"; D persistent infection and β-cell destruction: a) initiation of the β-cell destruction process by a 'predisposing' virus; and b) acceleration of β-cell destruction by 'precipitating' viruses.
Viral pathogenesis of T1D can thus ensue from a localized infection of β cells that provokes major inflammation with tissue destruction and release by the islets of sequestered antigens. Among the recruited cells, autoreactive T lymphocytes directed against these released antigens are stimulated and can then participate in disease development [96,97] (Fig. 1C). In this case, effector cells present in the pancreatic tissue are directed against autoantigens and are not reactive to viral antigens [98].

3.2.2.4. Activation of autoreactive T lymphocytes by superantigens?. The possible role of enterovirus superantigens cannot be excluded, as observations suggest that enteroviruses do contain such agents. Indeed, the preferential expression of TCR Vβ chains is observed, with a positive temporal correlation between enterovirus infections and increasing percentages of Vβ7 gene transcripts [99]. In another investigation, TCD4+ cells carrying TCR Vβ2, 7 and 8 were the most frequently activated following incubation with CV-B4 [100]. Such activation was significantly more frequent in recently diagnosed T1D patients than in controls.

Superantigens are known to bind directly to MHC class-II molecules outside of the conventional peptide-binding groove and to stimulate T cells regardless of antigen specificity via engagement with the TCR Vβ chain. Their interaction with MHC class-II molecules and subsequent T-cell activation does not require conventional antigen-processing and presentation by antigen-presenting cells (APC) and, thus, can be obtained with parafomaldehyde-fixed APC, for example. However, in this study, the capacity of CV-B4 lysates to induce T-cell proliferation required live and unfixed APC, suggesting that this selective expansion of TCD4+ cells carrying specific Vβ chains was the result of classical antigenic processing and presentation rather than superantigen activity. Other studies are needed to clarify the mechanism of the selective use of TCR Vβ and to investigate the possible existence of enterovirus superantigens.

3.2.2.5. Coxsackievirus B persistence. Enteroviruses and, in particular, CV-B are able to provoke persistent infections, a necessary property for triggering or aggravating chronic pathologies—in particular, T1D.

The idea of the possible involvement of CV-B persistence in β-cell destruction is supported by the detection by RT-PCR of CV-B4 E2 RNA for up to six months in CD-1 mouse islets with β-cell destruction and onset of diabetes [85], but also by the detection of enterovirus RNA by ISH in the pancreatic tissue of patients with recently or previously diagnosed T1D [45]. In another investigation, enterovirus VP1 and inclusions were observed in the pancreatic β cells of a multiorgan donor who accidently died nine months after diabetes onset [46].

The persistent infection of islets could induce destruction of β cells via several mechanisms (Fig. 1D). Continuous viral replication could damage and disrupt β-cell functions. Persistent infection could lead to prolonged activation of dendritic cells, with persistent presentation of viral antigens and autoantigens to T lymphocytes, and a consequent induction of an antiviral and autoimmune response that could provoke β-cell lysis [41]. Thus, enterovirus infections may initiate the process of β-cell destruction and also accelerate it later, due to reinfection that provokes a response of virus-specific Tc lymphocytes in the lymphoid tissue of the digestive tract (the primary site of replication). Antiviral T lymphocytes—whatever the enterovirus serotype that led to their proliferation—can, by cross-reactivity, participate in aggravation of the situation by destroying β cells persistently infected by another serotype [41]. Persistent viral replication, and the localized inflammatory reaction that accompanies it, may also lead to the recruitment in islets of autoreactive Tc lymphocytes, such as those directed against GAD65 and carrying the α4β7 integrin, a mucosal homing receptor found in islet infiltrates and in peripheral blood [41].

This hypothesis is supported by the results of experiments in mice infected by the lymphocytic choriomeningitis virus (LCMV) [101]. The authors of these tests support the idea of ‘viral déjà-vu’, in which innate immunity eliminates the (‘predisposing’) virus except at one site of the body, following which infections by the same or similar (‘precipitating’) viruses activate Tc lymphocytes directed against the initial viral epitope. In this way, T1D could be provoked by Tc lymphocytes directed against an epitope shared by both the ‘precipitating’ and ‘predisposing’ viruses (déjà-vu) persisting in the target tissues—in this case, pancreatic β cells. In support of this proposition and in the light of the results of epidemiological studies, CV-B4 or another enterovirus may well be candidates for acting as the ‘predisposing’ virus, with the same or other enteroviruses playing the role of ‘precipitating’ virus, in the pathogenesis of T1D.

The ability of CV-B4 strains to establish a persistent infection may be due to the substitution of amino acids located on the surface of the structural protein VP1. The infection can also impair the release of insulin; indeed, CV-B4-infected islets at 11–17 days post-infection failed to respond to high glucose levels [102].

3.3. Towards the primary prevention of T1D

If a causal relationship between enterovirus infections and T1D were to be finally established, it would be interesting to evaluate the feasibility of an enterovirus vaccine as a primary prevention of the disease. According to epidemiological data, nearly one third of all cases might be prevented by such a vaccine. The efficiency of vaccination against poliovirus is an encouraging example. However, it would be necessary to have more information concerning diabetogenic serotype distributions to guarantee vaccine efficiency and, to test vaccine safety, a greater understanding of the mechanisms of enterovirus-induced β-cell destruction [41].

4. Conclusion

Epidemiological studies strongly suggest an association between enterovirus infections, especially CV-B4 infections, and the emergence of T1D in genetically predisposed individuals. Such a possible association is supported by the detection of these viruses in the pancreas of patients with T1D.

Several pathogenic mechanisms of infection may participate in the β-cell destruction process, and we cannot exclude the pos-
sibility that the infection of tissues other than the pancreas—the thymus, for example, the central site of the development of self-tolerance—may have major consequences and, thus, a determining role in the autoimmune process. In addition, persistent enterovirus infection, as an initiating event, could be combined with reinfection by the same or other serotypes acting as accelerators.

Further studies are needed to clarify the relationship between enteroviruses—and especially CV-B4—and T1D pathogenesis before strategies to prevent the disease can be developed.

Conflicts of interest

No conflicts of interest.

Acknowledgments

The authors wish to thank the teams from the Laboratory of Virology/UPRES EA3610 (Pr D. Hober) and of the Laboratory of Virology/LR99ES27 (Prs Mahjoub Aouni and Jawhar Gharbi) and their collaborators (J. Weill, C. Stuckens, I. Cartigny, G.A. Loueil, G. Chauvet, P. Gronnier, I. Fajardy, C. Hober, M.C. Vantyghem, F. Brilot and V. Geenen). The present study was supported by EU FP6 Integrated Project EURO-THYMAIDE (Contract LSHB-CT-2003-503410), EU FP5 VIRDIAB Project supported by EU FP6 Integrated Project EURO-THYMAIDE (Contract QLK 2-CT-2001-01910), a grant from Nord-Pas-de-Calais Région (ArCir convention 2004/018), CHRU Lille, the ministère de l’Éducation nationale de la recherche et de la technologie, université de Lille-II, France, and ministère de la recherche scientifique, de la technologie et du développement des compétences, Tunisia. Hela Jaïdane was supported by the comité mixte de coopération universitaire franco-tunisien (CMCU 2004 N° 04/G0810) with scholarships from Égide Paris, Didier Hober was the ALFEDIAM/NOVARTIS 2002 and ALFEDIAM/ROCHE 2003 prize winner.

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Childhood Diabetes in Finland (DiMe) Study Group


