

Coxsackievirus Infections and NOD Mice

Relevant Models of Protection from, and Induction of, Type 1 Diabetes

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ABSTRACT: Human enteroviruses (HEVs) like the group B coxsackieviruses (CVBs) are prime candidates for infectious, environmental causes of human type 1 diabetes (T1D). Non-obese diabetic (NOD) female mice are well protected from T1D onset if inoculated with CVB when young. Older, prediabetic NOD mice can rapidly develop T1D following inoculation with CVB, mimicking clinical reports of disease-associated T1D onset. The ability to induce rapid T1D in NOD mice is linked to the rate of replication of the CVB strain in β cell cultures and pancreatic tissue, indicating that any CVB strain is potentially diabetogenic under the correct conditions. Rapid T1D onset is preceded by CVB replication in islet cells including β cells. Although CVB strains do not productively infect healthy islets of young mice, CVBs can replicate in healthy islets in the presence of murine IL-4. These models expand much of what is known or suspected regarding the etiologic role of HEVs in human T1D.

KEYWORDS: enterovirus; coxsackievirus; non-obese diabetic mice; NOD mice; type 1 diabetes; T1D; β cells; IL-4

INTRODUCTION

The group B coxsackieviruses (CVB1-6) are typical species B human enteroviruses (HEV-B).^{1,2} The CVBs have a demonstrated pancreatic biology in mice and in humans³⁻⁵ and are commonly implicated as causes of type 1 diabetes (T1D) (e.g., see Refs. 6-12). Other HEV-Bs (to date, all echoviruses¹³⁻¹⁸) have also been linked to T1D onset. Despite some studies that argue little or no association of CVBs with T1D onset,¹⁹⁻²² there

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Ann. N.Y. Acad. Sci. 1103: 143-151 (2007). © 2007 New York Academy of Sciences.
doi: 10.1196/annals.1394.009

is compelling evidence that HEVs play an etiologic role.^{23–25} However, the nature of this role(s) is not known; for example, if T1D does not occur when virus is isolable, HEV involvement could be difficult to prove.²⁶ Diverse HEVs replicate in cultured islets,^{27,28} indicating they may also have the capacity to infect and kill islets *in vivo*. On the other hand, *in vivo* studies of CVB-inoculated mice indicate that CVBs replicate only in healthy islet tissue of prediabetic non-obese diabetic (NOD) mice in which nearly every islet shows inflammation due to the autoimmune disease,^{29,30} but not in islets from healthy young mice in which insulinitis has yet to become evident.^{31,32} That CVBs do not productively replicate in healthy young islets *in vivo* indicates that isolation and/or culturing of islets must in some way alter islet cell gene expression in order that islets become permissive to HEV replication. At present, antiviral islet defenses *in vivo* are thought to center about the islet-specific expression of innate interferons.^{28,33,34}

That said, some HEVs may be unfairly tarred with this brush, having no role in T1D onset. There is no evidence, for example, that poliovirus (PV) infection, for example, is a player,³⁵ a very strong inference based on the ongoing, extensive natural experiment that has occurred throughout the 20th, and into the 21st, century with wild-type PV epidemics and worldwide application of PV vaccine strains. The only HEVs indicated to date as associated with T1D onset have been serotypes in the HEV-B species. Considering that HEVs circulate widely every year^{36–38} and the extreme rarity of epidemic or outbreak T1D events, it is apparent that HEV involvement in T1D etiology involves factors other than simple viral infection.

The NOD mouse is a relevant murine model for the study of human T1D,³⁹ although as with any model, there are limitations.⁴⁰ Because the CVBs replicate well in mice and because the CVBs are established as potential etiologic agents of T1D in humans, it is rational to use a highly T1D-prone host animal to test specific hypotheses regarding the role of CVBs in T1D etiology. (The CVBs do not replicate well or at all in rats, which unfortunately does not permit use of the BB rat model^{40,41}). Two basic models have been developed, which are described below.

CVBs AND PROTECTION FROM T1D ONSET IN NOD MICE

As CVBs are linked to T1D onset in humans, we initiated experiments using NOD mice to test the hypothesis that CVB infections would trigger T1D onset in advance of the naturally occurring autoimmune disease. Type 1 diabetes begins to manifest in female NOD mice at about 15–18 weeks of age, with 70–100% of mice becoming diabetic by 25–30 weeks of age.

Contrary to what might be expected if CVB infections initiated T1D, it was observed that inoculation of 4-week-old NOD mice with diverse CVB strains resulted in a significant level of protection from T1D onset compared

to age-matched, mock-infected controls.³¹ Despite being highly genetically predisposed to T1D, most CVB-infected NOD mice remained T1D free through 10 months of age. No evidence of islet infection was detected when islets were examined for the presence of virus *in situ*, consistent with failure to increase the rate or incidence of T1D. Criticism that nearly anything serves to lower T1D incidence in NOD mice⁴⁰ must also take into account that alone of near myriad treatments tried over the years,³⁹ the CVBs represent not only a commonly circulating human virus, but are considered to be naturally occurring triggering agents of the disease.

This is an apparent paradox: if CVBs indeed cause T1D, how does one explain lowered T1D incidences in CVB-inoculated NOD mice? Or is this merely a coincidence: are the CVBs no different than numerous other treatments³⁹ used to date? The indication that indeed, this is no coincidence came with the observation that some (but only a minority of) older (8 weeks of age) mice, inoculated with various CVB strains, developed T1D much earlier than naturally occurring T1D³¹ (it is important to note that those that did not develop T1D were well protected as they aged). A similar finding⁴² linked the phenomenon to the establishment of a critical mass of autoimmune inflammatory infiltration in the islets. Together, these two data points indicated that as NOD mice aged, and developed autoimmune anti-islet disease, mice could become more susceptible to CVB-induced T1D.

RAPID T1D INDUCTION BY CVB REPLICATION IN ISLETS OF PREDIABETIC, OLDER NOD MICE

Pursuing findings^{31,42} that somewhat older NOD mice became rapidly diabetic upon inoculation with CVB, we asked whether the key issue was the health state of the islets themselves. For this we used 12-week-old mice that show massive insulinitis in nearly every islet and are only a few weeks away from naturally beginning to develop T1D. When the mice were inoculated with various CVB strains, we observed two primary results.²⁹ CVB strains characterized as virulent (able to induce myocarditis, for example, in susceptible mice⁴) rapidly initiated T1D in mice, resulting in the majority of mice becoming diabetic within 1–2 weeks of virus exposure; but if mice were inoculated with poorly virulent strains, mice developed T1D either at the same rate as the mock-infected control mice or were delayed in developing T1D. When islets were examined within 2–4 days post inoculation, only islets in mice inoculated with virulent CVB strains showed productive virus replication, consistent with virus-mediated islet cell destruction immediately prior to T1D onset. These results cumulatively indicated that NOD mice could easily be tipped into the T1D abyss by an HEV infection, but only when their islets were already developing autoimmune insulinitis. Therefore, a link was established between the state of islet health and the outcome of a CVB infection:

healthy islets resist virus replication, while inflamed islets are permissive to virus infection.

The CVB are often termed “diabetogenic” because of observed connections between infections and T1D onset in humans. Indeed, CVB type 4 (CVB4) is often and specifically indicated as diabetogenic (perhaps historically due to the isolation of a CVB4 strain from a patient⁴³), while other CVBs (for example, CVB3) have been proposed to be unable to replicate in islets.⁴⁴ However, as there are no genetic grounds *a priori* to assume that such primary differences exist on the basis of CVB serotype, and understanding that replication rates differ between CVB strains of any serotype and that this appears to correlate with virulence phenotype expression,^{4,45–47} we asked whether inoculum dose per mouse affected the ability of two different CVB3 strains to induce T1D.³⁰ The results indicated that a virulent CVB3 strain, which replicates more rapidly to higher titer than a less virulent strain in pancreas and in an established murine β cell culture, induced the same incidence of T1D in NOD mice with up to 10^4 fewer infectious units inoculated per mouse than a poorly virulent strain: the latter required more than 10^6 TCID₅₀ of virus per mouse to achieve the same incidence of T1D that 50–500 TCID₅₀ of a virulent CVB3 strain achieved. Staining of islets in infected mice for insulin, glucagon, somatostatin, and pancreatic polypeptide in conjunction with viral protein, revealed that virus associates with all four primary islet cell types and even the poorly virulent strain associated with β cells. These results strongly indicate that any CVB strain can infect islets and initiate rapid T1D under the right conditions, data that moot speculations of diabetogenic CVB phenotypes associated with one but not other serotypes. All that would appear necessary and sufficient is either a rapid viral replication phenotype and/or sufficient inoculated dose, in association with a host with ongoing prediabetic insulinitis.

DISCUSSION

Somewhat akin to the Roman god of gates, Janus, who is depicted with a double-faced head, each looking in opposite directions, the CVBs have two opposite impacts upon the health outcome in NOD mice. When young mice are inoculated, CVB induces protection from T1D onset that varies as a function of the virus strain, while in older prediabetic mice, rapid T1D can occur following virus injection, again associated with virus strain. In each case, replication rate of the virus is key: in young mice, the immune system is altered most efficiently with virulent virus exposure, likely through an increased virus load, while in older mice, β cell destruction is seen as an acute islet infection, which results in T1D if sufficient numbers of β cells are destroyed prior to the rise of the antiviral adaptive immune response.

How does this relate to the human disease? High T1D incidences are a phenomenon of the 20th century and although described in antiquity,⁴⁸ T1D has

not been considered to be a sharply rising health problem until recently.⁴⁹⁻⁵¹ The CVBs, like most other HEVs, are transmitted primarily via a fecal oral route. This route of transmission was made easier with the poor/nonexistent hygiene and human waste-polluted water that was universal prior to the 20th century, an aspect of life much less common in the last 100 years. PV epidemics, also transmitted by the fecal oral route, become repeatedly annual in the 20th century with the advent of better hygiene, sewage treatment, and microbiologically safe water supplies.⁵²⁻⁵⁴ Instead of being exposed when young and protected by mothers' antibodies through passive transfer in breast milk, people were growing up without establishing protective PV immunity by natural exposure in water supplies and poor hygiene, creating renewing populations of susceptible individuals capable of supporting epidemic spread of PV-induced poliomyelitis. The CVBs are similar to PVs in this regard; we propose that exposure to CVBs (and many other HEVs) early in life became less common as the 20th century wore on. Prior widespread HEV exposure early in life had formerly suppressed autoimmune T1D incidences through an impact on the developing immune system. However, as societal hygiene improved and children were less frequently exposed to HEVs, T1D incidences were observed to be increasing. Analogous to the experimental NOD mouse system, HEV exposure may well suppress the development of a population of anti-islet autoimmune lymphocytes. If begun when the child is very young, such protection could last a lifetime with repeated, beneficial boosts from random, naturally acquired HEV infections. But with fewer exposures to HEVs, people have a less robust immune experience of HEV, which thereby may expose them to increased risk of T1D onset, especially if genetically predisposed to T1D.⁵⁵ Consistent with this hypothesis, well-developed and urban societies tend to higher T1D incidences than do others.⁵⁶⁻⁶⁴ Stochastic HEV infections under the right circumstances might also initiate T1D through destruction of β cell mass in some individuals with developing insulinitis.

ACKNOWLEDGMENTS

This work was supported in part by funding from the Juvenile Diabetes Research Foundation, the American Diabetes Association, the Edna Ittner Pediatric Research Foundation, the Multiple Sclerosis Society, and the National Institutes of Health.

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