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REVIEW

Enteroviruses, type 1 diabetes and hygiene: a complex relationship

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SUMMARY

Type 1 diabetes (T1D) is an autoimmune disease in which the immune system mounts an attack on the host's insulin-producing β cells. Because most cases of T1D cannot be attributed only to individual genetics, it is strongly inferred that there is a significant environmental contribution, such as infection, impacting disease development. The human enteroviruses (HEV) are common picornaviruses often implicated as triggers of human T1D, although precisely which of the numerous HEV may be involved in human T1D development is unknown. Experiments using non-obese diabetic (NOD) mice, commonly used to model T1D, show that induction of T1D by HEV infection in NOD mice is a multifactorial process involving both the virus and the host. Interestingly, results demonstrate that HEV infection of NOD mice can also induce long-term protection from T1D under certain conditions, suggesting that a similar mechanism may occur in humans. Based upon both experimental animal and observational human studies, we postulate that HEV have a dual role in T1D development and can either cause or prevent autoimmune disease. Whichever outcome occurs depends upon multiple variables in the host-virus equation, many of which can be deduced from results obtained from NOD mouse studies. We propose that the background to the sharply rising T1D incidences observed in the 20th century correlates with increased levels of hygiene in human societies. Viewing T1D in this perspective suggests that potential preventative options could be developed. Copyright © 2010 John Wiley & Sons, Ltd.

A ROLE FOR HUMAN ENTEROVIRUSES IN CAUSING TYPE 1 DIABETES

Environmental factors (e.g., infections; [1,2]) are proposed to explain T1D aetiology that cannot be ascribed solely to host-driven pathogenic autoimmunity [3–8]. Epidemiological and serological associations have long implicated human enteroviruses (HEV) in T1D aetiology [9–12]. The pancreas is not easily biopsied and so, directly linking HEV presence with T1D by live virus isolation from the pancreas is difficult. Nonetheless, a few HEV have been isolated from—and more have been associated with—cases of recent onset T1D [13–23]. While the group B coxsackieviruses (CVB1-6) are the

most commonly named viral triggers of T1D (e.g., [24,25,19,26,27,11,28]), numerous non-CVB HEV have been linked to T1D onset [13,15–17,20,22]. It is noteworthy that every HEV linked to date with T1D induction, belongs to the species B HEV (HEV-B; [13–18,29,30,19–23]). No HEV belonging to species A, C or D have been implicated as inducers of T1D. But because HEV-B circulate regularly in human populations, the importance of the association between HEV-B infection and T1D onset may be questioned. HEV-B serotypes are always among the top ten most common HEV that circulate annually [31–33] and represent about half of the known HEV serotypes [34]. Consequently, the observed relationship between HEV-B and T1D onset may be biased simply based on these two facts. The relatively few identifications of HEV in close association with T1D (identification of the

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virus from within pancreatic or islet tissue) requires more instances of molecular detection and sequence analysis to clearly implicate the HEV-B as the only HEV species responsible for triggering HEV-induced T1D. For the present, however, this apparent unique participation of the HEV-B in T1D onset is a well-supported working hypothesis.

Some HEV-B have been more commonly associated as inducers of T1D than others. Of the six closely-related CVB serotypes (CVB1-6; [34]), CVB type 4 (CVB4) is commonly termed 'diabetogenic' [35–40], although the rationale for this label is unclear. There is no evidence indicating that CVB4 strains are more involved in T1D onset than other CVB serotypes. Although CVB virulence phenotypes for myocarditis and other diseases have been genetically mapped [41], the genetics which control a diabetogenic phenotype have not been described despite the availability of sequence information and molecularly cloned cDNA copies of CVB4 genomes [42–44]. The focus on CVB4 as an inducer of T1D intensified with a report of a CVB4 isolated from a diabetic patient in 1979 [23]. This rare event—isolation of an HEV from the pancreas itself—implicated CVB4 in T1D aetiology, a connection made more convincing by previous studies in which CVB4 was shown able to replicate in mouse islets and induce T1D-like disease [45,9,46,47]. More recently, HEV capsid protein was detected within islets of diabetics [18]. These results were confirmed by others in a different patient set [48]. Setting this same study [18] apart was the reported isolation and sequence analysis of CVB4 from a patient (termed the 'Tuscany' strain). However, analysis of the sequence data indicated the Tuscany strain (isolated in 2007) differed by less than 1% at both the nucleotide and amino acid levels from the prototype CVB4 strain (JVB Benschoten) which was isolated—and last circulated—in 1951. Because of the rapid HEV evolution rate [49,50] and an unfathomable number of genome variations potentially available as defined by the concept of sequence space [51,52], it is extremely improbable that a CVB strain first isolated in the early 1950s, has remained in circulation with only minor genetic divergence since that time [34]. Yet despite this error, this basic study design represents a prime example of the sort of investigation needed to define which of the numerous HEV-B candidates are the most aetiologically important to T1D.

That not every HEV has a role in T1D aetiology is suggested by the polioviruses (PV), a typical and well-researched species C HEV [53]. Although annual PV epidemics became commonplace around the turn of the 20th century [54,55], neither T1D outbreaks nor increasing T1D incidences were observed as a function of annual polio epidemics, indicating no link between wild-type PV infection and T1D. T1D incidences began to increase from very low levels in the mid-20th century [28] while polio epidemics had been rife for at least 50 years. If widespread PV exposure suppressed T1D development, lowered T1D incidences should have been evident as the population became immune to PV due to naturally-occurring epidemics and the clinically-administered, global anti-PV vaccination effort. This was not observed. Efforts to link PV with T1D incidence have been negative [56,57].

It is evident that HEV populations and their infectious outcomes also vary widely [58–62,41]. Strain variation can determine whether CVB is detected in pancreatic islets *in vivo* [63] and can impact β cell function following islet infections *in vitro* [64]. Asymptomatic HEV infections are the most common outcome in humans [65]; these will not easily be associated with T1D onset. This is consistent with the observation that HEV-associated T1D 'outbreaks' don't occur despite regular HEV circulations. A similar conclusion could be drawn from the possibility that truly diabetogenic HEV strains are quite rare.

The number of HEV-linked T1D cases in individuals with T1D risk factors is unknown, although ongoing clinical studies such as TEDDY (The Environmental Determinants of Diabetes in the Young; [66]) should assist scientists in determining this key parameter. The annual number of HEV infections in the United States alone are estimated at 5–10 million [65] while the annual T1D incidence is approximately 16–18/100 000 in the United States [40]. Therefore, with significantly more HEV infections annually than new cases of T1D in the population, the inference is that HEV-induced T1D is not a common outcome of HEV infection as it is for other HEV infections that result in diverse diseases such as, e.g., aseptic meningitis, myocarditis or polio [67,65,68]. It is also possible that T1D may manifest long after the initiating HEV infection [11]; in such cases, proving HEV participation in disease

development would be expected to be rare or non-existent (e.g., [69]). The paucity of epidemic/outbreak T1D [70] despite annually circulating HEV [31–33,71], indicates that HEV-induced T1D is either itself rare and/or involves other factors. On the other hand, recent work [18,48] detecting HEV protein in diabetic pancreas samples indicates that sufficient viral RNA may persist in diabetic pancreata to permit amplification and identification of specific viruses.

LESSONS FROM MICE

Both CVB strain and dose are key to protection from, or rapid initiation of, T1D [72] in the NOD mouse. A series of experiments examined the impact of CVB strain and dosage differences on disease development in this animal model [63,72] in which older, prediabetic NOD mice were inoculated with two different well-characterised CVB3 strains. A poorly virulent and slowly replicating strain (CVB3/GA; [73]) slowed T1D initiation and lowered its incidence in older, prediabetic NOD mice relative to controls when inoculated at 5×10^5 $TCID_{50}$ per mouse. However, by increasing the virus dose inoculated into older NOD mice, earlier than expected T1D onset—not protection—was triggered [72]. Conversely, a rapidly replicating strain (CVB3/28; [74]) triggered T1D onset in most older NOD mice within a week of inoculation with 5×10^5 $TCID_{50}$ but by lowering the dose of this virus to 50 $TCID_{50}$ per mouse, fewer became diabetic. The dose of virus administered correlated with the virus titer in the pancreas (K. Kono, S. Tracy, unpublished data). These results indicate that infecting dose and resultant host virus load, are a key determinants of T1D initiation and that virus load is affected by the replication rate of the virus strain.

Host factors define whether CVB induces, or protects from (see below), T1D in the NOD mouse. A key difference between old and young NOD mice is the extent of insulitis (inflammation of the islets). Naturally-occurring autoimmune insulitis becomes more severe as NOD mice age. The ability of CVB to protect NOD mice from T1D onset when virus is inoculated at a young age (when islets are still healthy; [74]) is also observed in older mice [63], although fewer mice are protected due to the advanced stage of deleterious insulitis in older mice. Insulitis must be present in the NOD mouse at the time of viral infection

for rapid T1D onset to be triggered by the virus [63]: the greater the extent of insulitis in the NOD mice, the faster the rate with which mice become diabetic as a function of virus infection. This is a crucial point: inoculating NOD mice with CVB does not trigger T1D when insulitis is absent [74]. The virus exploits a changed host environment (insulitis) to productively replicate in islets and trigger T1D. Because both the dose and rate with which CVB strains replicate define a virus' ability to initiate rapid T1D in prediabetic NOD mice, it implies that any CVB strain, regardless of serotype, could trigger T1D in the older (prediabetic) NOD mouse under the right conditions. The impact of CVB on T1D development in the NOD mouse is determined by a combination of the dose inoculated into the mouse, the virus replication rate, and the extent of pre-existing insulitis. Therefore, based on available data, we postulate that HEV 'diabetogenicity' is not merely a characteristic inherent in the virus itself but rather, a complex but definable function of these variables. By inference, this same situation may also apply in humans. That is, an HEV-B infection may be unable to trigger T1D in a human with healthy islets and only with pre-existing insulitis in place, could the virus infection initiate rapid T1D onset. As naturally-occurring infectious HEV doses vary widely but are generally low, a 'diabetogenic' HEV-B strain in humans can then be defined as a rapidly replicating virus strain which can quickly produces a high virus load in the islets, triggering T1D onset even at a low dose provided that insulitis is present.

Individual human genetics determine whether a host is predisposed to T1D development, although genetics alone account for less than 50% of T1D cases [3,75,5,6]. Recent results from genome-wide association studies [76–79] may have identified a new candidate gene involved in T1D development. These studies identified a strong association between T1D and single nucleotide polymorphisms (SNPs) in the region encoding IFIH1 (interferon induced with helicase C domain 1), a protein postulated to be a helicase with the capacity to recognise double-stranded viral RNA [80]. Enteroviral replication in host cells produce both positive and negative strand RNA which can exist as (full or partially) double-stranded RNA [81,82]. The mechanism by which specific IFIH1 mutations would modulate autoimmune disease

development is as yet undefined, although mice with specific IFIH1 mutations that decrease IFIH1 levels, also have decreased proinflammatory cytokine levels [83]. The possibility exists that a similar downregulation of the inflammatory response may also occur in humans. Were this the case, individuals with decreased IFIH1 function might have decreased T1D risk, while normal IFIH1 function might predispose individuals to a higher risk of disease development, perhaps due to a decreased inflammatory response to an HEV infection or by slowing the development of autoimmune insulitis. Perhaps counterintuitively, it suggests that such mutations could be protective, not deleterious. This raises an intriguing possibility: might such mutations help to explain why certain populations [84,85] are at higher risk for T1D onset than others? One could postulate that 'normal' levels of proinflammatory mediators produced in a host with normal IFIH1 levels, in response to HEV infections contribute to damaging the target tissue (in this case, pancreatic islets). Such a mechanism could operate together with genetically-driven insulitis as well. Further exploration of this and possible other links between T1D and HEV infections are needed.

A ROLE FOR HEV IN PREVENTING HUMAN T1D

While the utility of the NOD mouse model of T1D for discovering treatments or agents that can also suppress T1D in humans has been widely discussed and sometimes dismissed [86–88], it is the best animal model for studying a relevant HEV infection (the CVB) and the impact of these viruses upon a naturally-occurring, autoimmune disease influenced by host genetics. The CVB, nearly alone of the HEV, replicate well in mice because of the close similarity between the murine and human coxsackie-adenovirus receptor (CAR) proteins [89]. We first considered the possibility that CVB and potentially other HEV, could protect the host from developing T1D following a series of experiments in NOD mice [74]. Testing the hypothesis that CVB infection can rapidly induce T1D in a genetically-prone host using young (4–6 week old) NOD mice that have yet to develop autoimmune insulitis, we were surprised to observe T1D development in CVB-inoculated mice was significantly lower than in control mice; this protection from T1D was maintained

for at least 10 months of age [74]. Of the various diverse viruses used to study T1D in the NOD mouse model (arenaviruses [90]; coronaviruses [91]; cardioviruses [92]; arteriviruses [93]), *only* the CVB have been implicated as causative agents of T1D in *humans*, while arena, corona-, cardio- and arterivirus infections in humans are not implicated as modulators of T1D incidence. The observation that the CVB not only failed to trigger rapid onset T1D in mice without noticeable autoimmune insulitis but provided long-term protection from T1D in these genetically prone NOD mice, suggested the possibility that there may be correlates with the natural history of HEV infections in humans. Importantly, the potential mechanism of an HEV-induced anti-T1D protective effect in humans has been given strong support by recent work [94] that has shown an increase in protective regulatory T cells is stimulated by CVB inoculation of NOD mice. This finding has direct correlations with ongoing human treatment trials to reverse T1D onset [95–97]. Cumulatively, these observations indicate that the role of HEV in preventing T1D merits continued examination in detail.

HUMAN ECOLOGY, HYGIENE, HEV AND T1D INCIDENCE

Relatively little consideration has been given to the potentially pivotal relationship between rising T1D incidences and significant changes in human living standards (or social environments) in the past 100 years. Prior to the 20th century, sewage treatment was uncommon, open sewers routine, the availability of microbiologically clean water to wash after defecation rare, and the need to place privies well away from sources of drinking water a poorly understood concept. Because HEV are commonly transmitted through a fecal-oral pathway, it is likely that immunity to numerous HEV serotypes—at least those serotypes circulating in the local community—was common in the past. As hygiene standards have improved, routine and repeated HEV exposure early in life have become less common (e.g., [98]).

Strachan proposed the 'hygiene hypothesis' to account for increased rates of asthma in highly developed societies [99], suggesting that exposure to a large number of infections early in life appropriately shapes the adaptive immune system. Failure of this process can result in autoimmunity or

inappropriate immune responses to environmental triggers. Although aspects of the hygiene hypothesis as originally stated and how it relates to T1D aetiology, are controversial, the fact that human societies are living in increasingly cleaner (from a microbiological standpoint) environments than before, is not in dispute. T1D incidence is increasing worldwide [85] particularly in highly-developed societies. The incidence of T1D in Finland was 2–3 cases/100 000 in the early 1950s but is currently much higher. During the past 50 years, HEV exposure rates have fallen in Finland [28]. Was the rarity of T1D *then* linked to early and regular exposures to HEV through poor hygiene? Could the incidence be rising *now* due to fewer *early* HEV exposures? On the face of it, these questions are non-intuitive: if HEV cause T1D, why are T1D incidences rising when HEV exposure rates are falling?

Examination of pertinent data suggest compelling answers. Lessons learned from understanding how increased hygiene levels were linked to annual epidemics of PV-induced poliomyelitis in the 20th century are relevant to this hypothesis [100,55]. While substantial data on other non-PV HEV circulations and diseases are rare prior to the mid-20th century, we can presume that our understanding of the mechanics involved in PV circulations and disease epidemics, pertain to other HEV. Not just polio epidemics, but *annual* polio epidemics [101] were linked to decreased PV exposure due to increased hygiene norms, better sewage containment and treatment and microbiologically clean(er) water supplies [102,103]. Because of improved water quality, children were less frequently exposed (and immune) to PV, thus placing more people at risk for polio at a later age whenever PV infection occurred. We hypothesise that a similar story has been unfolding for at least the last 60 years regarding T1D. With more people practicing improved hygiene, fewer are exposed as often to HEV, resulting in more humans who have not been exposed to HEV (other than PV through vaccination) at an early age. An HEV infection in an individual who is genetically predisposed to developing T1D with developing insulitis, might therefore destroy sufficient numbers of β cells to trigger T1D onset.

The age when one is first exposed to an HEV infection may be critical in determining how the virus(es) interacts with the host immune system as shown recently in a small animal model [94] and ultimately, whether T1D develops. HEV infections in the first year of life correlate with protection from T1D onset [104,105]. A study of Finns and Estonians during the first year of life found that HEV infections inversely correlated with T1D risk: while the Estonian cohort had a higher incidence of HEV infections than the Finns, T1D incidence in Estonia was 5 times lower than in Finland (7 versus 36/100 000) [106]. First-born children are at greater T1D risk than younger siblings [107] and diabetic children tend to have fewer siblings (fewer infections?) than non-diabetic children [57,108]. Well-developed countries generally have higher T1D incidences (from ~8–10 to 36 cases/100 000) than less developed countries (between 0–8 cases/100 000; [109,84,85,110,111]). Cultural conditions (more people in the home, higher population density] also tend to correlate with lower T1D risk [112,109,113]). The consistency of these observations relative to the primary tenet of the hygiene hypothesis and how it may apply to T1D onset, are striking, have been remarked upon by others [36,114], and provide compelling arguments to support a role for our environment in the HEV-linked aetiology of T1D.

A MODEL FOR HEV INVOLVEMENT IN HUMAN T1D

Individual human genetics alone do not account for the majority of T1D cases and a growing body of data suggest that HEV infections are a primary environmental influence in T1D aetiology. Based upon experimental and clinical observations and what we infer from our human history, we suggest a testable model for the role of HEV in T1D. At least 5 identified factors must align [115,116] in order for HEV to initiate T1D: (i) ongoing autoimmune insulitis; (ii) an infection by an HEV-B strain; (iii) a relatively rare infectious encounter with a rapidly replicating (virulent; [41]) virus strain; (iv) an infectious dose sufficient to quickly destroy significant numbers of insulin-producing β cells before a complete adaptive immune response can develop and (v) no protective immunity to the infecting HEV-B type. This is outlined in Figure 1.

The role of HEV-B infections in the aetiology of T1D in humans is proposed to be of two parts: either protective or deleterious. We hypothesise that HEV cannot replicate in healthy islets based
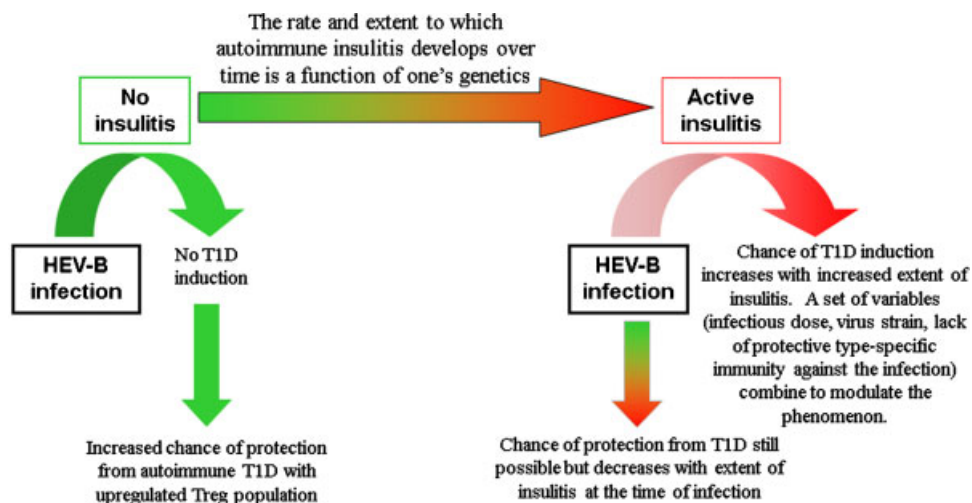
Figure 1. Enterovirus infections may play two roles with respect to human T1D. Whether protection from, or induction of, T1D occurs, depends upon the presence of active insulitis in the individual at the time of infection. Unable to replicate productively in islets with no insulitis, the virus does not induce T1D. However, the infection still can induce a population of protective regulatory T cells (Treg) that protect from autoimmune T1D onset. With insulitis in place, whether or not HEV rapidly induces T1D, depends upon the extent of insulitis, the dose and strain of the infecting virus, and whether the host has protective type-specific antiviral immunity

on observations that CVB do not replicate productively in healthy, non-inflamed pancreatic islets of NOD mice but do impart protection from developing T1D due to the host's own autoimmune predilections. If genetically predisposed to T1D but as yet without (significant) insulitis, an HEV-B infection could impart the benefit of provoking a protective Treg population that holds the development of pathogenic autoimmune islet-specific T cells in check, thereby diminishing the individual's chance of developing autoimmune T1D. Over the lifetime of the individual, other HEV-B exposures could help boost this protection. However, in the absence of a protective population of Tregs, the extent of insulitis continues to grow and deplete β cells with the outcome that there is a higher risk of developing autoimmune T1D. An HEV-B infection could kill sufficient β cells in such an individual before the rise of a protective antiviral type-specific immune response, causing rapid, virus-induced T1D onset (that is, T1D prior to the time the autoimmune response might have accomplished this on its own). The relative odds of developing T1D in this individual versus being protected from developing autoimmune T1D, is a function of the virus and infectious dose, as well as the extent of insulitis at time of infection. A primary requirement of this mechanism is pre-existing host-driven, anti-islet autoimmunity. Therefore,

depending upon the environment in which the virus finds itself upon infection, it can either induce protection against autoimmune T1D onset or, with insulitis present, induce T1D onset as a function of its replication in, and damage to, the islets.

The 'fertile field hypothesis', proposed to account for numerous observations in various autoimmune diseases including T1D [117], suggests that virus infections make the tissue a 'fertile ground' for autoaggressive lymphocytes to invade, expand and cause the specific disease. Here, we suggest that in the case of HEV-induced T1D, the converse scenario holds. Pre-existing, host-driven autoimmune insulitis is a necessary first step before a CVB infection can take hold and damage islets in NOD mice. For T1D, it is the host's own autoimmunity that prepares the new environment (inflamed islets) in which the virus can now replicate productively and induce damage. We propose that HEV either aids the host by preventing autoimmune T1D onset or colludes with the host's pathogenic autoimmune disease, by taking advantage of an abnormal islet environment to trigger virus-induced T1D. In either case, the virus simply uses the 'field' it encounters to induce quite different outcomes.

The requirement for a close temporal association of all of these characterised factors (and there may be more) helps to explain why most individuals

never develop T1D despite varying genetic predispositions to T1D. Assuming that the relative rarity of virulent HEV strains, random infectious dose size and the chance of having ongoing insulitis at the time of infection have not significantly changed over at least recent human history, an attention to the benefits of good hygiene and the resultant impact it has had upon HEV exposure chances is the primary variable in this hypothetical equation which has changed in many societies. Because this significant change in human habits has decreased the probability of individuals encountering protective HEV infections early in life, the chance for virus-triggered T1D in an individual with insulitis caused by a chance HEV-B infection, is now higher than before.

CONCLUDING REMARKS

The issue of the role(s) of HEV in T1D development remains an open and vigorous debate [36]. Based on a convincing collection of clinical and experimental data, it is likely that the HEV-B will be found at the top of any list of suspect environmental agents to be compiled from clinical surveys. Viewing the role of HEV in the processes that lead to protection from or causation of T1D in humans from a historical perspective that acknowledges the roles of personal and social hygiene, we gain a unique understanding of how this one group of viruses may have quite diverse impacts on the development of T1D. Future work need not, in our opinion, focus on viral genetics to understand how HEV can protect from T1D, but might more productively ask if the host protective immune mechanism can be initiated by viral antigens alone or if host antigenic stimuli are also required in concert. Whether specific HEV epitopes are sufficient, or whether replicating antigen is required to stimulate protection from T1D, remains to be determined but will impact how a potentially protective vaccine could be devised. Understanding these requirements will help to solve the problem of generating protective immunity to both the pathogenic autoimmune T1D process itself as well as induction of T1D by HEV.

ACKNOWLEDGEMENTS

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