The mucosal interface between 'self' and 'non-self' determines the impact of environment on autoimmune diabetes

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Abbreviations:

IEC, intestinal epithelial cells; MHC, major histocompatibility complex; TLA, thymus leukaemia antigen; IEL, intraepithelial lymphocytes; TCR, T-cell receptor; NOD, non-obese diabetic; VNTR, variable number of tandem repeats; APC, antigen presenting cells; Tr, regulatory T cells; NKT, natural killer T; LCMV, lymphocytic choriomeningitis virus; NTX, neonatal thymectomy; OVA, ovalbumin; BB, bio-breeding; HLA, human leukocyte antigen; BSA, bovine serum albumin; ; ICA, islet cell antibodies; IAA, insulin autoantibodies; GAD, glutamic acid decarboxylase; IA2, tyrosine phosphatase islet antigen 2; NADH, ; VP, virus protein

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Introduction - the mucosa

The primary role of the immune system is defence against pathogens, within the context of maintaining homeostasis between 'self' and 'non-self'. The mucosal surfaces, especially of the gastrointestinal, naso-respiratory and genitourinary tracts represent critical physical and functional interfaces between internal 'self' and external 'non-self'. At these sites, the mucosal immune system plays a seminal role in maintaining the delicate balance between defence against pathogens (immunity) and accommodation of non-pathogenic resident bacteria and a host of potentially immunogenic dietary or inhaled proteins (mucosal tolerance). Given this gatekeeper function of the mucosa at the interface between 'self' and 'non-self', the role of environmental factors in predisposing to or triggering autoimmune diabetes must be considered within the context of mucosal physiology.

Internal 'self' and external 'non-self' are separated by a single layer of epithelial cells covering mucosal surfaces. In addition to being a physical barrier, mucosal epithelial cells are actively involved in mucosal immunity. Intestinal epithelial cells (IEC) constitutively express major histocompatability complex (MHC) class II [1] as well as the non-classical MHC class I-like molecules CD1d [2] and thymus leukaemia antigen (TLA) [3], and interact directly with intraepithelial lymphocytes (IEL) via cadherin E - αEβ7 integrin, respectively [4]. In vitro, IEC can process and present dietary antigen to primed CD4 T cells [5]. Although they do not form a discrete, organised lymphoid tissue, IEL are distributed between and at the basement of IEC in numbers equivalent to that of all T cells present in the spleen and lymph nodes [6]. IEL are the first lymphoid cells to contact external 'non-self', are constitutively cytotoxic, and have a primary role in mucosal immune responses. In mice, half the IEL express Thy-1, αβ T-cell receptor (TCR) and CD8αβ heterodimer; the others express γδ (~40%) or αβ TCR (~10%) and CD8αα-

homodimer, and are unique in having an extrathymic ontogeny (reviewed in [7]). In humans, γδ T cells constitute a lesser proportion of small intestinal IEL, although this increases in the large intestine. In addition to IEL, the mucosal immune system comprises the loosely organised lamina propria lymphocytes located directly beneath the epithelium, lymphoid nodules called Peyer's patches and mesenteric lymph nodes that interface with the systemic immune system.

Mucosal immunity is required to prevent autoimmune diabetes

Paradoxically, the mucosa would be a place of great danger, if it wasn't so dirty. By dirty we mean colonisation by bacteria, which is necessary for the development of the mucosal immune system [8]. The critical role of normal mucosae in regulating autoimmunity is aptly illustrated by the effects of germ-free versus dirty environments on diabetes incidence in the autoimmune non-obese diabetic (NOD) mouse, the model of human type 1 diabetes. The incidence of spontaneous diabetes in NOD mice differs greatly in colonies around the world and appears to be inversely correlated with exposure to microbial infection [9]. The high incidence of diabetes in NOD mice housed under specific pathogen-free conditions is reduced by conventional conditions of housing and feeding [10]. Under such conventional 'dirty' conditions, bacterial colonisation of the intestine is accompanied by an increase in the number of IEL, particularly CD8αα αβ T cells [11], and by maturation of mucosal immune function [12]. Without natural bacterial colonisation and consequent development of mucosal immunity, animals have defective systemic immune tolerance and develop autoimmune disease.

Mechanisms of immune tolerance

Discrimination between 'self' and 'non-self' is an essential property of the immune system. For T cells, this is first achieved during their development in the thymus by clonal deletion of cells that recognise self-peptide presented by MHC molecules. However, this editing mechanism is imperfect. T cells with low avidity for self, or with specificity for self-antigens not expressed in the thymus, can escape this central tolerance mechanism and mature to be potentially autoreactive. Indeed, a diverse T-cell repertoire and therefore an effective immune system requires that 'self' and 'non-self' overlap at this level. The price paid, however, is potential for autoimmune disease – if peripheral regulatory mechanisms fail.

(Pro)insulin is the only β-cell specific autoantigen in type 1 diabetes in humans and in NOD mice. In the human thymus decreased expression of proinsulin mRNA is associated with a risk allele for type 1 diabetes that maps to a VNTR regulatory region 5' of the insulin gene [13]. It is likely that reduced expression of proinsulin epitopes in the thymus leads to failure of central tolerance of proinsulin-reactive T cells and therefore susceptibility to autoimmune-mediated destruction of insulin-producing β cells. Prevention of diabetes in the NOD mouse by expression of proinsulin as a transgene in antigen presenting cells (APC) [14] lends support to this view.

Lymphocytes that recognise islet antigens are found in some healthy individuals, as well as at higher frequency in individuals with autoimmune diabetes, implying that their activation in the periphery is normally regulated. Peripheral tolerance of 'self' has several explanations. First, it may be due simply to passive 'ignorance', whereby self-antigens remain cryptic or sequestered or their presentation by MHC molecules remains below a critical threshold for T-cell activation. This can be overcome by tissue damage or local 'danger' resulting in release and/or enhanced presentation of self-antigens, usually with upregulation of co-stimulatory molecules (CD80,

CD86 and CD40) on APC. Non-recognition of self may also be overcome by immune cross-reactivity with non-self (molecular mimicry). Second, depending on the quality or quantity of antigen presentation, responding T cells may be anergised or deleted by apoptosis. Third, tolerance can be active and mediated by regulatory T cells whose secreted anti-inflammatory products (eg IL-4, TGF-β, IL-10) or competition for antigen presentation can antagonise autoreactive T cells.

Peripheral tolerance mechanisms are especially important because they are potentially inducible for the prevention and treatment of autoimmune disease. While immune response genes such as those within the MHC determine the susceptibility to and the outcome of autoreactivity, autoimmune disease susceptibility is not simply 'deterministic'. Family and twin studies in type 1 diabetes (reviewed in [15]) reveal that the genetic component contributes less than half of the lifetime risk of the disease; in discordant monozygotic twins the probability of the second twin developing diabetes is no greater than 36% after 20 years follow-up [16]. Thus, susceptibility to type 1 diabetes must be shaped by environmental factors that impinge on peripheral tolerance mechanisms.

Mucosal immunity is a significant generator of peripheral tolerance. Feeding a soluble protein antigen suppresses subsequent systemic priming to the antigen ('oral tolerance'); a similar phenomenon occurs after antigen delivery to the naso-respiratory tract and other mucosae (reviewed in [17, 18]). Mucosal tolerance is attributed to several mechanisms (Figure 1) that are not mutually exclusive but may overlap, one or other predominating depending on conditions such as antigen dose, physical form and route of delivery. Cells that survive antigen-induced activation and apoptosis may be anergic and/or exhibit properties of regulatory T cells (Tr) (reviewed in [18]). Tr are triggered in an antigen-specific manner but exert antigen non-specific

'bystander suppression' in response to recognition of specific antigen locally in the tissues or draining lymph nodes. Bystander suppression due to anti-inflammatory cytokines (IL-4, TGF-β, IL-10) can act as a brake on autoimmunity to multiple self antigens often seen in human autoimmune disease.

Regulatory T cells

A balance between immune pathogenic and regulatory mechanisms in autoimmune diabetes was first suggested by the effect of cyclophosphamide [19] or of irradiation of recipients of diabetogenic T cells [20] to accelerate diabetes onset in young NOD mice. A regulatory role for CD4 T cells in young NOD mice was implied by the finding that they prevented diabetes when co-transferred with spleen cells from older diabetic mice to young, irradiated mice [21, 22]. Anti-diabetogenic T-cell lines and clones have been isolated from the islets of pre-diabetic NOD mice (eg Chosich & Harrison [23]). Lymphocytic infiltration of islets (insulitis) is present for months (mice) to years (humans) before the near-total destruction of β cells leads to diabetes. This protracted pre-clinical stage is consistent with immunoregulatory mechanisms that hold β-cell destruction in check.

Specific subsets of regulatory CD4 T cells [24-31], CD8 γδ cells [32] induced by mucosal administration of islet autoantigen protein or peptide, as well as CD4 NKT cells [33], have been shown to prevent diabetes in NOD mice. The potential importance of the mucosa in generating Tr that protect against diabetogenic environmental agents is illustrated by an elegant study of Homann *et al* [34] in which IL-4 and IL-10-secreting CD4 T cells generated by oral insulin prevented diabetes triggered by lymphocytic choriomeningitis virus (LCMV) in mice expressing an LCMV antigen transgenically in β cells.

gd **IEL – first line of mucosal defence**

γδ TCR are present on < 1% of peripheral T cells, compared to ~40% and ~15% of IEL in rodents and humans, respectively. Their selective localisation and relative abundance in the mucosa suggests a key role in the regulation of responses to environmental antigens. Several lines of evidence indicate that γδ IEL regulate autoimmune and other inflammatory disorders. First, γδ T cells have been demonstrated to suppress experimental autoimmune myocarditis [35], airway hyperreactivity to inhaled antigen [36], Lyme arthritis [37], contact sensitivity [38], type 1 diabetes [32], uveitis [39] and orchitis [40]. Second, as discussed above, germ-free NOD mice have an accelerated onset of diabetes, which is reduced by conventional 'dirty' conditions of housing and feeding that lead to bacterial colonisation of the intestine and an associated increase in numbers of IEL. Third, neonatal (3-day) thymectomy (NTX) of mice induces organ-specific autoimmune diseases such as gastritis, insulitis, thyroiditis and oophoritis/orchitis (reviewed in [41]) in association with failure to develop IEL [42]. In NOD mice, NTX significantly accelerates the onset and increases the incidence of diabetes in both male and female mice (NR Solly and LC Harrison, manuscript submitted). Fourth, TCR δ-/- mice and treatment with anti-γδ TCR antibody have been used to demonstrate that oral tolerance is dependent on CD8 γδ T cells [43, 44]. Finally, γδ -/- mice have deficient mucosal IgA synthesis [45] indicating that γδ T cells determine the nature of the mucosal B-cell response.

Holt and co-workers were the first to show that γδ Tr could be induced by mucosal antigen [46]. Administration of OVA to the naso-respiratory tract before systemic immunisation suppressed subsequent airway hyper-responsiveness to inhaled OVA and OVA-specific IgE and IL-4 responses [47]. Notably, these effects were transferable to untreated mice by small

numbers of CD8 γδ T cells. In NOD mice, aerosol delivery of insulin to the naso-respiratory mucosa delayed the onset of diabetes and induced CD8 γδ Tr that blocked adoptive transfer of diabetes [32]. Induction of these CD8 γδ Tr requires recognition of intact (but not necessarily hormonally active) insulin [48]. Although classical class I MHC molecules are not involved, how insulin is presented to these CD8 γδ T cells is unknown. Aerosol insulin treatment is followed by the appearance of γδ T cells producing IL-10 specifically in pancreatic lymph nodes [48], indicating that they function as Tr, at least in part via the anti-inflammatory effects of IL-10, analogous to Tr1 cells that suppress experimental inflammatory bowel disease [49].

In summary, several independent studies have implicated γδ T cells as mediators of cellular and humoral mucosal tolerance. Environmental influences on the generation and function of these cells may therefore have a major impact on autoimmune disease risk. Furthermore, IEL cannot be considered without reference to the IEC to which they are intimately physically and functionally related. The integrity of IEC is dependent on IEL [50-53]. Thus, if IEL development or function is impaired, the mucosal permeability barrier is breached, exposing the body to exogenous pathogens and antigens.

Impaired mucosal function and development of autoimmune diabetes

Genetic or environmental factors that influence mucosal immune function may predispose to autoimmune disease. Under conventional but not germ-free conditions, IL-2, IL-10 or TGF-β deficient mice develop chronic intestinal inflammation resembling human inflammatory bowel disease [54-57]. Thus, in the absence of anti-inflammatory cytokines characteristic of mucosal immune responses (reviewed in [17]), normal intestinal microflora evoke pathological responses in the underlying lamina propria. Patients with type 1 diabetes may show evidence of chronic

intestinal inflammation ([58], reviewed in [59]) indicating that abnormalities in mucosal function or intestinal permeability may contribute to the development of diabetes. Meddings *et al.* [60] have shown that diabetes-prone bio-breeding (BB) rats have increased gastrointestinal permeability compared with diabetes-resistant BB rats. Abnormal gastrointestinal permeability occurred before the development of insulitis and was not induced by dietary diabetogens indicating that impaired exclusion of dietary and bacterial antigens is an inherent defect in these animals which could allow the environment to influence the development of disease.

Celiac disease, associated with increased gut permeability in the acute phase [61, 62] is strongly linked with the human leukocyte antigen (HLA) DQB1*0201 (DQ2) allele that is also associated with risk for type 1 diabetes. Approximately one third of type 1 diabetes patients homozygous for the DQ2 risk allele have evidence of underlying celiac disease, compared with less than 2% of patients lacking DQ2 [63]. Many investigators report enhanced immune responses to dietary proteins such as gluten [64, 65] and the cows' milk proteins bovine serum albumin (BSA) [66], β-casein [67, 68], and β-lactoglobulin [69, 70] in individuals with or at risk for type 1 diabetes. However, analysis reveals that this association is with the predisposing HLA haplotype A1-B8-DR3-DQ2 and not necessarily the disease itself [71]. This haplotype is associated not only with celiac disease and type 1 diabetes, but with common variable IgA deficiency [72]. Thus genes on this haplotype may influence the maturation or function of the mucosa, and enhanced responses to dietary antigens could reflect impaired mucosal immune function.

Candidate diabetogenic agents and the mucosa

Dietary components

Cows' milk proteins

Cows' milk is usually the first source of dietary xenogeneic antigens to which the human infant is exposed, at a stage when the mucosal immune system may not have fully matured. Several investigators have noted a high correlation between per capita consumption of cows' milk and the prevalence of type 1 diabetes between [73, 74] and within [75] countries. However, this observation relates to milk consumption across all ages not just in infancy, and correlations at least as high are reported for coffee and sugar consumption [76]. Sources of cows' milk protein in infancy include dairy products that end up in maternal breast milk, hydrolyzed cows' milk protein in infant formulae and supplements, dairy products such as custard, cheese and yogurt, and cows' milk itself. Cows' milk contains five principal proteins: caseins (70-80%), β-lactoglobulin (10%) which is not present in human milk, α-lactalbumin (5%), γ-globulin (2%) BSA (1%). IgG antibodies to cows' milk proteins are present in virtually all infants exposed to cows' milk [77, 78] and have even been considered physiological [78]. The significance of increased immune responses to cows' milk proteins in recently-diagnosed patients with type 1 diabetes has been reviewed and debated ([71, 79]) and, as discussed, may be associated with impaired oral tolerance to dietary antigens associated with particular HLA haplotypes, such as A1-B8-DR3-DQ2 and with the absence of the protective DQB1*0301 allele, rather than with disease itself [68, 80].

Early introduction of cows' milk to the infant diet was first suggested as an etiological agent in type 1 diabetes by Borch-Johnsen *et al.* [81] in 1984, who reported an inverse relationship between breast-feeding frequency/duration and type 1 diabetes prevalence. This study heralded a rash of over 20 similar studies, all but three strictly retrospective. In a meta-analysis of the first

13 studies, Gerstein [82] concluded that there was only a small protective effect of breastfeeding, lack of which or exposure to cows' milk resulted in a relative risk no greater than 1.5. This was subsequently confirmed in a larger meta-analysis by Norris and Scott [83], who concluded that the apparent weak association could be explained by recall bias in retrospective studies or by disparate control groups. From an immunogenetic perspective, two of the studies analysed are interesting. Kostraba *et al* [76] and Perez-Bravo *et al* [84] reported that the relative risks were higher, 11.3 and 13.1, respectively, in children with HLA susceptibility genes for type 1 diabetes, who had early exposure to cows' milk or shorter periods of breastfeeding. The question of cows' milk and type 1 diabetes was then addressed by prospective studies of individuals at highest genetic risk.

Norris *et al.* [85], in the Denver-based Diabetes Autoimmunity Study in the Young (DAISY), retrospectively analysed infant feeding patterns during the first 6 months of age in relation to the development of islet autoantibodies, markers of type 1 diabetes, up to 7 years of age. They found no significant associations. In the Australian BabyDiab Study, Couper *et al.* [86] prospectively analysed infant feeding patterns and the development of islet autoimmunity in high-risk infants. They looked at the duration of exclusive and total breastfeeding as well as the times at which infant formula, dairy products or cows' milk itself were introduced. Newborns with a first-degree relative with type 1 diabetes were followed for a median of 29 (9-73) months. Home diaries recorded infant feeding, but no systematic feeding advice was given. Islet cell antibodies (ICA), insulin autoantibodies (IAA), glutamic acid decarboxylase (GAD) antibodies and tyrosine phosphatase IA2 antibodies were measured six-monthly. Cox proportional hazards survival analysis revealed no association between infant feeding and detection of a single antibody once, a single antibody repeatedly, or two or more antibodies. The same lack of

association was also found in a preliminary report from the German Baby-Diab Study [87] and a prospective study of at-risk infants in Finland [88] found that early introduction of cow's milk protein was not a significant risk factor for diabetes. However, while most epidemiological studies have focused only on diet in infancy, Virtanen *et al.* [88] also investigated childhood diet. Their results suggest that long-term exposure to cows' milk is required to increase the risk of diabetes; the relative risk of developing diabetes associated with high consumption of cows' milk during childhood (>3 glasses per day) was 5.4 in HLA-DQB1 matched children. The result of an ongoing trial of nutritional intervention in Finland in which cows' milk is omitted from the diet of at-risk infants is awaited. At this time, we conclude that there is insufficient evidence for the induction of islet autoimmunity by cows' milk in genetically-susceptible infants.

The development of spontaneous diabetes in either NOD mice or BB rats is not dependent on exposure to cows' milk proteins [89, 90]. Despite the fact that in the BB rat and NOD mouse, synthetic amino acid and casein hydrolysate diets were associated with a lower incidence of diabetes than standard intact casein-containing diets [91, 92], the addition of 25% casein as the only protein source, or BSA or whole cows' milk protein, did not reverse this protection in the BB rat [90]. Feeding a semi-purified diet containing 10% skim milk powder (a source of BSA) and 20% casein to NOD mice inhibited development of diabetes [93]. Therefore, in neither rodent model of type 1 diabetes is there support for the hypothesis that exposure to cows' milk proteins is involved in the pathogenesis of diabetes.

While the evidence does not support a role for cows' milk in the pathogenesis of type 1 diabetes, it is difficult to separate early exposure to bovine antigens from a lack of breast milk. Breast milk contains a host of growth factors and cytokines, mostly species specific, many of which appear to have a role in the maturation of intestinal mucosal tissues [94, 95]. Thus, early

withdrawal of breast milk could impair development of mucosa-mediated tolerance and promote immunity to dietary antigens (including islet antigens such as insulin). In addition, a lack of passively-transferred immunity via breast milk (including lactadherin, IgA, IgG, IgM antibodies, cytokines such as TGF-β and lymphocytes) may predispose the infant to potentially diabetogenic enteric infections (see below). Shorter duration of breast-feeding may be a surrogate marker of the time of introduction and amount of weaning foods (which may contain other diabetogens).

Increased immunity to cows' milk proteins may occur in certain individuals predisposed to type 1 diabetes, but is likely to reflect impaired function of mucosa-associated lymphoid tissue associated with specific genotypes [71]. It follows, therefore that cows' milk is not unique, but simply the first dietary antigen encountered, and that predisposed individuals would also exhibit increased immunity to substitutes such as goat and soy milk.

Wheat proteins

The incidence of type 1 diabetes is highest in Scandinavia and Finland and lowest in oriental countries such as Japan [96], where dietary wheat flour is replaced by rice. Dietary plant components dramatically affect the incidence of diabetes in the BB rat and NOD mouse. Proteins of plants, specifically those from wheat and soya-bean, appear to be the major dietary diabetogens in the BB rat and exert an effect even when animals are first exposed after weaning [92, 97, 98]. Diabetes-prone BB rats are protected from diabetes when fed alternate amino acid sources such as casein, hydrolysed casein, hydrolysed soy protein or fish meal [98]. Li *et al.* [91] showed that plant-derived diabetogens induce overexpression of MHC class I molecules on murine β cells as early as 24 days of age, about 10 days after pups begin to nibble on solid food. No increase in MHC class I expression was seen in diabetes-resistant BB rats fed diabetogenic

diets. This may relate to the increased gastrointestinal permeability of diabetes-prone compared with diabetes-resistant BB rats [60]. Although diabetogens may enhance β-cell antigenicity very early on, this does not appear to be sufficient to induce diabetes because long-term exposure to diabetogenic diets are required. Studies in the BB rat by Scott *et al.* [99], in which a diabetogenic diet was delayed until either 50 or 100 days or switched to a non-diabetogenic diet at 50 days of age, have shown that exposure to food diabetogens at age 50 to 100 days, corresponding to the period of early puberty to late adolescence, is critical for the dietary modulation of diabetes development.

Gluten, a major protein in wheat flour, and the environmental agent that induces celiac disease, has been implicated in the development of diabetes. When added to a basic semi-synthetic diet at weaning, gluten increased the incidence of diabetes from 15% to 35% in BB rats [100]. The early introduction of a gluten-free diet to NOD mice significantly delayed the onset of diabetes and decreased the incidence of diabetes from 64% to 15% [101]. While there is an increased prevalence of anti-gliadin antibodies and celiac disease in patients with type 1 diabetes [63, 102], there is little evidence that gluten is diabetogenic in humans. Weak peripheral blood T-cell responses to gluten are detectable in only a low percentage of recently-diagnosed type 1 diabetes patients [64] and a prospective trial in patients indicated no effect of a gluten-free diet on the control of diabetes [103].

NADH ubiquinone reductase in both wheat and soya beans has an identical sequence [104] to a dominant T-cell epitope in the islet antigen, tyrosine phosphatase IA-2 [104, 105], raising the possibility of molecular mimicry. Antibodies from recently-diagnosed type 1 diabetes patients are able to recognise peptides with similarities to ubiquinone reductases (J.Davies, pers comm) lending support to this concept.

Insulin

Immunoreactive insulin is present in human breast milk at concentrations of up to 5 ng/ml (LC Harrison, unpublished data). It is conceivable that tolerance could be generated to insulin (in breast milk) as part of the normal developmental process and that variation in the level of insulin produced in maternal breast milk may affect induction of such tolerance. Also, if maturation of mucosa-associated lymphoid tissue was impaired or delayed, this could lead not only to failure to develop tolerance but to active immunisation. In neonatal mice, the ontogeny of mucosal (oral) tolerance is strain dependent with a clear temporal profile. Intragastric administration of antigen before the first 7-10 days of life does not generally elicit oral tolerance and may in fact prime for systemic immunity [106, 107]. It would be of interest to evaluate breast milk insulin levels and parameters of mucosal function in NOD mice compared to non-diabetes prone genetically-similar NOR mice. Vaarala *et al.* [108, 109] proposed that bovine insulin in cows' milk could generate cross-reactive immunity (to human insulin) and demonstrated that IgG antibodies to bovine insulin crossreacted with human insulin. Although bovine and human insulin differ by only three amino acids, bovine insulin is known to be immunogenic in humans [110].

Viruses

Viruses could theoretically initiate autoimmunity to islets in multiple ways, by direct infection of the target tissue or by a range of indirect means [111-115]. Enteroviruses (Coxsackie, Polio and Echoviruses, all single stranded RNA) and more recently rotaviruses (double stranded RNA) that infect intestinal mucosa have been associated with type 1 diabetes. However direct evidence

is sparse due to the difficuly of isolating RNA, particularly double stranded RNA, from the pancreas. Signs of persisting infection are the presence of interferon-alpha (IFN-α) in the pancreas [116], or less directly, in the blood of newly-diagnosed children [117], and increased levels of 5' oligo-adenylate synthase, a marker of IFN-α/β, in blood mononuclear cells of individuals with type 1 diabetes [118, 119]. Single, recurrent or chronic infection of the pancreas or islet cells could either be directly cytolyic, or could induce expression of normally sequestered or non-expressed self antigens and upregulate MHC and co-stimulator molecules, initiating bystander inflammatory responses. Enteric viruses might also be transported to the pancreas by infected mucosal lymphocytes. Enteric viruses localised to the gut could exert indirect effects via molecular mimicry, production of superantigens or polyclonal B-cell activators, and alteration of mucosal immune function or permeability.

Enteroviruses

Enteroviruses have a high rate of mutation and recombination, eg $>10^4$ variants of coxsackie B4, which accounts for multiple infections despite the development of cross-reactive antibody responses, the persistence of the viruses in nature, and different clinical syndromes associated with infection [120]. Epidemics occur in summer, but there is rapid inactivation of enteroviruses by heat, drying and ultraviolet light. Virus survival in the environment is thus greatest in cold areas of the world where there is a high incidence of type 1 diabetes compared to warmer areas [121]. Furthermore, in Finland, Norway and Poland, the diagnosis of type 1 diabetes does not have the marked summer trough in incidence seen in more southerly countries such as France, the U.K. and Mediterranean countries [122].

Coxsackie viruses enter the intestine via the mouth and replicate in the cervical lymph nodes and Peyer's patches of the pharynx and gut, causing lymphoid hyperplasia and inflammation [120]. Following a 1-2 week incubation period in lymphoid tissue, the virus may spread in the blood to organs such as spinal cord and brain, meninges, myocardium and skin, and can be detected in the feces for several weeks [120]. Infections occur early in life. Coxsackie B viruses may be acquired transplacentally, but more commonly infection in a neonate occurs with contact in the newborn nursery [120]. Twelve percent of infants acquire non-polio enteroviruses in the first month of life [123] with isolation rates for all enteroviruses highest among infants aged 1-2 months [124]. Infections at this age are, however, largely asymptomatic [123] due to neutralising IgG transplacental antibodies and/or IgA antibodies in breast milk [123]. Antibody epitopes are located on the VP1, 2 and 3 structural proteins, while T-cell epitopes are within the non-structural proteins, which include the p2C protein [125]. In coxsackie B3,4,5 and A9, p2C contains a sequence (amino acids [aa] 32-47) with strong similarity to aa 250-265 of the type 1 diabetes autoantigen, GAD65.

Coxsackie B viruses have been associated with both the onset of clinical diabetes and the initiation of islet autoimmunity (see Chapter by Manns), and sero-epidemiologically associated with rises in islet autoantibodies [126] but this evidence is mainly circumstantial. Molecular mimicry has been suggested between p2C and GAD65 [127] where virus p2C-reactive T cells could traffic from the gut and recognise the similar GAD peptide in the pancreas or pancreatic lymph node, initiating islet autoimmunity leading to type 1 diabetes. Although the similar sequences in p2C and GAD bind to the diabetes susceptibility HLA-DR3 [128] and are thus both potentially T-cell epitopes, there is little evidence for these sequences being naturally processed and presented T-cell epitopes [71, 129]. On the other hand, systemic and organ-specific

infection by enteroviruses is demonstrated by innoculation of mice with coxsackie B4 virus. This leads to infection of the pancreas and type 1 diabetes [130]. Enteroviral RNA has been found in the blood of up to a half of children with newly-diagnosed diabetes [117, 131, 132] and in a quarter of those who subsequently developed diabetes [133, 134]. Coxsackie B4 viral RNA was detected in the pancreata of children with recently-diagnosed type 1 diabetes [135], but this was not subsequently confirmed [136]. Coxsackie B infections have also been shown not to be associated with diabetes in children [137] and to generate only transient immune responses to GAD that do not lead to type 1 diabetes [138]. In NOD mice, while coxsackie B4 infection accelerates the onset of type 1 diabetes, it does not initiate islet autoimmunity and depends on a pre-existing critical mass of autoreactive T cells around the islets [139]. This study also suggested that infection with coxsackie B virus prior to islet autoimmunity could block the development of diabetes. Intriguingly, Finns have a sharply rising incidence of diabetes in the very young but very little enteroviral infection, whereas their genetically-similar Estonian neighbours have a strikingly lower incidence of diabetes and high levels of enteroviral infections in the very young [140]. If anything, this implies that enterovirus infection, in humans at this age, is protective. Could Finns and Estonians be the human counterparts of germ-free and dirty NOD mice, respectively? Bjorksten *et al.* [141] have shown that gut flora differ quantitatively and qualitatively in Scandinavians and Estonians, and have suggested that the increase in number and diversity of gut microbial flora drives maturation of the immune system. Thus the immunological effects of a coxsackie B virus infection could be modified by the age at which the infection occurs in the gut and by the flora populating the gut at the time. The nature of gut flora also reflects whether or not the child is being breast-fed [142].

Rotavirus

Rotavirus is a genus of double-stranded RNA viruses of the Family Reoviridae, which includes reovirus. It has been extensively studied as the single most important cause of diarrhoea in infants and young children world-wide and also as a model for enteric viral infections. Rotavirus is ubiquitous, most children being regularly infected predominantly in winter epidemics [143]. The seasonality of diabetes diagnosis with the summer trough is again worthy of note in this regard [122]. Clinical symptoms are rare after the age of five, by which time sufficient IgA antibodies have developed to neutralise the virus in the gut. Rotavirus is transmitted from feces to the mouth, but is not infectious until activated by trypsin, a product of the exocrine pancreas. Thus the virus is activated in the duodenum, just outside the pancreatic and bile ducts, after which it infects IEC of the small intestine at the mature villus tip [144]. Rotavirus double-stranded RNA induces the IEC to secrete the chemokines IL-8, growth-related peptide alpha and RANTES [145], which are potent attractors of $\alpha 4\beta 7$ integrin$^+$ B cells and CD4 and CD8 T cells [146]. The major site of action of rotavirus is the gut, with little evidence of systemic infection. However, there are reports of pancreatitis [147, 148] and biliary atresia [149] associated with rotavirus infection, possibly due to infection of macrophages, B cells and dendritic cells, in which rotavirus RNA has been found [150]. Furthermore, as integrins $\alpha 2$ and $\alpha 4$ can mediate viral attachment and entry into cells [151], cells in or close to the gut mucosa bearing these integrins, eg IEL ($\alpha 4$) or pancreatic exocrine cells ($\alpha 2$) have the potential to carry infection beyond the IEC or provide additional infective sites .

Rotavirus was associated with type 1 diabetes by the finding of strong peptide sequence similarities between VP7, the most prevalent rotavirus coat protein, and T-cell epitopes in both GAD65 and tyrosine phosphatase IA-2 [104, 152]. Analysis of sera from children at-risk of type

1 diabetes followed from birth revealed that islet antibodies first appeared or increased temporally with rotavirus infection [153, 154]. However, whether the mechanism is molecular mimicry, direct infection of the pancreas, or both, is not yet determined. The implications for rotavirus vaccines differ depending on the mechanism: if only mimicry is involved, live vaccines could have the potential to trigger islet autoimmunity via the gut, but if the virus enters and infects the pancreas without mimicry, then vaccines could be protective against type 1 diabetes.

Rotavirus infection may also influence other candidate environmental agents, in particular dietary components, by increasing intestinal permeability [155-157]. Breast milk, which contains both neutralising IgA and IgG antibodies and lactadherin that binds and inactivates rotavirus, affords symptomatic protection [158]. In the gut of diabetes-susceptible children, low IgA due to cessation of breast feeding and/or IgA deficiency linked to the diabetes-predisposing HLA-A1-B8-DR3-DQ2 haplotype, could promote rotaviral infectivity, gut permeability and mucosal (non-IgA) immunity to dietary proteins.

In the last decade, hospitals have instituted changes to prevent the previously high level of rotavirus infection in neonates, in whom high titer anti-rotavirus IgG transplacentally transmitted to the serum is not protective [159]. However, infection is now rife in day-care centres and pre-schools. At the same time, the incidence of type 1 diabetes in Western Europe and Australia has increased sharply in under 5-year olds. Daycare attendance, before the age of three (with increased exposure to enteric viruses) has been cited as predisposing to type 1 diabetes [160], but before the age of one has been shown to be protective against type 1 diabetes [161]. Exposure to viruses may elicit different mucosal immune responses depending on age. It is critical to determine the age-related patterns of exposure of susceptible children to enteric viruses, and the consequences.

Concluding remarks

The mucosa is the gatekeeper between 'self' and infectious as well as non-infectious 'non-self', the interface between the genetic and environmental components of type 1 diabetes susceptibility. The immune and non-immune barrier functions of the mucosa actively and passively prevent the development of autoimmune disease. It is highly likely that by developing tools to investigate the very early development of mucosal immunity and function in humans we will gain a far deeper insight into how the pre-natal thymus-selected immune repertoire is conditioned to maintain homeostasis of self-reactivity and thereby avert diseases such as type 1 diabetes.

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Figure I: Mechanisms of mucosal tolerance