

## **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, nicotinamide adenine dinucleotide, reduced form; VP, virus protein.

### **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 histocompatibility 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 -  $\alpha E\beta 7$  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 number 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,  $\alpha\beta$  T-cell receptor (TCR) and CD8 $\alpha\beta$  heterodimer; the others express  $\gamma\delta$  (~40%) or  $\alpha\beta$  TCR (~10%) and CD8 $\alpha\alpha$ -homodimer, and are unique in having an extrathymic ontogeny (reviewed in [7]). In humans,  $\gamma\delta$  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 CD8 $\alpha\alpha$   $\alpha\beta$  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  $\beta$ -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  $\beta$  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- $\beta$ , 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.

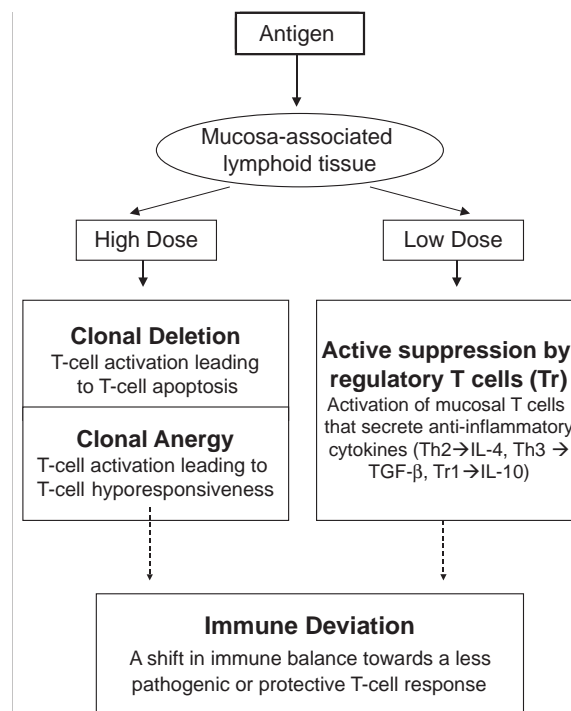
Mucosal immunity is a significant generator of peripheral tolerance. Feeding a soluble protein antigen suppresses subsequent systemic priming to the antigen ('oral tolerance'); a similar phenomenon occurs after antigen delivery to the naso-respiratory tract and other mucosae (reviewed in [17, 18]). Mucosal 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- $\beta$ , 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 [23]). Lymphocytic infiltration of islets (insulinitis) is present for months (mice) to years (humans) before the near-total destruction of  $\beta$  cells leads to diabetes. This protracted pre-clinical stage is consistent with immunoregulatory mechanisms that hold  $\beta$ -cell destruction in check.

**Figure 1: Mechanisms of mucosal tolerance**



Specific subsets of regulatory CD4 T cells [24-31], CD8  $\gamma\delta$  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  $\beta$  cells.

### **$\gamma\delta$ IEL – first line of mucosal defence**

$\gamma\delta$  TCR are present on < 1% of peripheral T cells, compared to ~30% 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  $\gamma\delta$  IEL regulate autoimmune and other inflammatory disorders. First,  $\gamma\delta$  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, insulinitis, 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  $\delta^{-/-}$  mice and treatment with anti- $\gamma\delta$  TCR antibody have been used to demonstrate that low dose oral tolerance is dependent on CD8  $\gamma\delta$  T cells [43, 44]. Finally,  $\gamma\delta^{-/-}$  mice have deficient mucosal IgA synthesis [45] indicating that  $\gamma\delta$  T cells determine the nature of the mucosal B-cell response.

Holt and co-workers were the first to show that  $\gamma\delta$  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  $\gamma\delta$  T cells. In NOD mice, aerosol delivery of insulin to the naso-respiratory mucosa delayed the onset of diabetes and induced CD8  $\gamma\delta$  Tr that blocked adoptive transfer of diabetes [32]. Induction of these CD8  $\gamma\delta$  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  $\gamma\delta$  T cells is unknown. Aerosol insulin treatment is followed by the appearance of  $\gamma\delta$  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  $\gamma\delta$  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- $\beta$  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 insulinitis 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],  $\beta$ -casein [67, 68], and  $\beta$ -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%),  $\beta$ -lactoglobulin (10%) which is not present in human milk,  $\alpha$ -lactalbumin (5%),  $\gamma$ -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- $\beta$  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  $\beta$  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  $\beta$ -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. Intra-gastric 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 difficulty of isolating RNA, particularly double stranded RNA, from the pancreas. Signs of persisting infection are the presence of interferon-alpha (IFN- $\alpha$ ) 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- $\alpha/\beta$ , 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 cytolytic, 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 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 seen after by inoculation of mice with coxsackie B4 virus; infection of the pancreas leads to 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]. On the other hand, 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<sup>+</sup> 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]. 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 [152, 153]. 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 [154-156]. Breast milk, which contains both neutralising IgA and IgG antibodies and lactadherin that binds and inactivates rotavirus, affords symptomatic protection [157]. 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 [158]. 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 [159], but before the age of one has been shown to be protective against type 1 diabetes [160]. 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.

## References

1. Gorvel JP, Sarles J, Maroux S, Olive D and Mawas C. Cellular localization of class I (HLA-A, B, C) and class II (HLA-DR and DQ) MHC antigens on

- the epithelial cells of normal human jejunum. *Biol Cell* 1984; 52:249-52
2. Blumberg RS, Terhorst C, Bleicher P, McDermott FV, Allan CH, Landau SB, Trier JS and Balk SP. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. *J Immunol* 1991; 147:2518-24
  3. Wu M, van Kaer L, Itohara S and Tonegawa S. Highly restricted expression of the thymus leukemia antigens on intestinal epithelial cells. *J Exp Med* 1991; 174:213-8
  4. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL and Brenner MB. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature* 1994; 372:190-3
  5. Kaiserlian D. Epithelial cells in antigen sampling and presentation in mucosal tissues. *Curr Top Microbiol Immunol* 1999; 236:55-78
  6. Rocha B, Vassalli P and Guy-Grand D. The V beta repertoire of mouse gut homodimeric alpha CD8+ intraepithelial T cell receptor alpha/beta + lymphocytes reveals a major extrathymic pathway of T cell differentiation. *J Exp Med* 1991; 173:483-6
  7. Lefrancois L, Fuller B, Olson S and Puddington L. Development of intestinal intraepithelial lymphocytes. *Journal* 1996; 183-193
  8. Cebra JJ, Periwal SB, Lee G, Lee F and Shroff KE. Development and maintenance of the gut-associated lymphoid tissue (GALT): the roles of enteric bacteria and viruses. *Dev Immunol* 1998; 6:13-8
  9. Pozzilli P, Signore A, Williams AJ and Beales PE. NOD mouse colonies around the world--recent facts and figures. *Immunol Today* 1993; 14:193-6
  10. Suzuki T. Diabetogenic effects of lymphocyte transfusion on the NOD or NOD nude mouse. *Journal* 1987; 112-116
  11. Imaoka A, Matsumoto S, Setoyama H, Okada Y and Umesaki Y. Proliferative recruitment of intestinal intraepithelial lymphocytes after microbial colonization of germ-free mice. *Eur J Immunol* 1996; 26:945-8
  12. Kawaguchi-Miyashita M, Shimizu K, Nanno M, Shimada S, Watanabe T, Koga Y, Matsuoka Y, Ishikawa H, Hashimoto K and Ohwaki M. Development and cytolytic function of intestinal intraepithelial T lymphocytes in antigen-minimized mice. *Immunology* 1996; 89:268-73
  13. Pugliese A, Zeller M, Fernandez A, Jr., Zalberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST and Patel DD. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. *Nat Genet* 1997; 15:293-7
  14. French MB, Allison J, Cram DS, Thomas HE, Dempsey-Collier M, Silva A, Georgiou HM, Kay TW, Harrison LC and Lew AM. Transgenic expression of mouse proinsulin II prevents diabetes in nonobese diabetic mice [published erratum appears in *Diabetes* 1997 May;46(5):924]. *Diabetes* 1997; 46:34-9
  15. Harrison LC, Colman PG, Honeyman MC and Kay TWH. Type 1 diabetes - from pathogenesis to prevention. *Journal* 1999; 85-100
  16. Redondo MJ, Rewers M, Yu L, Garg S, Pilcher CC, Elliott RB and Eisenbarth GS. Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study. *BMJ* 1999; 318:698-702
  17. Faria AM and Weiner HL. Oral tolerance: mechanisms and therapeutic applications. *Adv Immunol* 1999; 73:153-264
  18. Harrison LC and Hafler DA. Novel therapeutic approaches to autoimmune

- disease: antigen specific. *Current Opinion in Immunology* 2000 (in press);
19. Harada M and Makino S. Promotion of spontaneous diabetes in non-obese diabetes-prone mice by cyclophosphamide. *Diabetologia* 1984; 27:604-6
  20. Bendelac A, Carnaud C, Boitard C and Bach JF. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells. *J Exp Med* 1987; 166:823-32
  21. Boitard C, Yasunami R, Dardenne M and Bach JF. T cell-mediated inhibition of the transfer of autoimmune diabetes in NOD mice. *J Exp Med* 1989; 169:1669-80
  22. Hutchings PR and Cooke A. The transfer of autoimmune diabetes in NOD mice can be inhibited or accelerated by distinct cell populations present in normal splenocytes taken from young males. *J Autoimmun* 1990; 3:175-85
  23. Chosich N and Harrison LC. Suppression of diabetes mellitus in the non-obese diabetic (NOD) mouse by an autoreactive (anti-I-Ag7) islet-derived CD4+ T-cell line. *Diabetologia* 1993; 36:716-21
  24. Bergerot I, Fabien N, Maguer V and Thivolet C. Oral administration of human insulin to NOD mice generates CD4+ T cells that suppress adoptive transfer of diabetes. *J Autoimmun* 1994; 7:655-63
  25. Daniel D and Wegmann DR. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23). *Proc Natl Acad Sci U S A* 1996; 93:956-60
  26. Tian J, Clare-Salzler M, Herschenfeld A, Middleton B, Newman D, Mueller R, Arita S, Evans C, Atkinson MA, Mullen Y, Sarvetnick N, Tobin AJ, Lehmann PV and Kaufman DL. Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat Med* 1996; 2:1348-53
  27. von Herrath MG, Dyrberg T and Oldstone MB. Oral insulin treatment suppresses virus-induced antigen-specific destruction of beta cells and prevents autoimmune diabetes in transgenic mice. *J Clin Invest* 1996; 98:1324-31
  28. Han HS, Jun HS, Utsugi T and Yoon JW. Molecular role of TGF-beta, secreted from a new type of CD4+ suppressor T cell, NY4.2, in the prevention of autoimmune IDDM in NOD mice. *J Autoimmun* 1997; 10:299-307
  29. Ploix C, Bergerot I, Fabien N, Perche S, Moulin V and Thivolet C. Protection against autoimmune diabetes with oral insulin is associated with the presence of IL-4 type 2 T-cells in the pancreas and pancreatic lymph nodes. *Diabetes* 1998; 47:39-44
  30. Maron R, Melican NS and Weiner HL. Regulatory Th2-type T cell lines against insulin and GAD peptides derived from orally- and nasally-treated NOD mice suppress diabetes. *J Autoimmun* 1999; 12:251-8
  31. Lepault F and Gagnerault MC. Characterization of peripheral regulatory CD4+ T cells that prevent diabetes onset in nonobese diabetic mice. *J Immunol* 2000; 164:240-7
  32. Harrison LC, Dempsey-Collier M, Kramer DR and Takahashi K. Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes. *J Exp Med* 1996; 184:2167-74
  33. Baxter AG, Kinder SJ, Hammond KJ, Scollay R and Godfrey DI. Association between abTCR+CD4-CD8- T-cell deficiency and IDDM in NOD/Lt mice. *Diabetes* 1997; 46:572-82
  34. Homann D, Holz A, Bot A, Coon B, Wolfe T, Petersen J, Dyrberg TP, Grusby MJ and von Herrath MG. Autoreactive CD4+ T cells protect from autoimmune diabetes via bystander

- suppression using the IL-4/Stat6 pathway. *Immunity* 1999; 11:463-72
35. Cardillo F, Falcao RP, Rossi MA and Mengel J. An age-related gamma delta T cell suppressor activity correlates with the outcome of autoimmunity in experimental *Trypanosoma cruzi* infection. *Eur J Immunol* 1993; 23:2597-605
36. McMenamin C and Holt PG. The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8+ T cell-mediated but MHC class II-restricted CD4+ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J Exp Med* 1993; 178:889-99
37. Vincent MS, Roessner K, Lynch D, Wilson D, Cooper SM, Tschopp J, Sigal LH and Budd RC. Apoptosis of Fas<sup>high</sup> CD4<sup>+</sup> synovial T cells by Borrelia-reactive Fas-ligand<sup>high</sup> gamma delta T cells in Lyme arthritis. *J Exp Med* 1996; 184:2109-17
38. Szczepanik M, Anderson LR, Ushio H, Ptak W, Owen MJ, Hayday AC and Askenase PW. Gamma delta T cells from tolerized alpha beta T cell receptor (TCR)-deficient mice inhibit contact sensitivity-effector T cells in vivo, and their interferon-gamma production in vitro. *J Exp Med* 1996; 184:2129-39
39. Wildner G, Hunig T and Thurau SR. Orally induced, peptide-specific gamma/delta TCR+ cells suppress experimental autoimmune uveitis. *Eur J Immunol* 1996; 26:2140-8
40. Mukasa A, Yoshida H, Kobayashi N, Matsuzaki G and Nomoto K. Gamma delta T cells in infection-induced and autoimmune-induced testicular inflammation. *Immunology* 1998; 95:395-401
41. Bonomo A, Kehn PJ and Shevach EM. Post-thymectomy autoimmunity: abnormal T-cell homeostasis. *Immunol Today* 1995; 16:61-7
42. Lin T, Matsuzaki G, Kenai H, Nakamura T and Nomoto K. Thymus influences the development of extrathymically derived intestinal intraepithelial lymphocytes. *Eur J Immunol* 1993; 23:1968-74
43. Mengel J, Cardillo F, Aroeira LS, Williams O, Russo M and Vaz NM. Anti-gamma delta T cell antibody blocks the induction and maintenance of oral tolerance to ovalbumin in mice. *Immunol Lett* 1995; 48:97-102
44. Ke Y, Pearce K, Lake JP, Ziegler HK and Kapp JA. Gamma delta T lymphocytes regulate the induction and maintenance of oral tolerance. *J Immunol* 1997; 158:3610-8
45. Fujihashi K, McGhee JR, Kweon MN, Cooper MD, Tonegawa S, Takahashi I, Hiroi T, Mestecky J and Kiyono H. Gamma/delta T cell-deficient mice have impaired mucosal immunoglobulin A responses. *J Exp Med* 1996; 183:1929-35
46. Holt PG. Suppression of IgE responses by antigen inhalation: studies on the role of genetic and environmental factors. *Immunology* 1987; 60:97-102
47. McMenamin C, Pimm C, McKersey M and Holt PG. Regulation of IgE responses to inhaled antigen in mice by antigen-specific gamma delta T cells. *Science* 1994; 265:1869-71
48. Hanninen A and Harrison LC. Gamma delta T cells as mediators of mucosal tolerance: the autoimmune diabetes model. *Immunol Rev* 2000; 173:109-19
49. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE and Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 1997; 389:737-42
50. Boismenu R and Havran WL. Modulation of epithelial cell growth by intraepithelial gd T cells. *Science* 1994; 266:1253-1255
51. Komano H, Fujiura Y, Kawaguchi M, Matsumoto S, Hashimoto Y, Obana S, Mombaerts P, Tonegawa S, Yamamoto

- H, Itohara S and et al. Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A* 1995; 92:6147-51
52. Taylor CT, Murphy A, Kelleher D and Baird AW. Changes in barrier function of a model intestinal epithelium by intraepithelial lymphocytes require new protein synthesis by epithelial cells. *Gut* 1997; 40:634-40
53. Matsumoto S, Nanno M, Watanabe N, Miyashita M, Amasaki H, Suzuki K and Umesaki Y. Physiological roles of gammadelta T-cell receptor intraepithelial lymphocytes in cytoproliferation and differentiation of mouse intestinal epithelial cells. *Immunology* 1999; 97:18-25
54. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D and et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992; 359:693-9
55. Kuhn R, Lohler J, Rennick D, Rajewsky K and Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993; 75:263-74
56. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC and Horak I. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* 1993; 75:253-61
57. Berg DJ, Lynch NA, Lynch RG and Lauricella DM. Rapid development of severe hyperplastic gastritis with gastric epithelial dedifferentiation in *Helicobacter felis*-infected IL-10(-/-) mice. *Am J Pathol* 1998; 152:1377-86
58. Savilahti E, Ormala T, Saukkonen T, Sandini-Pohjavuori U, Kantele JM, Arato A, Ilonen J and Akerblom HK. Jejuna of patients with insulin-dependent diabetes mellitus (IDDM) show signs of immune activation. *Clin Exp Immunol* 1999; 116:70-7
59. Vaarala O. Gut and the induction of immune tolerance in type 1 diabetes. *Diabetes Metab Res Rev* 1999; 15:353-61
60. Meddings JB, Jarand J, Urbanski SJ, Hardin J and Gall DG. Increased gastrointestinal permeability is an early lesion in the spontaneously diabetic BB rat. *Am J Physiol* 1999; 276:G951-7
61. Vogelsang H, Schwarzenhofer M and Oberhuber G. Changes in gastrointestinal permeability in celiac disease. *Dig Dis* 1998; 16:333-6
62. Smecuol E, Bai JC, Vazquez H, Kogan Z, Cabanne A, Niveloni S, Pedreira S, Boerr L, Maurino E and Meddings JB. Gastrointestinal permeability in celiac disease. *Gastroenterology* 1997; 112:1129-36
63. Bao F, Yu L, Babu S, Wang T, Hoffenberg EJ, Rewers M and Eisenbarth GS. One third of HLA DQ2 homozygous patients with type 1 diabetes express celiac disease-associated transglutaminase autoantibodies. *J Autoimmun* 1999; 13:143-8
64. Klemetti P, Savilahti E, Ilonen J, Akerblom HK and Vaarala O. T-cell reactivity to wheat gluten in patients with insulin-dependent diabetes mellitus. *Scand J Immunol* 1998; 47:48-53
65. Catassi C, Guerrieri A, Bartolotta E, Coppa GV and Giorgi PL. Antigliadin antibodies at onset of diabetes in children [letter]. *Lancet* 1987; 2:158
66. Karjalainen J, Martin JM, Knip M, Ilonen J, Robinson BH, Savilahti E, Akerblom HK and Dosch HM. A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus [published erratum appears in *N Engl J Med* 1992 Oct 22;327(17):1252]. *N Engl J Med* 1992; 327:302-7
67. Cavallo MG, Fava D, Monetini L, Barone F and Pozzilli P. Cell-mediated immune response to beta casein in recent-onset insulin-dependent diabetes: implications for disease pathogenesis [published erratum

- appears in *Lancet* 1996 Nov 16;348(9038):1392]. *Lancet* 1996; 348:926-8
68. Ellis TM, Ottendorfer E, Jodoin E, Salisbury PJ, She JX, Schatz DA and Atkinson MA. Cellular immune responses to beta casein: elevated in but not specific for individuals with Type I diabetes mellitus. *Diabetologia* 1998; 41:731-5
  69. Vaarala O, Klemetti P, Savilahti E, Reijonen H, Ilonen J and Akerblom HK. Cellular immune response to cow's milk beta-lactoglobulin in patients with newly diagnosed IDDM. *Diabetes* 1996; 45:178-82
  70. Savilahti E, Saukkonen TT, Virtala ET, Tuomilehto J and Akerblom HK. Increased levels of cow's milk and b-lactoglobulin antibodies in young children with newly diagnosed IDDM. The Childhood Diabetes in Finland Study Group. *Diabetes Care* 1993; 16:984-9
  71. Harrison LC and Honeyman MC. Cow's milk and type 1 diabetes: the real debate is about mucosal immune function. *Diabetes* 1999; 48:1501-7
  72. Ambrus M, Hernadi E and Bajtai G. Prevalence of HLA-A1 and HLA-B8 antigens in selective IgA deficiency. *Clin Immunol Immunopathol* 1977; 7:311-4
  73. Scott FW. Cow milk and insulin-dependent diabetes mellitus: is there a relationship? *Am J Clin Nutr* 1990; 51:489-91
  74. Dahl-Jorgensen K, Joner G and Hanssen KF. Relationship between cows' milk consumption and incidence of IDDM in childhood. *Diabetes Care* 1991; 14:1081-3
  75. Fava D, Leslie RD and Pozzilli P. Relationship between dairy product consumption and incidence of IDDM in childhood in Italy. *Diabetes Care* 1994; 17:1488-90
  76. Kostraba JN, Cruickshanks KJ, Lawler-Heavner J, Jobim LF, Rewers MJ, Gay EC, Chase HP, Klingensmith G and Hamman RF. Early exposure to cow's milk and solid foods in infancy, genetic predisposition, and risk of IDDM. *Diabetes* 1993; 42:288-95
  77. Ferguson A. Immunogenicity of cows' milk in man. (Influence of age and of disease on serum antibodies to five cows' milk proteins). *Ric Clin Lab* 1977; 7:211-9
  78. Keller KM, Burgin-Wolff A, Lippold R, Wirth S and Lentze MJ. The diagnostic significance of IgG cow's milk protein antibodies re-evaluated. *Eur J Pediatr* 1996; 155:331-7
  79. Kolb H and Pozzilli P. Cow's milk and type I diabetes: the gut immune system deserves attention. *Immunol Today* 1999; 20:108-10
  80. Saukkonen T, Virtanen SM, Karppinen M, Reijonen H, Ilonen J, Rasanen L, Akerblom HK and Savilahti E. Significance of cow's milk protein antibodies as risk factor for childhood IDDM: interactions with dietary cow's milk intake and HLA- DQB1 genotype. Childhood Diabetes in Finland Study Group. *Diabetologia* 1998; 41:72-8
  81. Borch-Johnsen K, Joner G, Mandrup-Poulsen T, Christy M, Zachau-Christiansen B, Kastrup K and Nerup J. Relation between breast-feeding and incidence rates of insulin-dependent diabetes mellitus. A hypothesis. *Lancet* 1984; 2:1083-6
  82. Gerstein H. Cow's milk exposure and type 1 diabetes mellitus. *Diabetes Care* 1994; 17:13-19
  83. Norris JM and Scott FW. A meta-analysis of infant diet and insulin-dependent diabetes mellitus: do biases play a role? *Epidemiology* 1996; 7:87-92
  84. Perez-Bravo F, Carrasco E, Gutierrez-Lopez MD, Martinez MT, Lopez G and de los Rios MG. Genetic predisposition and environmental factors leading to the development of insulin-dependent diabetes mellitus in Chilean children. *J Mol Med* 1996; 74:105-9

85. Norris JM, Beaty B, Klingensmith G, Yu L, Hoffman M, Chase HP, Erlich HA, Hamman RF, Eisenbarth GS and Rewers M. Lack of association between early exposure to cow's milk protein and b-cell autoimmunity. Diabetes Autoimmunity Study in the Young (DAISY). *Jama* 1996; 276:609-14
86. Couper JJ, Steele C, Beresford S, Powell T, McCaul K, Pollard A, Gellert S, Tait B, Harrison LC and Colman PG. Lack of association between duration of breast-feeding or introduction of cow's milk and development of islet autoimmunity. *Diabetes* 1999; 48:2145-9
87. Hummel M, Schenker M, Ziegler AB and Group TB-DS. Appearance of diabetes-associated antibodies in offspring of parents with type 1 diabetes is independent from environmental factors (Abstract). *Diabetologia* 1998; 41 (supp 1):A91
88. Virtanen SM, Laara E, Hypponen E, Reijonen H, Rasanen L, Aro A, Knip M, Ilonen J and Akerblom HK. Cow's milk consumption, HLA-DQB1 genotype, and type 1 diabetes: a nested case-control study of siblings of children with diabetes. Childhood diabetes in Finland study group. *Diabetes* 2000; 49:912-7
89. Paxson JA, Weber JG and Kulczycki A, Jr. Cow's milk-free diet does not prevent diabetes in NOD mice [published erratum appears in *Diabetes* 1998 Jan;47(1):144]. *Diabetes* 1997; 46:1711-7
90. Malkani S, Nompleggi D, Hansen JW, Greiner DL, Mordes JP and Rossini AA. Dietary cow's milk protein does not alter the frequency of diabetes in the BB rat. *Diabetes* 1997; 46:1133-40
91. Li XB, Scott FW, Park YH and Yoon JW. Low incidence of autoimmune type I diabetes in BB rats fed a hydrolysed casein-based diet associated with early inhibition of non-macrophage-dependent hyperexpression of MHC class I molecules on b cells. *Diabetologia* 1995; 38:1138-47
92. Hoorfar J, Buschard K and Dagnaes-Hansen F. Prophylactic nutritional modification of the incidence of diabetes in autoimmune non-obese diabetic (NOD) mice. *Br J Nutr* 1993; 69:597-607
93. Coleman DL, Kuzava JE and Leiter EH. Effect of diet on incidence of diabetes in nonobese diabetic mice. *Diabetes* 1990; 39:432-6
94. Xanthou M, Bines J and Walker WA. Human milk and intestinal host defenses in newborns: an update. *Adv Paediatrics* 1995; 42:171-208
95. Srivastava MD, Srivastava A, Brouhard B, Saneto R, Groh-Wargo S and Kubit J. Cytokines in human milk. *Res Commun Mol Pathol Pharmacol* 1996; 93:263-87
96. Onkamo P, Vaananen S, Karvonen M and Tuomilehto J. Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. *Diabetologia* 1999; 42:1395-403
97. Atkinson MA, Winter WE, Skordis N, Beppu H, Riley WM and Maclaren NK. Dietary protein restriction reduces the frequency and delays the onset of insulin dependent diabetes in BB rats. *Autoimmunity* 1988; 2:11-9
98. Scott FW. Food-induced type 1 diabetes in the BB rat. *Diabetes Metab Rev* 1996; 12:341-59
99. Scott FW, Cloutier HE, Kleemann R, Woerz-Pagenstert U, Rowsell P, Modler HW and Kolb H. Potential mechanisms by which certain foods promote or inhibit the development of spontaneous diabetes in BB rats: dose, timing, early effect on islet area, and switch in infiltrate from Th1 to Th2 cells. *Diabetes* 1997; 46:589-98
100. Elliott RB and Martin JM. Dietary protein: a trigger of insulin-dependent diabetes in the BB rat? *Diabetologia* 1984; 26:297-9

101. Funda DP, Kaas A, Bock T, Tlaskalova-Hogenova H and Buschard K. Gluten-free diet prevents diabetes in NOD mice. *Diabetes Metab Res Rev* 1999; 15:323-7
102. Vitoria JC, Castano L, Rica I, Bilbao JR, Arrieta A and Garcia-Masdevall MD. Association of insulin-dependent diabetes mellitus and celiac disease: a study based on serologic markers. *J Pediatr Gastroenterol Nutr* 1998; 27:47-52
103. Kaukinen K, Salmi J, Lahtela J, Siljamaki-Ojansuu U, Koivisto AM, Oksa H and Collin P. No effect of gluten-free diet on the metabolic control of type 1 diabetes in patients with diabetes and celiac disease. Retrospective and controlled prospective survey [letter]. *Diabetes Care* 1999; 22:1747-8
104. Honeyman MC, Stone NL and Harrison LC. T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA-2: potential for mimicry with rotavirus and other environmental agents. *Mol Med* 1998; 4:231-9
105. Hawkes CJ, Schloot NC, Marks J, Willemsen SJ, Drijfhout JW, Mayer EK, Christie MR and Roep BO. T-cell lines reactive to an immunodominant epitope of the tyrosine phosphatase-like autoantigen IA-2 in type 1 diabetes. *Diabetes* 2000; 49:356-66
106. Strobel S and Mowat AM. Immune responses to dietary antigens: oral tolerance. *Immunol Today* 1998; 19:173-81
107. Miller A, Lider O, Abramsky O and Weiner HL. Orally administered myelin basic protein in neonates primes for immune responses and enhances experimental autoimmune encephalomyelitis in adult animals. *Eur J Immunol* 1994; 24:1026-32
108. Vaarala O, Paronen J, Otonkoski T and Akerblom HK. Cow milk feeding induces antibodies to insulin in children--a link between cow milk and insulin-dependent diabetes mellitus? *Scand J Immunol* 1998; 47:131-5
109. Vaarala O, Knip M, Paronen J, Hamalainen AM, Muona P, Vaatainen M, Ilonen J, Simell O and Akerblom HK. Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes. *Diabetes* 1999; 48:1389-94
110. Reeves WG and Kelly U. Insulin antibodies induced by bovine insulin therapy. *Clin Exp Immunol* 1982; 50:163-70
111. Menser MA, Forrest JM and Bransby RD. Rubella infection and diabetes mellitus. *Lancet* 1978; 1:57-60
112. Harrison LC and McColl G. Infection and autoimmune disease. *Journal* 1998; 127-140
113. Tlaskalova-Hogenova H, Stepankova R, Tuckova L, Farre MA, Funda DP, Verdu EF, Sinkora J, Hudcovic T, Rehakova Z, Cukrowska B, Kozakova H and Prokesova L. Autoimmunity, immunodeficiency and mucosal infections: chronic intestinal inflammation as a sensitive indicator of immunoregulatory defects in response to normal luminal microflora. *Folia Microbiol (Praha)* 1998; 43:545-50
114. Moller E. Mechanisms for induction of autoimmunity in humans. *Acta Paediatr Suppl* 1998; 424:16-20
115. Rose NR. The role of infection in the pathogenesis of autoimmune disease. *Semin Immunol* 1998; 10:5-13
116. Foulis AK, Farquharson MA and Meager A. Immunoreactive alpha-interferon in insulin-secreting beta cells in type 1 diabetes mellitus. *Lancet* 1987; 2:1423-7
117. Chehadeh W, Weill J, Vantyghem MC, Alm G, Lefebvre J, Watre P and Hober D. Increased level of interferon-alpha in blood of patients with insulin-dependent diabetes mellitus: relationship with coxsackievirus B infection. *J Infect Dis* 2000; 181:1929-1939
118. Petrovsky N, Harrison LC, Kyvik KO, Beck-Nielsen H, Green A and

- Bonnevie-Nielsen V. Evidence for the viral aetiology of IDDM [letter]. *Autoimmunity* 1997; 25:251-2
119. Bonnevie-Nielsen V, Martensen PM, Justesen J, Kyvik KO, Kristensen B, Levin K, Beck-Nielsen H, Worsaa A and Dyrberg T. The antiviral 2',5'-oligoadenylate synthetase is persistently activated in type 1 diabetes. *Clin Immunol* 2000; 96:11-8
120. Melnick J. Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses, and Newer Enteroviruses. *Journal* 1996; 655-712
121. Wendorf MA. Diabetes and enterovirus autoimmunity in glacial Europe. *Med Hypotheses* 1999; 52:423-9
122. Levy-Marchal C, Patterson C and Green A. Variation by age group and seasonality at diagnosis of childhood IDDM in Europe. The EURODIAB ACE Study Group. *Diabetologia* 1995; 38:823-30
123. Jenista JA, Powell KR and Menegus MA. Epidemiology of neonatal enterovirus infection. *J Pediatr* 1984; 104:685-90
124. Maguire HC, Atkinson P, Sharland M and Bendig J. Enterovirus infections in England and Wales: laboratory surveillance data: 1975 to 1994. *Commun Dis Public Health* 1999; 2:122-5
125. Juhela S, Hyoty H, Hinkkanen A, Elliott J, Roivainen M, Kulmala P, Rahko J, Knip M and Ilonen J. T cell responses to enterovirus antigens and to beta-cell autoantigens in unaffected children positive for IDDM-associated autoantibodies. *J Autoimmun* 1999; 12:269-78
126. Hiltunen M, Hyoty H, Knip M, Ilonen J, Reijonen H, Vahasalo P, Roivainen M, Lonrot M, Leinikki P, Hovi T and Akerblom HK. Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. Childhood Diabetes in Finland (DiMe) Study Group. *J Infect Dis* 1997; 175:554-60
127. Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL and Maclaren NK. Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. *J Clin Invest* 1994; 94:2125-9
128. Vreugdenhil GR, Geluk A, Ottenhoff TH, Melchers WJ, Roep BO and Galama JM. Molecular mimicry in diabetes mellitus: the homologous domain in coxsackie B virus protein 2C and islet autoantigen GAD65 is highly conserved in the coxsackie B-like enteroviruses and binds to the diabetes associated HLA-DR3 molecule. *Diabetologia* 1998; 41:40-6
129. Endl J, Otto H, Jung G, Dreisbusch B, Donie F, Stahl P, Elbracht R, Schmitz G, Meinl E, Hummel M, Ziegler AG, Wank R and Schendel DJ. Identification of naturally processed T cell epitopes from glutamic acid decarboxylase presented in the context of HLA-DR alleles by T lymphocytes of recent onset IDDM patients. *J Clin Invest* 1997; 99:2405-15
130. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J and Sarvetnick N. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med* 1998; 4:781-5
131. Nairn C, Galbraith DN, Taylor KW and Clements GB. Enterovirus variants in the serum of children at the onset of Type 1 diabetes mellitus. *Diabet Med* 1999; 16:509-13
132. Lonrot M, Salminen K, Knip M, Savola K, Kulmala P, Leinikki P, Hyypia T, Akerblom HK and Hyoty H. Enterovirus RNA in serum is a risk factor for beta-cell autoimmunity and clinical type 1 diabetes: a prospective study. Childhood Diabetes in Finland (DiMe) Study Group. *J Med Virol* 2000; 61:214-20
133. Andreoletti L, Hober D, Hober-Vandenberghe C, Fajardy I, Belaich S, Lambert V, Vantighem MC, Lefebvre J

- and Wattle P. Coxsackie B virus infection and beta cell autoantibodies in newly diagnosed IDDM adult patients. *Clin Diagn Virol* 1998; 9:125-33
134. Hyoty H, Hiltunen M, Knip M, Laakkonen M, Vahasalo P, Karjalainen J, Koskela P, Roivainen M, Leinikki P, Hovi T and et al. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes* 1995; 44:652-7
135. Szopa TM, Titchener PA, Portwood ND and Taylor KW. Diabetes mellitus due to viruses--some recent developments. *Diabetologia* 1993; 36:687-95
136. Foulis AK, McGill M, Farquharson MA and Hilton DA. A search for evidence of viral infection in pancreases of newly diagnosed patients with IDDM. *Diabetologia* 1997; 40:53-61
137. Di Pietro C, Del Guercio MJ, Paolino GP, Barbi M, Ferrante P and Chiumello G. Type 1 diabetes and Coxsackie virus infection. *Helv Paediatr Acta* 1979; 34:557-61
138. Cainelli F, Manzaroli D, Renzini C, Casali F, Concia E and Vento S. Coxsackie B virus-induced autoimmunity to GAD does not lead to type 1 diabetes [letter]. *Diabetes Care* 2000; 23:1021-2
139. Serreze DV, Ottendorfer EW, Ellis TM, Gauntt CW and Atkinson MA. Acceleration of type 1 diabetes by a coxsackievirus infection requires a pre-existing critical mass of autoreactive T-cells in pancreatic islets. *Diabetes* 2000; 49:708-711
140. Lonrot M, Knip M, Marciulionyte D, Rahko J, Urbonaite B, Moore WP, Vilja P and Hyoty H. Enterovirus antibodies in relation to islet cell antibodies in two populations with high and low incidence of type 1 diabetes [letter]. *Diabetes Care* 1999; 22:2086-8
141. Bjorksten B. Environment and infant immunity. *Proc Nutr Soc* 1999; 58:729-32
142. Bjorksten B. The intrauterine and postnatal environments. *J Allergy Clin Immunol* 1999; 104:1119-27
143. Coulson BS, Grimwood K, Masendycz PJ, Lund JS, Mermelstein N, Bishop RF and Barnes GL. Comparison of rotavirus immunoglobulin A coproconversion with other indices of rotavirus infection in a longitudinal study in childhood. *J Clin Microbiol* 1990; 28:1367-74
144. Rott LS, Rose JR, Bass D, Williams MB, Greenberg HB and Butcher EC. Expression of mucosal homing receptor alpha4beta7 by circulating CD4+ cells with memory for intestinal rotavirus. *J Clin Invest* 1997; 100:1204-8
145. Gromkowski SH, Mama K, Yagi J, Sen R and Rath S. Double-stranded RNA and bacterial lipopolysaccharide enhance sensitivity to TNF-alpha-mediated cell death. *Int Immunol* 1990; 2:903-8
146. Ebert EC. Human intestinal intraepithelial lymphocytes have potent chemotactic activity. *Gastroenterology* 1995; 109:1154-9
147. De La Rubia L, Herrera MI, Cebrero M and De Jong JC. Acute pancreatitis associated with rotavirus infection [letter]. *Pancreas* 1996; 12:98-9
148. Nigro G. Pancreatitis with hypoglycemia-associated convulsions following rotavirus gastroenteritis. *J Pediatr Gastroenterol Nutr* 1991; 12:280-2
149. Gilger MA, Matson DO, Conner ME, Rosenblatt HM, Finegold MJ and Estes MK. Extraintestinal rotavirus infections in children with immunodeficiency. *J Pediatr* 1992; 120:912-7
150. Brown KA and Offit PA. Rotavirus-specific proteins are detected in murine macrophages in both intestinal and extraintestinal lymphoid tissues. *Microb Pathog* 1998; 24:327-31
151. Hewish MJ, Takada Y and Coulson BS. Integrins alpha2beta1 and alpha4beta1 can mediate SA11 rotavirus attachment and entry into cells. *J Virol* 2000; 74:228-36

152. Honeyman MC, Coulson BS, Stone NL, Gellert SA, Goldwater PN, Steele CE, Couper JJ, Tait BD, Colman PG and Harrison LC. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. *Diabetes* 2000; 49:1319-24
153. Honeyman MC, Coulson BS and Harrison LC. A novel subtype of type 1 diabetes mellitus [letter]. *N Engl J Med* 2000; 342:1835; discussion 1837
154. Jalonen T, Isolauri E, Heyman M, Crain-Denoyelle AM, Sillanaukee P and Koivula T. Increased beta-lactoglobulin absorption during rotavirus enteritis in infants: relationship to sugar permeability. *Pediatr Res* 1991; 30:290-3
155. Johansen K, Stintzing G, Magnusson KE, Sundqvist T, Jalil F, Murtaza A, Khan SR, Lindblad BS, Mollby R, Orusild E and et al. Intestinal permeability assessed with polyethylene glycols in children with diarrhea due to rotavirus and common bacterial pathogens in a developing community. *J Pediatr Gastroenterol Nutr* 1989; 9:307-13
156. Obert G, Peiffer I and Servin AL. Rotavirus-induced structural and functional alterations in tight junctions of polarized intestinal Caco-2 cell monolayers. *J Virol* 2000; 74:4645-51
157. Newburg DS, Peterson JA, Ruiz-Palacios GM, Matson DO, Morrow AL, Shults J, Guerrero ML, Chaturvedi P, Newburg SO, Scallan CD, Taylor MR, Ceriani RL and Pickering LK. Role of human-milk lactadherin in protection against symptomatic rotavirus infection. *Lancet* 1998; 351:1160-4
158. Bishop RF, Cameron DJ, Veenstra AA and Barnes GL. Diarrhea and rotavirus infection associated with differing regimens for postnatal care of newborn babies. *J Clin Microbiol* 1979; 9:525-9
159. Verge CF, Howard NJ, Irwig L, Simpson JM, Mackerras D and Silink M. Environmental factors in childhood IDDM. A population-based, case-control study. *Diabetes Care* 1994; 17:1381-9
160. McKinney PA, Okasha M, Parslow RC, Law GR, Gurney KA, Williams R and Bodansky HJ. Early social mixing and childhood Type 1 diabetes mellitus: a case-control study in Yorkshire, UK. *Diabet Med* 2000; 17:236-42\

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Figure I: Mechanisms of mucosal tolerance