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DIETARY INSULIN AND THE GUT IMMUNE SYSTEM IN TYPE 1 DIABETES

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Academic dissertation

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ABSTRACT

Type 1 diabetes is considered to be an autoimmune disease in which T-cells destroy the insulin-secreting β-cells. A genetic background predisposes to the disease, but environmental factors are thought play a role in the pathogenesis. Early exposure to cow`s milk (CM) proteins and enteroviral infections are the two major putative environmental factors associated with the risk for type 1 diabetes, both of which trigger the gut immune system. Indeed, direct involvement of the gut immune system in the pathogenesis of autoimmune diabetes has been shown in experimental models of human disease. Of the major autoantigens (insulin, GAD65, and IA-2), insulin has been suggested to be the primary one, one reason for which is that insulin autoantibodies are the first autoantibodies to appear in children who show signs of autoimmunity.

To study the involvement of the gut immune system in human type 1 diabetes, we analyzed the expression of gut-associated homing receptor α4β7 integrin in a peripheral blood mononuclear cell (PBMC) population reactive to GAD65 in patients with newly diagnosed type 1 diabetes and in one prediabetic subject. We depleted α4β7-positive cells by immunomagnetic separation from PBMCs, and compared the proliferation response to GAD65 and to a control antigen (tetanus toxoid) in the whole PBMC population and in the PBMC population depleted of α4β7-positive cells. In addition, we studied the induction of an insulin-specific immune response by exposure to the bovine insulin (BI) present in infant formulas. Development of cellular immune responses to BI and human insulin (HI) by proliferation test and the emergence of insulin-binding antibodies by enzyme immunoassay in relation to CM formula exposure and family history of type 1 diabetes were followed in children at genetic risk for the disease.

Reactivity to GAD65 in type 1 diabetes was found in the PBMC population expressing the gut-associated homing receptor α4β7. In contrast, reactivity to parenteral tetanus toxoid was not associated with this population. These results link cellular reactivity against an autoantigen in type 1 diabetes to the gut-associated lymphoid tissue. Exposure to dietary insulin primed insulin-specific cellular and humoral immune responses in infants at genetic risk for type 1 diabetes. At 3 months, cellular and humoral immune responses to BI were highest in infants exposed to CM formula than in infants who received either hydrolyzed casein-based formula or who were fully breast fed. In infants exposed to CM formula early on, the cellular reactivity to insulin was observed at 9 months of age to mount a response to HI, as well. We suggest that

this insulin-specific immune response may in some circumstances turn autoaggressive and in some genetically susceptible children lead to β-cell destruction and type 1 diabetes. The factors that lead to the activation of the insulin-primed T-cells are unknown, but may be associated with the regulation of the gut immune system. Interestingly, we found lower immune responses to insulin in children of diabetic mothers than in children of diabetic fathers or with an affected sibling. This may explain the epidemiological finding of lower risk of type 1 diabetes in children with a diabetic mother than in children with an affected father. Our results suggest that, in offspring of diabetic mothers, exposure to maternal diabetes/insulin therapy during pregnancy and early infancy results in tolerization to insulin.

In sum, our findings on primary immunization to a β-cell-specific antigen – insulin – in the gut, and the expression of gut-associated homing receptor on auto(GAD)-reactive T-cells suggest a link between the gut immune system and β-cell autoimmunity in humans.

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications referred to in the text by their Roman numerals (I-V).

I Paronen J, Vaarala O, Savilahti E, Saukkonen T, Åkerblom HK. Soluble adhesion molecules and oral antigen feeding in infants. Pediatr Res 1996;40:276-279.

II Paronen J, Klemetti P, Kantele JM, Savilahti E, Perheentupa J, Åkerblom HK, Vaarala O. Glutamate decarboxylase-reactive peripheral blood lymphocytes from patients with IDDM express gut-specific homing receptor α4β7-integrin. Diabetes 1997;46:583-588.

III Vaarala O, Paronen J, Otonkoski T, Åkerblom HK. Cow milk feeding induces antibodies to insulin in children – a link between cow milk and insulin-dependent diabetes? Scand J Immunol 1998;47:131-135.

IV Paronen J, Knip M, Savilahti E, Virtanen SM, Ilonen J, Åkerblom HK, Vaarala O, and the Finnish Trial to Reduce IDDM in the Genetically at Risk Study Group. Effect of cow´s milk exposure and maternal type 1 diabetes on cellular and humoral immunization to dietary insulin in infants at genetic risk for type 1 diabetes. Diabetes 2000;49:1657-1665.

V Paronen J, Björkstén B, Hattevig G, Åkerblom HK, Vaarala O. The effect of maternal diet during lactation on the development of bovine insulin-binding antibodies in children at risk for allergy. J Allergy and Clin Immunol 2000;106:302-306.

ABBREVIATIONS

APC	Antigen-presenting cell
APD-I	Autoimmune polyendocrine disease type 1
BB	Biobreeding
BF	Breast fed
BI	Bovine insulin
BLG	β-lactoglobulin
BSA	Bovine serum albumin
CBV	Coxsackie B virus
CM	Cow`s milk
CPM	Counts per minute
CTL	Cytotoxic lymphocyte
D	Diet
DC	Dendritic cell
EIA	Enzyme immunoassay
GAD	Glutamic acid decarboxylase
GALT	Gut-associated lymphoid tissue
HC	Hydrolyzed casein
HI	Human insulin
HLA	Human leucocyte antigen
IAA	Insulin autoantibodies
IEL	Intraepithelial lymphocyte
ICA	Islet cell antibodies
ICAM- 1	Intercellular adhesion molecule-1
IL	Interleukin
IFN	Interferon
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal vascular addressin-1
MHC	Major histocombatibility complex
MLN	Mesenteric lymph node
NOD	Nonobese diabetic
ND	Nondiet

OD	Optical density
PBMC	Peripheral blood mononuclear cell
PNAd	Peripheral lymph node addressin
PP	Peyers patches
RIA	Radio immunoassay
SI	Stimulation index
TCR	T-cell receptor
TGF-β	Transforming growth factor β
TRIGR	Trial to reduce IDDM in genetically at risk
TT	Tetanus toxoid

INTRODUCTION

Type 1 diabetes is considered to be an autoimmune disease in which insulin-secreting pancreatic β-cells are destroyed (Eisenbarth 1986, Castano and Eisenbarth 1990). The genetic background predisposes to the disease, but environmental factors are also thought to play a role in the pathogenesis. Early exposure to CM proteins and childhood enterovirus infections have been suggested to act as these environmental agents (reviewed by Åkerblom and Knip 1998).

The incidence of type 1 diabetes has been rising over the recent decades (EURODIAB ACE Study Group 2000). The highest incidence of type 1 diabetes in the world exists in Finland: over 50 new cases / 100,000 children / year. This disease is the second most common chronic disease in children, and it increases morbidity and mortality not to mention the burden to the individual and his/her family. The disease is also a financial load on society since the lifetime costs for one patient with type 1 diabetes rise to one million US dollars.

Of the diseases in childhood allergies have also become more common (Hanson and Telemo 1997). Food allergies manifest in the first years of life, and involve disproportionate immune responses to dietary antigens. This is a reflection of disturbance in gut immunity and in oral tolerance. In type 1 diabetes, disturbance in oral tolerance has been suggested as well, and the involvement of the gut immune system in the pathogenesis of type 1 diabetes is currently under discussion (reviewed by Harrison and Honeyman 1999, Kolb and Pozzilli 1999, Vaarala 1999).

In the present study the role of the gut immune system and oral tolerance were explored in children who participated in the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) and in children with newly diagnosed type 1 diabetes. In addition, immunity to dietary insulin was studied in the TRIGR children and in children at risk for allergy. A pilot phase of TRIGR began in Finland in the early 1990s. It is a double-blind randomized study to discover the effect of early CM exposure in the emergence of diabetes-associated autoantibodies in children at genetic risk for type 1 diabetes. When pathogenic mechanisms of type 1 diabetes are better understood, perhaps one day the disease can be prevented.

REVIEW OF THE LITERATURE

TYPE 1 DIABETES

Epidemiology

Type 1 diabetes (insulin-dependent diabetes mellitus) typically manifests during childhood. During the first year of life the disease is rare, but from there the incidence increases. Although type 1 diabetes is mainly diagnosed in childhood, a proportion of the disease is also found in adolescents and young adults (Laakso and Pyörälä 1985, Blohmè et al 1992). Over the past decades the onset of type 1 diabetes has moved to younger age groups (Karvonen et al 1999).

Altogether, over the last decades the incidence of type 1 diabetes has risen significantly worldwide (3.0% per year) (Onkamo et al 1999), and in Finland the rise has been from 12 cases per 100,000 children a year in the beginning of the 1950s to 50 per 100,000 children a year now (Antti Reunanen, personal communication). There is a worldwide difference in incidence of type 1 diabetes ranging from this record-high incidence of diabetes in Finland to the lowest reported values of 0.6 per 100,000 children / year in Korea and Mexico. A north-to-south gradient in this incidence rate also exists, with no countries below the equator having an incidence over 15 per 100,000 (Karvonen et al 1993). An interesting exception to this geographical variation is Sardinia with the second highest incidence of type 1 diabetes in the world, after Finland. A temporal variation also exists with a lower number of new cases during warm summer months than in the cooler winter months (Karvonen et al 1993). In addition to these worldwide differences in incidence rates, a higher rate of diabetes has been reported in offspring of diabetic fathers vs. mothers (Warram et al 1984), and a male excess in newly diagnosed diabetic children especially in high-incidence countries (Tuomilehto et al 1992, Lévy-Marchal et al 1995).

Pathogenesis

Genetic background

Type 1 diabetes is a polygenic disease in which environmental factors also play a role, so that the genetic background of an individual is essential but not sufficient by itself in causing the disease. The average risk for type 1 diabetes among first-degree relatives (siblings and children) of diabetic patients is 5 to 7 % (Tillil and Köbberling 1987) compared to a 0.3 to 0.5% risk in the general population. Family and twin studies have revealed that the genetic background contributes to less than half of the lifetime risk; in twin studies the concordance rate for monozygotic twins has been only around 30% (Olmos et al 1988, Kaprio et al 1992). The greatest susceptibility to type 1 diabetes is mapped to the human major histocompatibility complex (MHC) or the human leucocyte antigen complex (HLA) gene region in the short arm of chromosome 6, which contributes about 40% to the familial clustering of the disease (Davies et al 1994).

The first evidence of an association between HLA coding genes and type 1 diabetes came from studies reporting that the HLA class I alleles B8 and B15 were more frequent in patients with diabetes than in controls (Singal and Blajchman 1973, Nerup et al 1974). Since then it has become evident that class II alleles of the HLA complex confer the major susceptibility to type 1 diabetes. Over 90% of type 1 diabetes patients of Caucasian origin possess either HLA-DR3 or -DR4, compared to about 50% of the normal population. The highest risk is associated with the heterozygous DR3/4 phenotype, which is found in 30 to 50% of patients but in only 1 to 6% among healthy individuals. In contrast, the HLA-DR2 allele seems to confer protection from the disease (Ilonen et al 1978, Wassmuth and Lernmark 1989, Deschamps and Khalil 1993).

Following the introduction of advanced analyzing techniques in molecular biology, alleles of the DQ locus were identified and shown to affect the susceptibility to type 1 diabetes even more strongly (Owerbach et al 1983, Nepom et al 1986). HLA DQB1*0302 was shown to be a susceptibility allele together with DBQ1*02, and the heterozygosity of these alleles was noticed to confer the highest risk for type 1 diabetes. The DQB1*0302 and/or DQB1*02 alleles are found in 95% of children with the disease compared to about 50% of healthy individuals, and the most closely associated genotype, DQB1*02/302, in 46% of the new-onset diabetic children compared to 3% of the background population (Lernmark 1994). Due to a strong linkage disequilibrium between the DR and DQ alleles, it seems that DR4-DQA1*301-DQB1*0302 and DR3-DQA1*501-DQB1*02 are the major type 1 diabetes susceptibility haplotypes in

Caucasians. On the other hand, the DQB1*0602 allele present in DR2-DQA1*0102-DQB1*0602 haplotype is associated with the strongest protection against the disease (Kockum et al 1993, Deschamps and Khalil 1993). Moreover, it has been suggested that the ability to confer susceptibility or resistance to type 1 diabetes resides within a single amino acid residue of the HLA DQ β-chain (Todd et al 1987) and/or in the DQ α-chain (Khalil et al 1990). Albeit this susceptibility is linked to the MHC locus, MHC-identical siblings are only 13% concordant for the disease (Thompson et al 1988).

HLA molecules play a major role in controlling the immune responses because they bind antigenic peptides of foreign or endogenous origin and present them to T-lymphocytes. The class I genes (HLA-A, -B, -C) encode for HLA I molecules, which are expressed on most nucleated cells. Peptides presented by these class I molecules are recognized by CD8+ cells responsible for cytotoxic activity and suppressor function. Class II antigens are expressed only on antigen- presenting cells (APCs): 1) professional APCs = macrophages, B-lymphocytes, activated T-lymphocytes, dendritic cells, 2) non-professional APCs = endothelial cells, thymic epithelial cells, mucosal epithelial cells. The critical event in immune recognition is to discriminate self from non-self in being able to defend against foreign agents without self-destruction. This is achieved through tolerance induction (Abbas et al 1997). Selection processes are responsible for the survival of T-cells that express only “useful” TCRs, i.e., TCRs that recognize peptides derived from foreign proteins in association with self-allelic forms of MHC molecules. In the thymus, T-cells with receptors that fail to bind the self HLA-peptide complex die from “neglect”, whereas T-cells with receptors that engage the self HLA-peptide complex receive signals that trigger a functional process called positive selection in which T-cells mature and become immunocompetent. Those peptides, bound by the HLA molecules and engaged by T-cell receptors (TCRs) during selection, are derived from self proteins within the thymic APCs. Thus there is an inherent process of generating autoreactive T-cells. To control autoreactivity, high-affinity interactions between the TCR and the HLA-self-peptide complex trigger deletion of the involved T-cells (=negative selection). This thymic T-cell “education” achieves a state called central tolerance. The lack of tolerance due to poor binding capacity of self-peptides of certain HLA class II (namely DQ) molecules may explain the association between the HLA molecules and risk for type 1 diabetes (Altmann et al 1991, Sheehy 1992, Nepom and Kwok 1998).

In the periphery, tolerance is maintained through deletion or anergy of antigen-reactive cells or through induction of certain regulatory cells. Sasazuki et al (1989) have proposed that in various human T-cell responses, dominant immune suppression genes operate, and that these gene products are generally HLA-DQ molecules which stimulate suppressor-inducer cells. Sheehy (1992) also proposed this theory concerning the HLA molecules and a failure to maintain tolerance in the periphery in type 1 diabetes. In peripheral immune responses, antigen presentation is mainly achieved through HLA-DR molecules. It is possible that in individuals susceptible to type 1 diabetes the HLA class II molecules may bind islet-cell antigen with a particularly high affinity (Nepom 1990, Nepom and Kwok 1998) or may react to environmental stimuli inappropriately, initiating or sustaining an autoimmune response (Behar and Porcelli 1995). Recent in vivo evidence for the contribution of HLA molecules to the development of autoimmune diabetes comes from a study using transgenic mice with human HLA-DQ molecules and showing that spontaneous diabetes develops in such mice (Wen et al 2000).

Of other genes associated with the risk for type 1 diabetes, the polymorphism of the insulin gene region (IDDM2) located on chromosome 11 has been implicated in disease susceptibility (Owerbach and Nerup 1982, Bell et al 1984). This IDDM2 is calculated to confer 10% of the familial clustering of the disease (Davies et al 1994). The highly polymorphic region near the human insulin gene is composed of a variable number of tandemly repeating (VNTR) sequences, and can be described as a locus with at least three classes of alleles: a common small “class I” allele averaging 570 base pairs, a rare intermediate “class II” allele of about 1,320 base pairs, and a large “class III” allele averaging 2,470 base pairs in size. In Caucasians, a strikingly higher frequency of class I alleles and genotypes containing the two class I alleles are observed in patients with type 1 diabetes, whereas two class III alleles seem to be rare (Owerbach and Nerup 1982, Bell et al 1984, Hitman et al 1985). Although relative disease risk for insulin gene region polymorphism has been reported to be increased in HLA-DR4 positive diabetics in some studies (Julier et al 1991, Lucassen et al 1993), in Finland the insulin gene region-encoded susceptibility to type 1 diabetes exerts maximal effect when there is a low HLA-DR associated risk (Metcalfe et al 1995).

The level of transcription of insulin mRNA in the pancreas correlates with the allelic variation within the VNTR so that the disease-associated haplotype (class I) produces higher insulin mRNA levels than does the negatively associated (class III) haplotype (Bennett et al 1995, Lucassen et al 1995). The persistently high rate of insulin transcription and translation may

make the β-cell more vulnerable to oxidative stress, making it more susceptible to autoimmune damage. Alternatively, insulin may be the primary autoantigen, and immunological tolerance to insulin may be broken via overproduction. Similarly, increased transcription may lead to secretion of more immunogenic forms of insulin precursors or aberrantly folded forms of the molecule, which both may lead to loss of tolerance. In addition, insulin expression in human thymus is likewise modulated by the VNTR alleles so that, in contrast to the pancreas, protective class III alleles are associated with higher insulin mRNA levels than are predisposing class I alleles (Pugliese et al 1997, Vafiadis et al 1997). This may affect central tolerance induction, because higher antigen levels in the thymus are reportedly associated with tolerance, whereas lower levels determine positive selection possibly leading to autoreactive T-cells in the periphery (Sebzda et al 1994). The possible role of other genes (altogether 15 suggested loci in several chromosomes) in type 1 diabetes susceptibility has been discussed in papers by Owerbach and Gabbay (1996) and Concannon et al (1998), but evidence as to the role of these genes in risk for type 1 diabetes is low when compared to that of the HLA molecules and the insulin gene.

Autoimmunity

Description of autoimmune diabetes

Type 1 diabetes is considered to be an autoimmune disease in which insulin-secreting β-cells are destroyed. The autoimmune nature of the disease is supported by several findings: 1) presence of activated T-cells in the inflamed islets (Bottazzo et al 1985), 2) disease association with the HLA molecules (Becker 1999), 3) efficacy of T-cell interventions in interfernce with the disease process (Carel et al 1996), 4) recurrence of the disease after pancreatic graft transplantation (Sibley et al 1985), 5) transfer of autoimmune diabetes after allogenic bone marrow transplantation (Lampeter et al 1993), 6) presence of autoantibodies (Verge et al 1996). In addition, type 1 diabetes is associated with other diseases described to be of autoimmune origin (Maclaren and Riley 1985, Savilahti et al 1986). The lesion in the diabetic pancreas was first described by Gepts (1965) – namely the striking lack of β-cells without any effect on other endocrine cells, and an inflammatory cell infiltrate termed "insulitis." Later, it was shown that the cellular infiltrate consists mainly of T- and B-lymphocytes and macrophages, of which the CD8+ T-cells appear to be most prevalent (Itoh et al 1993). Furthermore, an increased expression of HLA molecules, as a sign of inflammation, is detected in the β-cell lesion (Bottazzo et al 1985, Foulis et al 1987).

Cell-mediated immunity

Support for the role of T-cells in mediating the disease comes particularly from animal models of human type 1 diabetes, namely the non-obese diabetic (NOD) mouse and the Biobreeding (BB) rat. Purified T-cells from diabetic NOD donors transfer the disease to healthy NOD neonates, a process requiring both CD4+ and CD8+ lymphocytes (Bendelac et al 1987, Miller et al 1988). Islet-reactive T-cell clones propagated from the spleen and the lymph node cells (Haskins et al 1988) or from the cells in the insulitis lesion in the pancreas of diabetic animals (Daniel et al 1995) have been shown to induce β-cell destruction. On the other hand, development of overt diabetes can be reduced or prevented in experimental animals by in vivo administration of antibodies against T-cells (Hayward and Shreiber 1989) or CD4+ cells (Koike et al 1987, Wang et al 1987); and neonatal thymectomy (Like et al 1982) or intrathymic islet transplantation (Posselt et al 1992) in BB rats can protect from the disease. In addition, immune intervention therapies targeted against T-cells have been shown to alter the course of the disease in humans; i.e., immunosuppression by azathioprine or cyclosporin can delay the onset of the disease (Carel et al 1996) or induce transient remission (Harrison et al 1985, The Canadian-

European Randomized Control Trial Group 1988, De Filippo et al 1996). In recent-onset patients of juvenile type 1 diabetes a different immunomodulator, Linomide, which enhances T-cell function rather than having a suppressive effect and presumably generates suppressor cells, seems to improve β-cell function (Coutant et al 1998). This effect of Linomide may also be mediated through stimulation of macrophages, suppression of endogenous production of TNF-α by NOD beta cells, or induction of antigen-specific unresponsivenesss (Gross et al 1994).

The individual roles of CD4+ and CD8+ cells in beta cell destruction are still under debate. Consensus exists that in the disease process both cell types are required. In transfer experiments, diabetes is precipitated most efficiently when both CD4+ and CD8+ cell populations are used (Yagi et al 1992). Moreover, although transfer of NOD-derived activated CD4+ cell populations or clones into lymphocyte-deficient NOD recipients often results in insulitis and diabetes, an analogous transfer of NOD-derived CD8+ cells only rarely does. In a recent review, Dilts et al (1999) speculated that the CD4+ cell is the immunological effector which requires activity of the CD8+ cell, particularly in the early pathogenic process, whereas another review discusses the effector function of both cell types (Wong and Janeway 1999). NOD mice which lack beta 2-microglobulin, and thus MCH class I, are devoid of insulitis and diabetes, pointing to a role for CD8+ cells in the pathogenesis of type 1 diabetes (Wicker at al 1993). The majority of the cell infiltrate in the islets of diabetic patients consists of lymphocytes, mainly of CD8+ cells (Bottazzo et al 1985, Itoh et al 1993). It has also been shown that persistent activation of CD8+ cells characterizes prediabetic twins (Peakman et al 1996). In addition, a CD4+ cell response measured as enhanced cellular reactivity to islet antigens by in vitro proliferation test is found in diabetic patients and in individuals at risk for the disease (Durinovic-Bellò et al 1996, review by Roep 1996).

The role of B-lymphocytes in actual islet cell destruction is much weaker, because splenocytes from diabetic NOD mice depleted of B-lymphocytes are shown to be as efficient as whole spleen cells in transferring the disease to healthy neonates (Bendelac et al 1987), and adoptive transfer of autoimmune NOD mouse diabetes by T-cells does not require recruitment of host B-cells (Bendelac et al 1988). Autoantibodies produced by B-cells are considered as markers of β-cell destruction and are used for prediction of type 1 diabetes. They themselves are not considered to be able to cause the disease, because offspring of diabetic mothers with transplacentally acquired autoantibodies do not develop diabetes after birth. In a model of virus-

induced autoimmune diabetes, neither B-lymphocytes nor antibodies directed against self antigens of the islets of Langerhans were required for the development of diabetes (Holz et al 2000). Some studies in NOD mice stress, however, the contribution of B-cells that supposedly act as APCs in the disease process (Serreze et al 1996, Noorchashm et al 1997, Serreze et al 1998). In experimental animals, the role of macrophages and dendritic cells, which also act as APCs, seems to be in the initiation of β-cell inflammation (Lee et al 1988, Voorbij et al 1989, Jansen et al 1994). In addition, defects in maturation and function of APCs, found both in human and mouse autoimmune diabetes (Jansen et al 1995, Takahashi et al 1998, Serreze et al 1993), may be the basis for disturbed regulatory/suppressor cell activity and tolerance development, making an individual susceptible to cell-mediated β-cell destruction.

Th1/Th2 paradigm

T helper 1 (Th1) and Th2 patterns of cytokine production were originally described among mouse CD4+ T-cells, and later among human T-cells. Mouse Th1 cells produce interleukin-2 (IL-2), interferon γ (IFN-γ), and lymphotoxin, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Human Th1 and Th2 cells produce similar patterns, although the synthesis of certain cytokines is not as tightly restricted to a single subset as in mouse T-cells. The characteristic cytokine products of Th1 and Th2 cells are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. In addition to Th1 and Th2 cells are Th0 cells, which secrete cytokines of both patterns and which are precursors of Th1 and Th2 cells, and Th3 cells, which produce high amounts of transforming growth factor β (TGF-β) and which are linked to the development of oral tolerance (reviewed by Paul and Ceder 1994, Mosmann and Sad 1996). Th1 cells and their secreted cytokines are involved in classic cell-mediated functions such as clonal expansion of cytotoxic lymphocytes, macrophage activation, and class-switching to IgG isotypes that mediate complement lysis of sensitized cells. Accordingly, Th1 immunity functions especially in infections requiring intracellular eradication of pathogens. This Th1-type immunity is also associated with autoimmune disorders like multiple sclerosis (Voskuhl et al 1993), rheumatoid arthritis (Simon et al 1994), and type 1 diabetes (Kallmann et al 1997, Wilson et al 1998). By contrast, Th2 cells and cytokines contribute to the antibody-mediated immunity, activating B-cells to switch to neutralizing antibody and IgE. In addition, they enhance eosinophil proliferation and function. Th2 responses are thus associated with allergic manifestations (reviewed by von Hertzen 2000).

In the pathogenesis of type 1 diabetes, Th1/Th2 imbalance is under active discussion (Rabinovitch 1994, Liblau et al 1995). In NOD mice β-cell reactive T-cells and clones capable of mediating autoimmune destruction produce Th1-type cytokines IL-2 and IFN-γ, whereas Th2 cells producing IL-4 and IL-10 may be protective (Healey et al 1995, Katz et al 1995). The role of IFN-γ in disease pathogenesis is supported by the fact that in NOD mice and in BB rats the expression of this cytokine in islet infiltrating mononuclear cells (Rabinovitch et al 1995 and 1996) or in pancreatic tissue (Kolb et al 1996) is upregulated in destructive insulitis and diabetes. IFN-γ expression in the islets is also essential for disease development in a transgenic animal model (von Herrath and Oldstone 1997). In humans, IFN-γ containing lymphocytes have been found in islets of patients who died from recent-onset type 1 diabetes and in islets of one prediabetic individual (Foulis et al 1991). In addition, anti-IFN-γ antibodies can prevent diabetes in NOD mice (Debray-Sachs et al 1991). The production of IFN-γ is not restricted to CD4+ cells, but CD8+ cells are also involved, as shown during β-cell destruction in syngeneic islet grafts (Suarez-Pinzon et al 1996). In contrast, the Th2- related cytokines IL-4 and IL-10 are capable of preventing the disease in NOD mice (Rapaport et al 1993, Pennline et al 1994) by potentiation of regulatory Th2 cell function (Cameron et al 1997); and pancreatic IL-4 expression in NOD mice results in inhibitory Th2 activity and protection from diabetes (Gallichan et al 1999). Decreased IL-4 production by peripheral blood mononuclear cells has been shown in patients with newly diagnosed type 1 diabetes (Berman et al 1996). Indeed, this kind of failure in Th2-type immunity is suggested in the NOD mouse (Cameron et al 1998) and in humans (Kallmann et al 1999). The imbalance between Th1/Th2 responses may be due to the decreased number and function of normally IL-4 secreting NK-T-cells found in NOD mice (Gombert et al 1996, Baxter et al 1997) and in patients with type 1 diabetes (Wilson et al 1998). In NOD mice, diabetes can be prevented by adoptive transfer of a cell population containing NK-T-cells obtained from non-autoimmune-prone donors (Baxter et al 1997), and this effect was mediated by IL-4 and/or IL-10 (Hammond et al 1998). Later it was shown that the functional deficiency found in NOD NK-T-cells rather results from the inability of these cells to switch to the IFN-γ secreting phenotype than from the defect in IL-4 secretion. This mechanism was suggested to be involved in the pathogenesis of autoimmune diabetes, because IFN-γ may also play a critical role in regulation of T-cell immunity (Falcone et al 1999). In addition to regulatory Th2 cells, other regulatory CD4 cell populations may exist. In normal rats which also harbor potentially autoreactive T-cells, a regulatory CD4+ cell population has been described

which produces IL-2 and IL-4 without IFN-γ and has the capacity to inhibit autoimmune reactivity (Fowell and Mason 1993).

Autoantigens and autoantibodies

ICA

The first autoantibodies described in type 1 diabetes were the islet cell antibodies (ICA) which were discovered when sera from patients with polyendocrine autoimmune disease including type 1 diabetes reacted with islets in frozen sections of human pancreas (Bottazzo et al 1974). ICA were found to be predictive markers of type 1 diabetes in first-degree relatives of patients with the disease (Bonifacio et al 1990) and in a general population with a high background incidence (Karjalainen 1990). It was later discovered that GAD (Atkinson et al 1993a) and IA-2 autoantibodies (Bonifacio et al 1995) contribute to the ICA-reactivity in sera of patients with newly diagnosed type 1 diabetes. In addition, this ICA-response comprises reactivity to other, yet not recognized β-cell antigens distinct from GAD and IA-2.

Table 1. Suggested autoantigens in type 1 diabetes (T1D) (modified from Atkinson and Maclaren 1993b)

Autoantigen	Characteristics
Insulin	β-cell-specific, target of IAA in human T1D and in animal models of autoimmune diabetes, cellular reactivity to insulin both in human and in NOD mouse diabetes, disease-modifying antigen
GAD65/GAD67	Synaptic-like microvesicle protein, target of 64kD antigen/GAD antibody in human T1D and in animal models of diabetes, cellular reactivity to GAD in human T1D and in animals models, disease-modifying antigen
IA-2/ICA512	Target of autoantibodies in human T1D, cellular immune antigen, identified by immunoscreening of islet cDNA, relation to 37K/40K antigen
ICA69	Target of autoantibodies in human T1D, cellular immune antigen, molecular mimic with bovine serum albumin
Glucose transporter	Target of autoantibodies in human T1D, GLUT-2 directed?
Heat shock protein 65	Target of autoantibodies and cellular immunity in NOD mice, contains p277 peptide, disease-modifying antigen
Carboxypeptidase H	Target of autoantibodies in human T1D, identified by immunoscreening of islet cDNA, neurosecretory granule protein
Ganglioside	Pancreatic islet cell antigen, target of autoantibodies in prediabetics and in NOD mice
Imogen 38 (38kD)	Mitochondrial antigen, cellular and humoral immune antigen in human T1D

Insulin

Of the suggested autoantigens in type 1 diabetes (Table 1) (Harrison 1992, Atkinson and Maclaren 1993b), the major ones are thought to be insulin, GAD, and IA-2 (reviewed by Leslie et al 1999, Wegmann and Eisenbarth 2000), of which insulin is the only β-cell specific antigen. Insulin is a 5.8 kD protein hormone, the most important regulator of fuel metabolism, together with glugacon. It is synthesized from a prehormone, proinsulin consisting of A- and B-chains and a C-peptide. The cleavage of the C-peptide results in an insulin molecule. Insulin is stored in secretory granules in the β-cells as hexamers, and the secretion is stimulated by glucose and the parasympathetic nervous system. It is an essential hormone, the shortage of which leads to a catabolic state and death, which was the clinical picture of type 1 diabetes before insulin therapy. In 1921, insulin was for the first time given to a few "preliminary patients", and 2 years later its large-scale production was started in the USA and Germany. This insulin was extracted from animal pancreas, and only at the end of the 1970s was biosynthetic human insulin brought on market. Although the amino acid sequence of insulin is well preserved between animal species, antibodies to animal insulins existed in insulin-treated patients, and the most immunogenic was beef insulin (Kurtz et al 1980), which differs from human insulin (HI) by three amino acids (A8, A10 and B30). When HI is used for treatment, antibody levels are lowest (Kumar 1993).

Insulin autoantibodies (IAA) were first found in 1983 in patients with newly diagnosed type 1 diabetes who had not yet received insulin therapy (Palmer at al 1983). These autoantibodies are commonly detected in diabetic patients, and predict the disease when combined with other islet cell antibodies (Srikanta et al 1986, Bingley et al 1994). Autoantibodies to proinsulin have also been detected in patients with type 1 diabetes before insulin treatment (Kuglin et al 1988). IAA are most frequent in young children affected with the disease (Arslanian et al 1985), and are most often the first autoantibodies to appear in subjects with beta cell autoimmunity (Ziegler et al 1999, Yu et al 2000). In addition, IAA levels correlate with the rate of progression to type 1 diabetes (Eisenbarth et al 1992), making IAA a good marker of ongoing autoimmunity. An association of IAA with the HLA DR4 haplotype has been described (Ziegler et al 1991). However, cellular reactivity to insulin is more complicated to measure, though enhanced responses of peripheral blood mononuclear cells (PBMC) to insulin or proinsulin have been detected, according to some studies, in prediabetic individuals (Keller at al 1990, Dubois-Laforgue et al 1999) and in patients with newly diagnosed type 1 diabetes before insulin treatment (MacCuish et al 1975). A HLA-DR-restricted human insulin-specific T-cell clone

with a Th1/Th0 cytokine profile has also been isolated and characterized from the peripheral blood of a patient with recent onset type 1 diabetes, providing evidence of insulin immunity at cellular level (Schloot et al 1998).

In NOD mice, IAA are also detected at an early age (Michel et al 1989) and are associated with early development of diabetes (Yu et al 2000). Interestingly, they are detected more often and at higher levels in the more diabetes-prone female NOD mice than in the males (Michel et al 1989). Moreover, it has been shown that the majority of islet infiltrating lymphocytes are insulin-reactive in prediabetic NOD mice (Wegmann et al 1994a), and that T-cell clones made from these insulin-specific cells are either capable of accelerating diabetes in young NOD mice or of inducing diabetes in NOD/SCID mice in adoptive transfer experiments (Daniel et al 1995). In addition, insulin-specific T-cells (Th1-like) are present in islet infiltrates from the early stages of insulitis until development of diabetes, pointing to a role for insulin in the pathogenesis of autoimmune diabetes and especially in the early phases of the disease process (Wegmann et al 1994b). A CD8+ T-cell clone that transfers diabetes in NOD mice has also been shown to recognize the same B-chain peptide of insulin (Wong at al 1999). In fact, tolerization to insulin in animal models by oral (Zhang et al 1991), intravenous (Hutchings and Cooke 1995), nasal (Daniel and Wegmann 1996, Harrison et al 1996), or subcutaneous routes (Gotfredsen et al 1985, Atkinson et al 1990, Daniel and Wegmann 1996) results in reduction of insulitis and diabetes incidence.

Glutamic acid decarboxylase (GAD)

Sera from newly diagnosed diabetic children were found to immunoprecipitate a protein having a molecular weight of 64,000 (64K) from detergent lysates of human islet cells (Baekkeskov et al 1982), and these anti-64K antibodies preceded the clinical onset of type 1 diabetes (Baekkeskov et al 1987). The 64K autoantigen was later characterized as the smaller isoform of glutamic acid decarboxylase (GAD65), an enzyme that catalyzes the synthesis of the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Baekkeskov et al 1990). Indeed, glutamate decarboxylase exists in two distinct isoforms, as GAD65 and GAD67 with molecular masses of 65 kDa and 67 kDa. This enzyme is mainly expressed in mammalian organisms in pancreatic β-cells and in GABA-secreting neurons in the brain. The tissue distribution of the two isoforms differs, however; in humans only GAD65 is expressed in the islet cells, whereas in the mouse the predominant form is GAD67 with little or undetectable GAD65, and in the rat both GAD isoforms are detected in the islets, with a predominance of GAD65. In all three

species, both GAD isoforms are expressed in the brain (Kim et al 1993). It is generally speculated that GAD may be an "endocrine transmitter" in the β-cells.

The role of GAD as an autoantigen in type 1 diabetes is supported by findings on GAD autoantibodies (Baekkeskov et al 1982 and 1987) and on T-cell reactivity to GAD in prediabetic individuals and in patients with newly diagnosed disease (Atkinson et al 1992). GAD autoantibodies (GADA) are found in the majority of patients with newly diagnosed type 1 diabetes, with a predominance in older children and adolescents (≥10 years) (Sabbah et al 1996), and GADA possess predictive value for the disease typically in older relatives of patients with type 1 diabetes (Greenbaum et al 1999). Accordingly, GADA are frequently found in type 1 diabetic patients diagnosed at an older age, and are not restricted to type 1 diabetes, but also appear in patients with type 2 diabetes (Littorin et al 1999) and in women with gestational diabetes (Füchtenbusch et al 1997). However, they also predict insulin-dependency in these patients. GADA are more prevalent in diabetic children with the HLA DR3 phenotype (Hagopian et al 1995) and in girls with the disease (Sabbah et al 1996). In addition, GADA are also detectable in patients with stiff-man syndrome and with autoimmune polyendocrine disease type I (APD I) (Björk et al 1994). These two rare autoimmune disorders are, however, associated with type 1 diabetes, and the prevalence of GADA is reported to be higher in those patients with stiff-man syndrome who also have type 1 diabetes (Solimena et al 1990) and also in APD-I patients with type 1 diabetes (Tuomi et al 1996). Even so, the high titers of GADA typical in stiff-man syndrome and also those found in APD I do not necessarily coincide with type 1 diabetes (Solimena et al 1990, Björk et al 1994).

In NOD mice, anti-GAD reactivity is detected very early in the disease process (Kaufman et al 1993, Tisch et al 1993), and GAD-reactive CD4+ Th1 cell lines are able to adoptively transfer insulitis and diabetes in NOD/SCID mice (Zekzer et al 1998). In addition, administration of GAD65 or its immunogenic peptides either intrathymically, intravenously, or intranasally to young NOD female mice can prevent insulitis and diabetes (Kaufman et al 1993, Tisch et al 1993, Tian et al 1996a).

Protein tyrosine phosphatase-like islet cell antigens IA-2 and IA-2β

A second islet membrane-associated autoantigen, distinct from GAD65, was found after trypsin treatment of immunoprecipitates from islet-membrane detergent lysates. The majority of patients with type 1 diabetes have antibodies to these 37/40K tryptic fragments of an islet cell antigen (Christie et al 1990, Christie et al 1993). An islet cell autoantigen (ICA) 512 (also known as IA-2) was independently identified by two groups as an autoantigen in type 1 diabetes by the following methods: 1) by screening a human islet cDNA expression library with a pool of type 1 diabetic patients´ sera (Rabin et al 1994), and 2) by searching for clones enriched in a human insulinoma cDNA library obtained by subtracting glucagonoma cDNAs (Lan et al 1994). The next year IA-2 was identified as the diabetes-related 37/40K autoantigen (Bonifacio et al 1995, Passini et al 1995, Payton et al 1995). IA-2 is a member of the protein tyrosine phosphatase family with a 3.6 kb cDNA showing a 979 amino acid protein homologous to the protein tyrosine phosphatase-2 (Lan et al 1994). It is a transmembrane protein found to have no enzymatic activity (Rabin et al 1994). Its exact biological role is still unknown. IA-2 is primarily expressed in neuroendocrine cells such as the islets of Langerhans and many parts of the central nervous system. The majority of newly diagnosed type 1 diabetic patients have autoantibodies to IA-2, and they can be used for prediction of the disease (Hawa et al 1997, Savola et al 1998, Leslie et al 1999). Contrary to initial expectation, none of the sera from patients with type 1 diabetes reacted with the extracellular domain, instead they all precipitated with the intracellular domain (Notkins et al 1997, Zhang et al 1997). The frequency of IA-2 antibodies varies with age and HLA genotype, being highest in the younger age groups (Hawa et al 1997) and in patients with the HLA DR4 alleles (Genovese et al 1996). In addition, enhanced T- cell responses to ICA512/IA-2 have been described in newly diagnosed patients and in individuals at risk for the disease (Durinovic-Bellò et al 1996, Ellis et al 1998a), and T-cell lines reactive to IA-2 have been generated from PBMC of children with newly diagnosed type 1 diabetes (Hawkes et al 2000).

Later, a second novel protein, a tyrosine phosphatase-like molecule, IA-2β, was isolated and shown to be an important autoantigen in type 1 diabetes (Lu et al 1996, Notkins et al 1997, Li et al 1997). It is similar in many respects to IA-2, especially in its intracellular domain, which is 74% identical to IA-2, and IA-2β is also enzymatically inactive, like IA-2. Overall, between 35 and 50% of patients with type 1 diabetes have autoantibodies to IA-2β (Lu et al 1996, Notkins et al 1997, Li et al 1997), which is considerably less than the 55 to 75% with autoantibodies to IA-

2. Moreover, since more than 95% of type 1 diabetic patients who have IA-2β antibodies also have antibodies to IA-2, screening for autoantibodies to IA-2β does not offer much advantage for disease prediction. In short, the best predictive value is reached with a combination of all these four autoantibodies (Bingley et al 1994, Verge et al 1996, Kulmala et 1998).

Environmental factors

Environmental factors are thought to play a role in the pathogenesis of type 1 diabetes in addition to genetic factors (reviewed by Leslie and Elliott 1994, Åkerblom and Knip 1998, Najk and Palmer 1999), because genetics alone cannot explain, for example, the less than 35% concordance rate in identical twins (Olmos et al 1988, Kaprio et al 1992) or the ever-rising incidence of type 1 diabetes worldwide (EURODIAB ACE Study Group 2000). The existence of temporal variation with a lower number of new cases (Karvonen et al 1993) during warm summer months than in the cooler winter months points to some environmental effect, as well. In addition, studies in transmigratory populations also suggest a role for environmental factors in type 1 diabetes (Elliott 1992, Bodansky et al 1992). These environmental factors include dietary triggers and viral infections.

Dietary factors

Cow`s milk proteins

The first two publications raising an interest in the possible association of cow´s milk (CM) proteins and type 1 diabetes were a BB rat study (Elliott and Martin 1984) and a breast feeding survey (Borch-Johnsen et al 1984). Since then, support for the role of CM proteins in the etiology of type 1 diabetes has accumulated from ecological and epidemiological studies as well as from studies in experimental animals. In addition, findings in humans on enhanced cellular and humoral immune responses to CM proteins in type 1 diabetes have suggested possible involvement of CM proteins in the pathogenesis of the disease or more likely in the mechanisms explaining the disease. An abrupt change in the incidence of type 1 diabetes after the migration of Polynesians to New Zealand was suggested most likely to result from the early introduction of dairy products into the infant diet, accompanied by an earlier weaning in New Zealand (Elliott 1992). A positive correlation has been reported between per capita consumption of unfermented milk proteins in populations worldwide (Scott 1990) or fluid cow milk consumption in children aged 0 to 14 years and incidence of type 1 diabetes (Dahl-Jørgensen et al 1991, Fava et al 1994). In addition, countries with the lowest prevalence of breast feeding at 3 months of age had the highest incidence of the disease (Scott 1990).

Many studies concerning the relationship between duration of breast feeding risk of type 1 diabetes have found an inverse association, but also found none or positive association (Table 2). Arguments against an association between breast feeding and risk for type 1 diabetes point to the fact that the incidence of type 1 diabetes is rising despite the increasing prevalence of breast feeding. In Finland, however, exclusive breast feeding in particular has decreased so that at 3 months less than 30% of infants are receiving only breast milk (Sairanen et al 1997). Indeed, Virtanen et al (1993) separated the association of breast feeding and risk for type 1 diabetes and found that introduction of dairy products at an early age was the most important risk factor. Two meta-analyses of case control studies confirmed that patients with type 1 diabetes were more likely to have been breast fed for < 3 months and to have been exposed to CM formula before 3 to 4 months of age when compared to healthy controls, and concluded that early CM exposure may increase the risk approximately 1.5 times (Gerstein 1994, Norris and Scott 1996). This risk related to early CM exposure was shown to be higher (~13) in individuals at high genetic risk (Kostraba et al 1993, Pérez -Bravo et al 1996). In addition, CM consumption in later childhood may also increase risk for type 1 diabetes (Verge et al 1994, Virtanen et al 1998 and 2000).

Table 2. Association between the duration of breast feeding and the risk for Type 1 diabetes (modified from Åkerblom and Knip 1998)

Inverse association	***No association***	***Positive Association***
Borch-Johnsen et al. 1984	Fort et al 1986	Nigro et al 1985
Glatthaar et al. 1988	Siemiatycki et al 1989	Kyvik et al 1992
Mayer et al. 1988	Kostraba et al 1992	Meloni et al 1997
Blom et al. 1989	Samuelsson et al 1993	
Dahlquist et al. 1991	Patterson et al 1994	
Virtanen et al. 1991	Bodington et al 1994	
Virtanen et al. 1992	Soltész et al 1994	
Metcalfe & Baum 1992	Thorsdottir et al 2000	
Kostraba et al.1993		
Verge et al. 1994		
Perez-Bravo et al 1996		
Gimeno & de Souza 1997		

The effect of diet on diabetes risk has been shown in experimental animals. The first study to suggest a dietary trigger in the BB rat model showed that the normal 50% incidence of diabetes in the colony was reduced to 15% in rats fed a semi-synthetic amino acid diet, and that CM supplementation to this diet restored the incidence of diabetes to 52% (Elliott and Martin 1984). A subsequent study in BB rats showed that the deleterious effect of CM proteins is established during a relatively narrow period at an early age (weaning period) (Daneman et al 1987). Similar experiments using a hydrolyzed CM protein-based formula for diabetes prevention have confirmed these results that exposure to intact CM proteins is a risk factor for autoimmune diabetes in the other animal model NOD mouse, as well (Elliott et al 1988, Coleman et al 1990). In both animals, the opposite findings also exist showing no effect from CM proteins on diabetes incidence (Malkani et al 1997, Paxson et al 1997).

Children with newly diagnosed type 1 diabetes have been reported to have increased levels of CM antibodies, particularly IgA and IgG antibodies to CM protein and to β-lactoglobulin (BLG) (Savilahti et al 1988, Dahlquist et al 1992, Savilahti et al 1993), while findings on anti-bovine serum albumin (BSA) antibodies are more controversial (Karjalainen et al 1992, Atkinson et al 1993c). IgA antibodies to BLG and CM have been independently shown to associate with increased risk for type 1 diabetes (Dahlquist et al 1992, Virtanen et al 1994a). In addition, differences in CM protein antibodies as well as BLG antibodies are more pronounced among children with an early onset (<3-4 years) of type 1 diabetes (Dahlquist et al 1992, Savilahti et al 1993). In a Finnish study, on 42 sibpairs identical for HLA-associated risk alleles, children with diabetes still had higher levels of CM antibodies than did their non-diabetic siblings (Saukkonen et al 1998). On the other hand, no difference was detected in antibodies to another dietary component, ovalbumin between children with newly diagnosed type 1 diabetes and healthy control children (Saukkonen et al 1994), but eggs are introduced to the infant diet much later than is CM. Studies on T-cells have confirmed the altered immunity to CM proteins in patients with newly diagnosed type 1 diabetes. These patients show enhanced responses of their PBMCs to BSA, BLG, and β-casein (Cheung et al 1994, Vaarala et al 1996, Cavallo et al 1996), although the specificity of these responses to type 1 diabetes has been questioned (Atkinson et al 1993c, Ellis et al 1998b). In sum, these findings have been suggested to result from one or more of the following: dysregulation of oral tolerance to CM proteins, a changed pattern of CM consumption, or altered permeability of the gut in children who will develop type 1 diabetes (Savilahti et al 1988). The exact mechanisms as to how CM proteins participate in the disease

process are unknown, but molecular mimicry has been suggested between individual CM proteins and tissue structures (Martin et al 1991, Karjalainen et al 1992, Cavallo et al 1996).

Plant proteins

Soy- and gluten- containing diets have been shown to be diabetogenic at an early age both in NOD mice (Hoorfar et al 1993) and in BB rats (Hoorfar et al 1991), and the effect of these has been considered at least as strong as the effect of CM proteins on diabetes incidence of the animals. Indeed, a gluten-free diet is able to prevent diabetes in NOD mice (Funda et al 1999). In addition, in one BB rat study, long-term exposure to a plant-based diet later in life, particularly around puberty, was an important risk factor for diabetes (Scott et al 1997) suggesting that later exposure to a dietary antigen, not just early exposure predisposes to autoimmune diabetes. In humans there is scant evidence that gluten is an important dietary trigger in autoimmune diabetes, although low cellular reactivity to gluten has been shown in patients with newly diagnosed type 1 diabetes without celiac disease (Klemetti et al 1998). Of interest, however, is that prevalence of autoimmune disorders including type 1 diabetes in patients with celiac disease was related to the duration of gluten exposure, i.e., the longer the exposure the higher the prevalence (Ventura et al 1999).

Nitrates and nitrites

Nitrates and nitrites are implicated as risk factors for type 1 diabetes. Parental ingestion at conception of N-nitroso compounds in smoked/cured mutton resulted in an increased incidence of diabetes in their male offspring (Helgason and Jonasson 1981). This finding has been confirmed by experiments in an animal model (Helgason et al 1982). Nitrosamine compounds in the diet were shown to increase the risk for childhood diabetes in a prospective population-based case control study (Dahlquist et al 1990). In addition, nitrate levels in community drinking water (Kostraba et al 1992) as well as dietary nitrite intake of children and their mothers (Virtanen et al 1994b) have been associated with the risk for type 1 diabetes. The fact that nitrates and nitrites may play a role in the etiology of diabetes relates to the endogenous formation of diabetogenic nitrosamines from them as has been shown in mice: N-nitroso compounds can cause immune-mediated diabetes (Kolb-Bachofen et al 1988).

Vitamin D deficiency

Vitamin D deficiency in childhood has been associated with the risk for type 1 diabetes and other autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis (reviewed by

Cantorna 2000). An ecological study within counties in Sweden showed an inverse correlation between a low number of sunshine hours and incidence of type 1 diabetes, suggesting a possible role for vitamin D, because ultraviolet light on the skin is the major source of vitamin D (Dahlquist and Mustonen 1994). Accordingly, the north-to-south gradient in type 1 diabetes incidence may at least in part be explained by vitamin D deficiency. Indeed, vitamin D supplement in early childhood was reported to be protective in a multinational case-control study (The EURODIAB Substudy 2 Study Group 1999). Vitamin D receptors have been identified in peripheral blood monocytes and in activated lymphocytes, a finding that has created interest in vitamin D as an immune system regulator (Provvedini et al 1983). The active form of vitamin D {1.25 $(OH)_2D_3$} has been shown to inhibit T-cell proliferation and to decrease production of Th1 cytokines IL-2, IFN-γ, and TNF-α in vitro. In addition, this hormone promotes suppressor cell activity and inhibits generation of cytotoxic cells in vitro (reviewed by Lemire 1992). In NOD mice, prevention of insulitis and diabetes was achieved by starting activated vitamin D supplementation early on, before insulitis, an effect shown to result from induction of certain suppressor cells (Mathieu et al 1992 and 1994). In addition, activated vitamin D analogs induced an immune shift locally in the inflamed islets of the native pancreas as well as in the transplanted islet grafts in NOD mice, a shift characterized by downregulation of Th1 cytokines (Casteels et al 1998). Likewise, in NOD mice immunized with GAD65 peptide, vitamin D analog supplementation induced an immune deviation (Th1 to Th2 shift) in the peripheral immune system after rechallenge, and these results led the authors to suggest a possible role for vitamin D in prevention of type 1 diabetes (Overbergh et al 2000).

Viral infections

Viral infections have been associated with type 1 diabetes, namely congenital rubella (Menser et al 1978), cytomegalovirus infection (Pak et al 1988), mumps (Gundersen 1927, Ludvigsson et al 1988), and especially mucosal-associated infections, e.g., enterovirus (Friman et al 1985, Frisk et al 1992) and rotavirus infections (Honeyman et al 2000). Several different mechanisms by which viral infections may mediate β-cell destruction are described (reviewed by Szopa et al 1993) including direct β-cell lysis (Yoon et al 1979, Roivainen et al 2000), persistent infection (Chehadeh et al 2000) and/or initiation and sustenance of the autoimmune reaction against β-cells (Ludvigsson et al 1988, Horwitz et al 1998) and more specifically molecular mimicry (Kaufman et al 1992, Honeyman et al 1998).

The role of enterovirus infections in the pathogenesis of type 1 diabetes has received attention (reviewed by Szopa et al 1993, Graves et al 1997). Direct involvement of Coxsackie B viruses in β-cell damage occurred in children who died of well-documented overwhelming Coxsackie B virus (CBV) infections (Jenson et al 1980), and the CBV4 in a pancreas isolate of a child with acute-onset diabetes was able to induce diabetes in mice (Yoon et al 1979). However, other mechanisms by which CBV infections may affect the disease process, like molecular mimicry, are thought to be more plausible, partly because no signs of enterovirus RNA were detected in the pancreases of patients who had died of recent-onset type 1 diabetes (Foulis et al 1997). A homologous sequence has been discovered between GAD65 and the 2C protein of CBV4 (Kaufman et al 1992). This sequence is also present in enterovirus serotypes other than CBV4 and has been shown to be a T-cell epitope both in man (Atkinson et al 1994) and in the NOD mouse (Kaufman et al 1993), and to induce immunological cross-reactivity between GAD65 and 2C at antibody level both in man and in mice (Hou et al 1994, Lönnrot et al 1996) and at T-cell level in mice (Tian et al 1994). In addition, in patients with recent-onset type 1 diabetes a relation has been shown between T-cell responses to GAD65 and to purified CBV4 without the homologous peptide of 2C protein, pointing to a role for enterovirus infections in general and for the gut immune system in type 1 diabetes (Klemetti et al 1999). A high frequency of CBV-specific IgM responses in patients with newly diagnosed type 1 diabetes has been reported in epidemiological studies (Banatvala et al 1985, Friman et al 1985). Moreover, seasonal variations in CBV infections and in type 1 diabetes are known to be similar (reviewed by Barret-Connor 1985). This association between enterovirus infections and type 1 diabetes has been confirmed in large prospective studies which have implied a role for enteroviruses in initiation and/or acceleration of the disease process (Dahlquist et al 1995, Hyöty et al 1995, Hiltunen et al 1997). An increased risk for type 1 diabetes has been associated with viral infections occurring long before manifestation of clinical disease, even associated with infections in utero. Findings on CBV and enterovirus RNA in serum of patients with new-onset disease and of prediabetic individuals have provided further evidence on the role for enterovirus infections in type 1 diabetes (Clements et al 1995, Lönnrot et al 2000a).

ADHESION MOLECULES

Adhesion molecules are needed for lymphocyte activation (T-cell differentiation and proliferation, T-cell dependent B-cell activation, activation of cytotoxic cells) and for trafficking cells. They also play a role in embryogenesis (reviewed by Adams and Shaw 1994). T-cells that have not yet encountered an antigen are termed naive and migrate almost exclusively through lymph nodes and other secondary lymphoid tissues. After activation by a specific antigen, the T-cell enters a resting state as a memory cell, primed to respond rapidly to the same specific antigen the next time an encounter occurs. The memory T-cell acquires a distinct ensemble of adhesion molecules on its surface that allows it to migrate preferentially through a particular tissue similar to or related to the site at which it was first activated, performing ongoing immune surveillance. This lymphocyte migration into a tissue is regulated by adhesion to endothelium; accordingly the phenotype and the function of the endothelium also differ between tissues and will regulate the influx of lymphocytes to a particular site. However, inflammation augments the influx of T-cells and reduces the selectivity that normally governs homing (Picker et al 1994).

The three major classes of adhesion molecules are based on their molecular structure: selectins, integrins, and the immunoglobulin superfamily (Picker 1994). The major tissue-specific adhesion molecule pairs are: 1) L-selectin and its endothelial counter-receptor, peripheral lymph node addressin (PNAd) which mediate lymphocyte homing prefentially to peripheral lymph nodes, 2) $\alpha 4\beta 7$ integrin and its endothelial ligand, mucosal vascular addressin (MAdCAM-1) which traffick lymphocytes mainly to gut-associated tissues, Peyers patches (PPs), mesenteric lymph nodes (MLNs), and intestinal lamina propria, and 3) skin homing receptor, cutaneous lymphocyte-associated antigen (CLA) and its endothelial ligand E-selectin. Adhesion receptor pairs that are more nonspecific include lymphocyte function-associated antigen-1 (LFA-1) and its ligand intercellular adhesion molecule-1 (ICAM-1), which pair also mediates lymphocyte activation (Fig. 1). In addition, the pair $\alpha 4\beta 1$ integrin and vascular cell adhesion molecule-1 (VCAM-1) exists, of which VCAM-1, in addition to ICAM-1, is associated with chronic inflammation. In addition to tissue specificity, selectins are involved in the first steps of the adhesion cascade, while integrins mediate a strong adhesion to endothelium (Fig. 2).

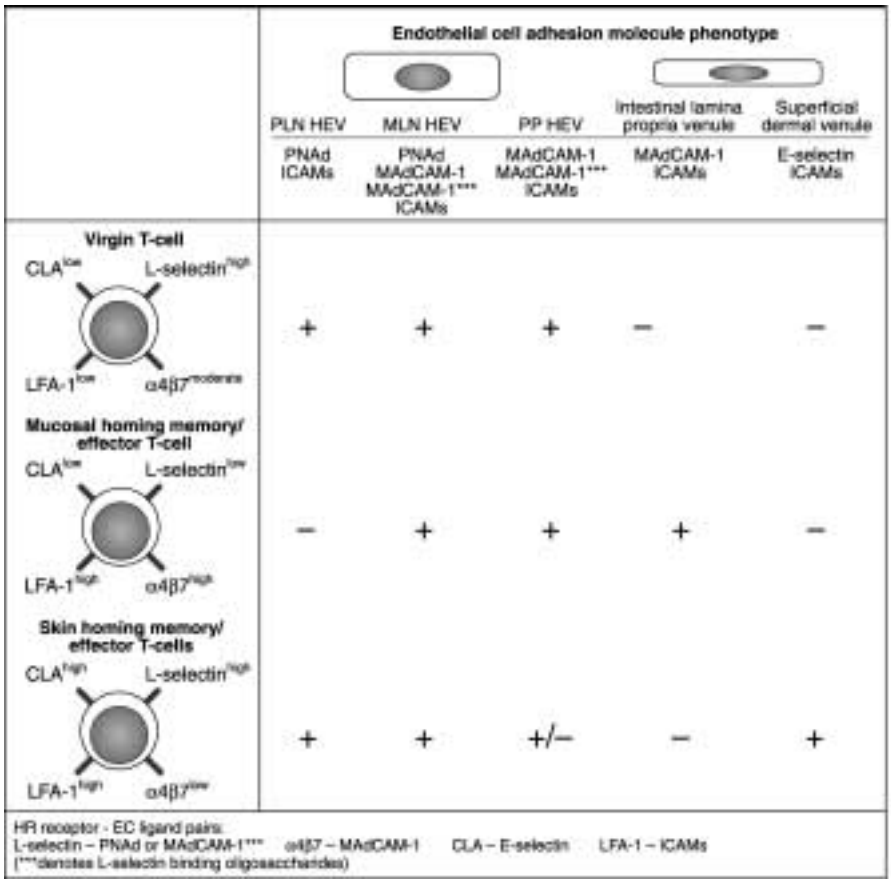

Figure. 1 A model of differential T-cell homing to secondary lymphoid tissues and to mucosal vs peripheral tertiary sites (modified from Picker 1994). CLA, cutaneoues lymphocyte-associated antigen; HEV, high endothelial venule; ICAM, intercellular adhesion molecule; LFA-1, lymphocyte function-associated antigen; MAdCAM-1, mucosal vascular addressin-1; MLN, mesenteric lymph node; PLN, peripheral lymph node; PNAd, peripheral lymph node addressin; PP, Peyers patches.

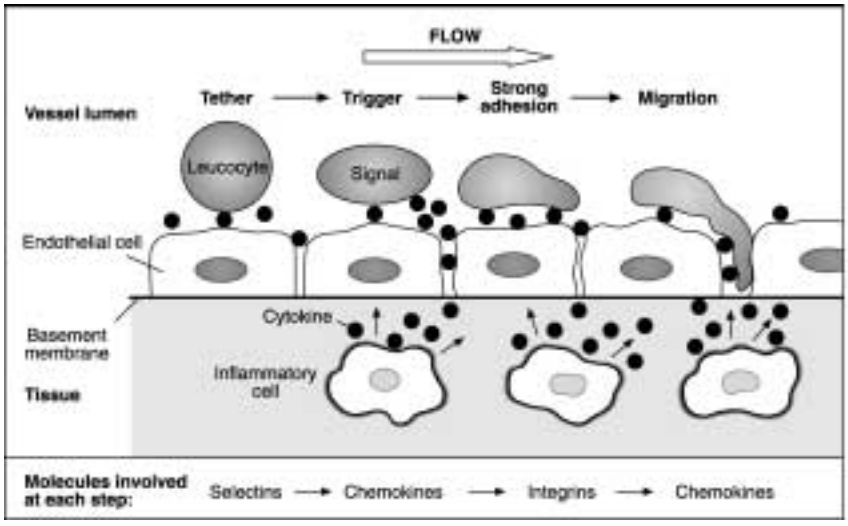

Figure. 2 Sequential steps in adhesion of leucocytes to endothelium (modified from Adams and Shaw 1994).

Gut-homing of lymphocytes is a distinct entity both in rodents and in man (Berlin et al 1993, Hamann et al 1994, Schweighoffer et al 1993, Erle et al 1994). The pairing of the gut-associated homing receptor α4β7 integrin with the mucosal endothelial ligand MAdCAM-1 which is expressed on high endothelial venules of mucosal lymphoid organs (PPs, MLNs) and on gut lamina propria venules is the major route directing normal lymphocyte traffic into the gut-associated lymphoid tissue (GALT). In addition, this lymphocyte trafficking is involved in inflammatory conditions in the gut as well. Accordingly, administration of anti-α4 integrin monoclonal antibody was shown in primates to attenuate acute spontaneous colitis (Podolsky et al 1993). In another model, in murine experimentally induced colitis, a concordant increase in MAdCAM-1 expression was associated with increased cellular infiltrates in the areas of intestinal inflammation (Viney et al 1996). Most or all naive lymphocytes express α4β7 integrin, and the intensity of expression of this homing receptor correlates with the activation of cells. In the GALT, activation of cells results in upregulation of this homing-receptor while downregulation of other receptors occurs (Mackay et al 1992, Berlin et al 1993, Picker 1994). However, it has also been shown that this receptor may be a lymphocyte activation marker (Lazarovits et al 1984). Support for the evidence that α4β7 integrin is associated with mucosal homing of lymphocytes in humans comes from two studies. Rott et al (1997) reprted that after natural rotavirus infection circulating CD4+ cells with memory for intestinal rotavirus expressed mucosal homing receptor α4β7. In another study, children with CM allergy had increased expression of this mucosal integrin in their BLG-reactive peripheral CD4+ lymphocytes (Eigenmann et al 1999).

The role of adhesion molecules in the development of type 1 diabetes has recently received attention (reviewed by Yang et al 1996). Vascular endothelium of the inflamed islets in NOD mice has been shown to express ICAM-1, MAdCAM-1, and PNAd (Hänninen et al 1993a, Faveeuw et al 1994, Yang et al 1994). Moreover, in such mice, counter-receptors of these: LFA-1, α4β7, and L-selectin, mediate lymphocyte homing to the pancreas (Hänninen et al 1993a and 1996a, Fabien et al 1996). Accordingly, inhibition of insulitis and prevention of diabetes, though at different magnitudes, has been achieved by treating NOD mice with monoclonal antibodies to these homing receptors as well as to their ligands ICAM-1 (Hasegawa et al 1994, Yang et al 1994, Yang et al 1997) and MAdCAM-1 (Yang et al 1997, Hänninen et al 1998). In addition, in NOD mice, soluble forms of ICAM-1 have inhibited insulitis and the onset of autoimmue diabetes (Martin et al 1998). The endothelium of the inflamed islets in a child who died shortly

after diagnosis of type 1 diabetes strongly expressed ICAM-1 but no PNAd (Hänninen et al 1992), and a T-cell line propagated from the islet-infiltrating cells was found to be both α4- and LFA-1-positive but negative for L-selectin (Hänninen et al 1993b), suggesting a different role for various adhesion molecules in human autoimmune diabetes. Lastly, increased levels of soluble adhesion molecules (sICAM-1 and sL-selectin) have been described in type 1 diabetes and also in subjects at risk for the disease, levels thought to reflect an ongoing immune activation (Lampeter et al 1992, Toivonen et al 2001).

GUT IMMUNITY AND ORAL TOLERANCE

Gut immune system

Structure and function

The intestine forms a barrier not only against invading agents but also againts dietary antigens and commensal bacteria, although allowing transport of nutrients across the epithelium. The mucosal barrier consists of an extrinsic barrier with non-specific defense mechanisms, i.e., gastrointestinal acidity, digestive enzymes, mucus, and peristalsis, and an immunological barrier with secretory IgA and IgM, whereas the intrinsic barrier is based on the integrity of the intestine itself. Uncontrolled penetration of antigens is one mechanism which may initiate pathological processes that may lead either to gastointestinal disease states or even to systemic manifestations of disease, for example food-induced ezcema in infancy (reviewed by Sanderson and Walker 1999).

The lymphoid tissues associated with the intestine are continuously exposed to antigens and are the largest part of the immune system (as reviewed by Mowat and Viney 1997). Lymphocytes are found in organized tissues such as Peyer´s patches (PP) and mesenteric lymph nodes (MLN) as well as scattered throughout the lamina propria and epithelium of the mucosa itself. The lamina propria contains most components of the immune system, with large numbers of B-cells, plasma cells, macrophages, dendritic cells, and T-cells of both CD4+ and CD8+ subsets. Approximately 10 to 15% of the cells in the normal epithelium are lymphocytes, the so called intraepithelial lymphocytes (IELs), of which >90% are T-cells and the majority of these (~80%) are of the CD8+ subset. A peculiar characteristic of IELs is that a proportion of these cells express γδ TCR (<10% in the small intestine, 40% in the colon in humans), and considerable expansion of these cells occurs in patients with active celiac disease or in individuals with food-induced gastrointestinal symptoms (Savilahti et al 1990, Kokkonen et al 2000). Indeed, postulated functions of mucosal γδ TCR+ T-cells include regulation of immune responses to food antigens via IFN-γ, production of epitheliotropic cytokines, stimulation of epithelial renewal during infection/enteropathy, and help for IgA antibody production.

Antigen presentation in the mucosal immune system is performed either by professional APCs (dendritic cells or macrophages) or by epithelial cells. After activation in the GALT, lymphocytes decrease their expression of L-selectin and upregulate the α4β7 integrin in order to be able to interact with the mucosal counter receptor MAdCAM-1 and thus recirculate within

mucosal tissues (Berlin et al 1993, Farstad et al 1995), whereas IELs expressing the αeβ7 integrin are thought to reside in the mucosa permanently. Cross-talk between epithelial cells and the mucosal lymphocytes (especially E-cadherin-αeβ7 interaction) and interaction of local immune cells, epithelial cells, and APCs may be critical for the induction of tolerance and expression of active mucosal immunity. In contrast to the PPs and MLNs, which probably are the principal sites for T- and B-cell priming, the lymphocytes in lamina propria and the IELs are the effector arm of the local response, inducing tolerance rather than immunity. Antigen presentation by epithelial cells, which do not under normal conditions express costimulatory molecules, may lead to down-regulation of T-cell responses, while under pathological conditions (inflammatory or infectious), activation of IELs occurs (reviewed by Mowat and Viney 1997).

Oral tolerance

The GALT is a well-developed immune network that not only evolved to protect the host from ingested pathogens, but also developed the inherent property of preventing the host´s pathological reactions to ingested dietary proteins. It is unique, as it favors the induction of cells which secrete suppressive and regulatory cytokines such as TGF-β or IL-4/IL-10, which provoke the induction of oral tolerance (reviewed by Weiner 1997). The term oral tolerance refers to systemic immunological unresponsiveness following oral antigen feeding. The preferential generation of Th2- and Th3-type responses in the gut may be related to the fact that IL-4 is the primary cytokine produced by cells from the mucosal immune system (Daynes et al 1990), and that this cytokine promotes the growth and differentation of these types of cell responses (reviewed by Weiner 1997). TGF-β serves as a switch factor for IgA responses in the gut (Coffman et al 1989). A state similar to oral tolerance may also be a critical component of homeostatic responses to commensal bacteria. In contrast, live organisms and particulate antigens stimulate active local and systemic cell-mediated and humoral immunity (reviewed by Mowat and Viney 1997, Strobel and Mowat 1998). The induction of tolerance to orally administered antigens depends on the nature and dose of the antigen, time-schedule of feeding (continuous or intermittent), on use of adjuvants, and especially on host factors such as age and genetic background of the individual, state of the GALT (normal vs inflammatory conditions), and pre-existing immune responses (Vaarala 1999, Hänninen 2000). Based on animal studies, passive tolerance is induced by large doses of dietary antigen, i.e., deletion of antigen-specific cells by very high doses or anergy of antigen-specific cells by high to intermediate doses, whereas low doses induce active suppression with involvement of regulatory cells wich provide

bystander suppression. However, very low doses of dietary antigens can even prime for immune responses (reviewed by Weiner 1997, Strobel and Mowat 1998) (Fig. 3). For humans, no data exist on antigen dosage in oral tolerance induction.

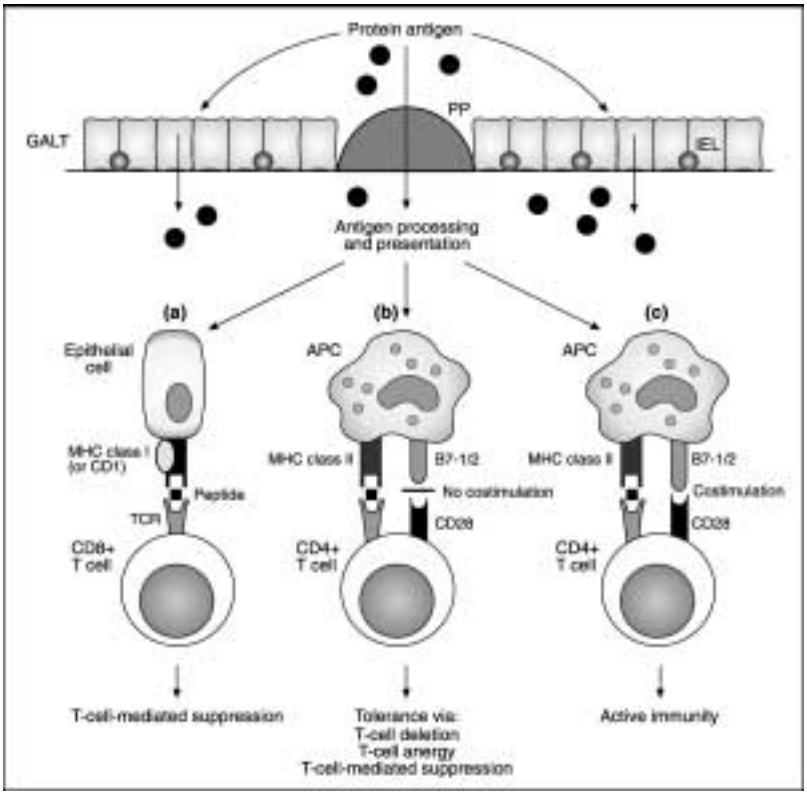

Figure 3. Immunoregulation after oral antigen administration (modified from Strobel and Mowat 1998). After passage through mucosa and local prosessing, antigen is presented in the context of major histocombatibility complex (MHC) class I and/or class II molecules. The immunological outcome (tolerance vs immunity) is dependent on the nature and dose of the antigen, its frequency of administration and a number of host factors. (a) Presentation of antigens in association with conventional and/or atypical class I MHC molecules may lead to activation of specific CD8+ T-cells and T-cell mediated suppression via transforming growth factor β (TGF-β) secretion. (b) When there is no activating costimulatory signals on class II antigen-presenting cells (APCs) (e.g. dendritic cells or enterocytes), anergy or deletion of CD4+ T-cells is induced. Alternatively, T-cell mediated suppression by production of inhibitory cytokines (e.g. TGF-β) may occur. (c) In contrast, during inflammation antigen presentation in association with class II molecules may activate CD4+ T-cells and lead to active immunity. GALT, gut-associated lymphoid tissue; IEL, intraepithelial lymphocyte; PP, Peyers patches; TCR, T-cell recptor.

Intraepithelial CD4+ T-cells play a role in the induction of oral tolerance. It is speculated that antigen presentation by epithelial cells may anergise antigen-specific CD4+ T-cells directly. Alternatively, oral antigen exposure may induce regulatory CD4+ T-cells in the PPs or MLNs. Induction of regulatory CD4+ T-cells after oral exposure has been shown in animal models in which suppression of autoimmune disease has been achieved by such regulatory cells. In mice susceptible to experimental autoimmune encephalomyelitis (EAE), feeding myelin basic protein, an antigen capable of producing the disease, has induced regulatory cells in MLNs which secrete TGF-β, IL-4, IL-10, and minimal IFN-γ. Moreover, CD4+ T-cell clones propagated from the

MLNs could suppress EAE and induced a shift from a Th1 to a Th2/Th3 cytokine pattern locally in the central nervous system (Chen et al 1994). Suppression of insulitis with a concomitant increase in IL-4, IL-10, prostaglandin E, and TGF-β expression and down-regulation of Th1 cytokines in the pancreas have been achieved in NOD mice by oral insulin administration, a process suggestive of induction of regulatory cells (Hancock et al 1995). Indeed, regulatory CD4+ cells from insulin-fed mice were able to prevent diabetes in cotransfer experiments (Bergerot et al 1994). In EAE it has been shown that the regulatory cytokines may be produced either by CD4+ or by CD8+ cells, and in this experimental model, TGF-β has been considered the major cytokine in oral tolerance induction (Khoury et al 1992, Chen et al 1994 and 1995). However, in NOD mice, CD4+ cells are considered the major regulatory subset in oral tolerance, and in cotransfer experiments, CD8+ cells from insulin-fed mice can even accelerate progression to diabetes (Bergerot et al 1994). In addition, in NOD diabetes, IL-4 may be the major regulatory cytokine involved in oral tolerance (Homann et al 1999a and b). These findings point to diversity involved in oral tolerance induction depending on the nature of the antigen and the host immune system. More recently, interest has arisen as to the role of mucosal CD8+ T-cells in oral tolerance (Panja et al 1993, Harper et al 1996, Ke and Kapp 1996), and especially as to the involvement of T-cells expressing the γδ TCR, which are T-cells that normally do not exhibit classical antigen-specific cytotoxic T-cell activity and do not proliferate well in response to mitogens or antigens (Mengel et al 1995, Ke et al 1997).

In humans, oral tolerance induction is largely unknown. It becomes especially relevant after birth when oral feeding begins. This a labile period in life, since mucosal defense mechanisms have not completely evolved. Maternal milk provides factors that modulate the infants´ gastrointestinal tract and offer passive defense mechanisms against infections (Goldman 2000). The beneficial effect of breast feeding in tolerance induction is based on immunomodulators in breast milk: sIgA, other milk immunoglobulins, cellular elements, cytokines, and non-immunologic defense factors, which affect antigen delivery and formation of the gut flora, both of which are associated with the development of oral tolerance (Hanson et al 1985). Indeed, the first exposure to dietary antigens through maternal milk, which contains small amounts of dietary antigens (Kilshaw and Cant 1984), is normally well tolerated by the infant, and the likelihood for sensitization through this route is small (Jakobsson and Lindberg 1979). On the other hand, early exposure to intact CM proteins in infancy (<3 months) induces enhanced cellular and humoral immune responses to these antigens, although the responses finally

decrease over the years suggesting the development of oral tolerance (Vaarala et al 1995, Jenmalm and Björkstén 1998). In one study performed in adults, oral antigen feeding was shown to decrease T-cell reactivity but to induce B-cell priming, resulting in higher titers of antibodies to this ingested antigen after parenteral challenge (Husby et al 1994). In mice, exposure to dietary antigens early in life may result in priming, whereas the same exposure later results in tolerance induction (Strobel and Ferguson 1984). These studies point to the importance of age in oral tolerance induction. A recent study in children and adolescents without any adverse reactions to foods reported that PP T-cells from the ileum show an enhanced proliferative response to dietary BLG compared with that of peripheral blood T-cells from the same patients, and that in this reponse a Th1-type cytokine profile predominated, contradicting the findings in rodents which typically show a local Th2/Th3 response following oral antigen feeding (Nagata et al 2000). In another study, oral administration of myelin basic protein to patients with multiple sclerosis induced circulating antigen-specific T-cells which secreted TGF-β, a result being in agreement with findings of animal studies (Fukaura et al 1996). All this points to the fact that many factors influence the development of oral tolerance and that only a little is known about this issue in humans.

The gut immune system, oral tolerance, and type 1 diabetes

In type 1 diabetes, attention is currently focused on the role of the gut immune system (Harrison and Honeyman 1999, Kolb and Pozzilli 1999, Vaarala 1999). Studies in the animal models of type 1 diabetes suggest involvement of the gut immune system in the pathogenesis of autoimmune diabetes. During embryogenesis, the pancreas develops in the mesoduodenum, which is derived from the foregut. Accordingly, the pancreas seems to belong to the same lymphocyte recirculation pool as the gut and thus seems to have the same homing characteristics. Direct involvement of the gut immune system in the pathogenesis of autoimmune diabetes has been described in NOD mice, where islet infiltrating lymphocytes were shown to express the gut-associated homing receptor α4β7 (Hänninen et al 1993a and 1996a, Faveeuw 1994). This receptor was predominant in lymphocytes in the early insulitis lesion, whereas L-selectin expression was initially low and increased with time. Likewise, the counter-receptor MAdCAM-1 became expressed on islet endothelium at the time when the first lymphocytes infiltrated the pancreas and it predominated in young NOD mice, suggesting a role for the gut immune system in the initiative phases of diabetes. Indeed, treatment of NOD mice with anti-MAdCAM-1 or anti-β7 has been shown to reduce insulitis and diabetes (Yang et al

1997, Hänninen et al 1998). The effect of anti-MAdCAM-1 treatment on spontaneous diabetes development was limited to very early age, being effective only when started at 3 weeks of age but not when started at 10 weeks (Hänninen et al 1998). In another study, however, treatment was still effective when started at 8 weeks (Yang et al 1997). In addition, treating recipient mice with anti-MAdCAM-1 was able to reduce the incidence of diabetes only with use of MLN cells from young NOD donors, whereas no effect occurred if MLN or spleen cells came from diabetic donors. These findings provide further support for the view that the gut immune system plays a specific role in the early phases of diabetes development (Hänninen et al 1998). Lastly, a defect in the gut has been suggested in diabetes-prone BB rats. The animals were shown to have increased gastrointestinal permeability measured by altered sucrose excretion and an altered lactulose-mannitol ratio, a defect not seen in non-diabetes-prone rats (Meddings et al 1999). In addition, this permeability defect was shown to be an early change, occurring before insulitis and clinical diabetes, and thus suggesting that this mechanism may affect antigen delivery to the mucosal immune system and may play a role in (auto)immune responses initiated in the gut.

In man, indirect evidence suggests involvement of the gut immune system in type 1 diabetes. One study has shown that type 1 diabetes patients with normal small-bowel mucosal histology have increased staining with anti-DR and -DP antibodies in the villous epithelium and more $\alpha 4\beta 7$-positive cells in the lamina propria, suggesting jejunal immune activation (Savilahti et al 1999). A permeability defect in the gut demonstrated by elevated mannitol and/or lactulose urinary excretion has also been reported in type 1 diabetes patients without signs of celiac disease or other gastrointestinal problems (Carratu et al 1999), and especially in diabetic patients with the HLA-DQB1*0201 gene (Kuitunen M, personal communication). These findings may point to the fact that a primary defect in the gut exists in type 1 diabetes, although a secondary insult cannot be excluded. In addition, in children with newly diagnosed type 1 diabetes, enhanced immune responses to CM proteins may suggest a disturbance in oral tolerance and a role for the gut immune system (Savilahti et al 1988, Vaarala et al 1996). Finally, the fact that a T-cell line propagated from the pancreatic islets of a child who died shortly after diagnosis of type 1 diabetes was $\alpha 4$ integrin-positive and showed strong adherence both to pancreatic and to mucosal endothelium but only weak adherence to peripheral lymph node endothelium implies involvement of GALT in the pathogenesis of type 1 diabetes in man as well (Hänninen et al 1993b).

The gut immune system and oral tolerance in other autoimmune diseases and in food allergy

The role of the gut immune system and oral tolerance has been implicated in several other autoimmune diseases. The best known autoimmune disease involving the gut immune system is celiac disease, in which tolerance to dietary gliadin is broken (reviewed by Schuppan 2000). Owing to continous gliadin exposure, the number of mucosal γδ T-cells is elevated and mucosal damage occurs, while an elimination diet allows restoration of the intestinal histology and improvement of the clinical picture, although the number of γδ T-cell count remains elevated (Savilahti et al 1990). In addition, a persistent defect in intestinal permeability has been shown in patients with celiac disease in whom strict gluten withdrawal has been accompanied by restoration of normal intestinal histology, a phenomenon pointing to a primary defect in the gut (Bjarnason and Peters 1983). Th1-type cells seem to play a major role in celiac process. Tissue transglutaminase (TGase), an enzyme expressed also in the gut wall, is the major autoantigen in celiac disease. Gut-derived T-cells are not reactive to TGase alone, but TGase mediates the gliadin modification in the gut important for HLA binding and critical for T-cell recognition of this antigen (Molberg et al 1998). On the other hand, TGase autoantibodies exist in celiac disease and can be used for screening of the disease. A prerequisite for production of these disease-associated autoantibodies is that hapten-carrier-like complexes of gliadin and TGase be formed in vivo; this enables (gliadin-specific) T-cell help to Tgase-specific B-cells. Celiac disease shares a predisposing allele, HLA DQB1*0201, with type 1 diabetes, and this may in part explain the higher incidence of type 1 diabetes in patients with celiac disease (Koivisto et al 1977). Early exposure of the immature gut immune system to gliadin is suggested to be a cofactor for the manifestation of clinical disease as is in type 1 diabetes early exposure to CM proteins (reviewed by Schuppan 2000). In addition, the duration of exposure to gliadin in celiac disease plays a role in spreading of autoimmunity (Ventura et al 1999). In mice, intravenous or intranasal administration of gliadin down-regulated the antigen-specific Th1-like cell proliferation after parenteral challenge, highlighting potential immunomodulatory therapies in celiac disease as well (Rossi et al 1999).

Some individual findings have suggested a failure in oral tolerance in other autoimmune diseases, as well. Patients with systemic lupus erythematosus (SLE) show elevated levels of antibodies to bovine γ-globulin and bovine milk casein, but not to ovalbumin. This specific failure in oral tolerance to bovine-derived proteins has been shown also in the animal model of

SLE (Carr et al 1987a), and in fact, a casein-free diet radically alters the course of murine SLE and decreases immunological markers related to this disease (Carr et al 1987b). Elevated levels of anti-BSA antibodies are not restricted to type 1 diabetes but have also been detected in patients with SLE, rheumatoid arthritis, and chronic autoimmune thyroiditis (Atkinson et al 1993c). These anti-BSA antibodies from patients with rheumatoid arthritis displayed specific reactivity to an epitope highly homologous with human collagen type I, suggesting possible molecular mimicry in this disease, as well (Perez-Maceda et al 1991). One study indicated that children with juvenile rheumatoid arthritis are less likely to have been breast fed than controls, and that the protective effect of breast feeding increased with a longer breast feeding time (Mason et al 1995). In genetically predisposed animals, CM feeding induced mild rheumatoid arthritis (Hanglow et al 1985). In addition, a correlation has been reported between liquid CM consumption and prevalence of multiple sclerosis (Malosse et al 1992).

Food allergy/hypersensitivity is common in childhood and especially during the first years of life. This failure in oral tolerance to dietary antigens may result in gastrointestinal symptoms, eczema, respiratory manifestations or anaphylaxis, and the symptoms can occur even during exclusive breast feeding (Jakobsson and Lindberg 1978, Lifschitz et al 1988). CM, eggs, cereals, and fish are considered to be the major allergens at an early age. This pathological reactivity in the gut may be associated with increased intestinal permeability (Barau and Dupont 1994, de Boisseau et al 1994, Majamaa and Isolauri 1996). In addition, some children with food-induced gastrointestinal symptoms exhibit lymphonodular hyperplasia in the small intestine and/or the colon (Kokkonen et al 1999). Detection of elevated levels of mucosal γδ+ T-cells (Kokkonen et al 2000) and of higher numbers of cytotoxic IELs (Hankard et al 1997, Augustin et al 2001) in the active phase of the disease suggests involvement of these cells in the pathogenesis of this type of food allergy. Recently, it has been shown that some children who have suffered from food allergy in infancy may at school age have gastrointestinal symptoms due to residual intestinal disease (Kokkonen et al 2001). In short, despite occurrence of symptoms in various other tissues, the pathologic reactivity seems to reside in the gut.

AIMS OF THE STUDY

Based on animal studies, the gut immune system seems to play a role in autoimmune diabetes pathogenesis. Indirect evidence also suggests involvement of the gut immune system in human type 1 diabetes. Based on this, we reasoned that reactivity to diabetes-associated autoantigens may be associated with the gut immune system. In this thesis, a novel hypothesis was explored that the potentially immunogenic bovine insulin present in CM formula may elicit an immune response in infants, because immunoreactivity to other CM proteins exists, as well. If such insulin immunity develops, and if in certain circumstances it turns autoaggressive, this may result in β-cell destruction and type 1 diabetes.

The objectives of this work were:

1. To assess markers of immune activation (sICAM-1, sL-selectin) in relation to CM exposure in infants at genetic risk for type 1 diabetes.

2. To investigate the expression of gut-associated integrin α4β7 on GAD-reactive peripheral blood lymphocytes in type 1 diabetes.

3. To evaluate whether CM contains immunoactive bovine insulin and to discover whether dietary insulin induces insulin-specific humoral and cellular immune responses in infants at genetic risk for type 1 diabetes.

4. To study the effect of a maternal CM-free diet during lactation on development of antibodies to dietary insulin in offspring at risk for allergy.

SUBJECTS AND METHODS

Subjects

Subjects in Studies I and III were those *children taking part in the first pilot study of the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) project*. Twenty newborn infants of mothers with type 1 diabetes in the Department of Obstetrics, University of Helsinki, were recruited into the study. Exclusive breast-feeding was encouraged; thereafter in a double-blind, randomized manner 10 infants received adapted CM-based formula (Enfamil®, Mead Johnson, Evansville, IN, USA, group I, CM group) and 10 infants extensively hydrolyzed casein-based formula with peptides of a molecular weight less than 1.2 kD (Nutramigen®, Mead Johnson, group II, HC group) until the age of 9 months. To ensure that the two study formulas could not be distinguished by taste or smell, 20% of the Enfamil® powder was replaced by Nutramigen®. The mothers of all infants were advised not to give CM- or beef-containing infant food to their children during the dietary intervention period. The infants visited the outpatient clinic at the Children´s Hospital, University of Helsinki, at 3, 6, 9, 12, and 24 months of age, and the dietary advisor maintained, in addition, phone contact with the mothers. The compliance of the family was inquired about each time and only two (one in each group) minor unintentional failures in the diet were recorded. Blood samples were drawn at these follow-up visits. One child in the HC group developed diabetes at the age of 14 months, and one child in the CM group CM allergy at the age of 7 months, after which time he was excluded from the study because of a break in the dietary intervention. One infant (CM group) who was exposed to the study formula only after 7.5 months of age was excluded from the analyses at 3 and 6 months of age. The study was approved by the ethics committees of both participating hospitals. There was no difference in gestational age, birth weight or in history of infections between the groups; neither was there any difference between the groups in the age at which the formula was started or in duration of breast feeding. In Study I, plasma samples were available at 3 months from eight and nine, at 6 months from eight and nine, at 9 months from eight and eight, at 12 months from nine and nine, and at 24 months from nine and nine children in the CM and HC groups, respectively. In addition to these samples taken at follow-up visits, a cord blood sample was available and analyzed from 13 infants: nine in the CM group and four in the HC group. In Study III, the plasma samples were studied for insulin-binding antibodies only from 6 months of age due to the possible confounding effect of maternal IAA at earlier time-points. At 6 months, samples were available from eight and eight, at 9 months from nine and seven, at 12 months from ten

and nine, and at 24 months of age from nine and eight children in the CM and HC groups, respectively. Six 6-month-old children who were fully breast fed were included as control subjects in that study (III).

Subjects in Study II had either *manifest type 1 diabetes or signs of diabetes-related autoimmunity*. For comparison, patients with autoimmune polyendocrine disease type 1 (APD-I) (Aaltonen et al 1994) and healthy individuals were included. Peripheral venous blood samples were obtained with parents´ consent from six patients with newly diagnosed type 1 diabetes and from one boy at risk for type 1 diabetes (258 JDF units of ICA) from the Children`s Hospital, University of Helsinki, Aurora Hospital, and Jorvi Hospital. The mean age of the six children with type 1 diabetes (five boys and one girl) was 9.6 years (range 3.8-15.3). Subjects were selected for the study because of their cellular immunity to GAD65 (stimulation index above 3.0 in a proliferation test). We also studied three female patients (ages 29, 33, 39 years) with APD-I, who had a cellular immune response to GAD65. One of the patients had been diagnosed with type 1 diabetes 4 years earlier. Three healthy adults without cellular reactivity to GAD65 served as controls. The study plan was approved by the ethics committees of the hospitals.

Subjects in Study IV were *children taking part in the second pilot study of the TRIGR project*. Newborn infants with a first-degree relative (mother, father or sibling) with type 1 diabetes were invited to the second pilot of TRIGR between 1995 and 1997, but only those at increased genetic risk (HLA-DQB1*02/*0302, *0302/x or *02/y genotypes, where x stands for alleles other than *02, *0602 or *0603 and y for alleles other than *0302, *0602 or *0603) entered the study. Consecutively recruited children (n=63) outside the Helsinki area were studied for humoral immunity to insulin only. In addition, 56 children from the Helsinki area were studied both for insulin-specific T-cell reactivity and for humoral immunity. Insulin was added to the T-cell analysis only from the beginning of the year 1996. Infants were randomized to receive after breast feeding in a doubled-blinded manner either adapted CM-based formula (Enfamil®, Mead Johnson, Evansville, IN, USA) supplemented with 20% Nutramigen® to make the two study formulas similar in taste and smell (CM group), or extensively hydrolyzed casein-based formula with peptides of molecular weight less than 1.2 kD (Nutramigen®, Mead Johnson; HC group) until the age of 6 to 8 months depending on when the formula was started. According to the protocol, all infants were supposed to receive the study formula for a minimum of 2 months. Breast feeding was encouraged, and the mothers were asked to add the study formula to their infant’s diet at the latest at 6 months of age. During the intervention period, they were advised to

eliminate all infant food products containing CM or beef from their infant's diet, but their own diet while lactating was not modified.

There was a difference between the two randomization groups in the age at introduction of the study formula, the mean being 1.9 months in the CM group (n=58) and 3.0 months in the HC group (n=61) (p=0.03, Mann-Whitney U-test). No difference was recorded in duration of total breast feeding between the groups (7.6 vs 8.3 months, p=0.53). Duration of study formula feeding was 4.8 and 3.6 months in the two groups (p=0.01). Deviations from this protocol also occurred. In this series, 14 children (12%) were not exposed to the study formula at all (five in the CM group, nine in the HC group), and these were included in the breast-fed (BF) group until 6 months old and excluded thereafter. Two infants (one in each group) who dropped out early (before the 3-month visit and before the 6-month visit) and whose feeding practice was unknown were excluded from analysis. Four children were diagnosed with CM allergy at 3, 4.5, 7, and 12 months of age; the first one was fully BF until diagnosis, the second received HC, the third received the CM study formula after 6 months of age, and the last one ordinary CM at 10.5 months of age and no study formula at all. The two infants diagnosed with CM allergy during the dietary intervention period were excluded from analysis after the diagnosis because of the change in their diet. Infants (n=6) of mothers with type 1 diabetes and high levels of antibodies to BI and HI in the cord blood (≥2x median optical density (OD) in the BF group at 3 months of age) were excluded from the analysis at 3 and 6 months of age due to transplacental transfer of insulin antibodies. Three infants with high initial levels of antibodies to BLG in cord blood and decreasing levels thereafter up to 3 to 6 months of age were excluded from the analysis of this variable at 3 and 6 months. The studies were approved by the ethics committees of all participating hospitals.

Subjects in Study V were taking part in *an allergy prevention trial*. Infants from families with atopic disease in at least two first-degree relatives (parents, siblings) or in one first-degree relative plus who had an elevated cord blood IgE level were recruited to a Swedish prospective study on prevention of food allergies. Patient selection and diet criteria for the two study groups have been previously reported in detail (Hattevig et al 1989). During the first 6 months, the infants received only breast milk or supplementation with a hydrolyzed casein-based formula (Nutramigen®, Bristol-Meyers, Stockholm, Sweden). CM was introduced into the diet after 6 months of age, and egg-containing products after 9 months. The nursing mothers from the diet (D) group avoided CM, egg white, and fish products during the first 3 months of lactation. The

diet period was limited to 3 months to minimize dietary mistakes and to attain maximal compliance. The mothers from the non-diet (ND) group consumed an unrestricted diet during their entire lactation. The two groups were comparable in regard to heredity for atopic disease, pattern of breast feeding, Nutramigen® supplementation, and number of dietary errors (Hattevig et al 1989). Serum samples from a total of 123 infants (78 in the D group and 45 in the ND group) were available for insulin-binding antibody analyses from the original Swedish cohort of 140 infants. No drop-outs occurred in the original study cohort. Samples were available from the D and ND groups from 69 and 42 infants at birth, from 71 and 40 infants at 3 months, from 74 and 36 infants at 6 months, from 66 and 28 children at 12 months, from 62 and 28 children at 18 months, and from 73 and 36 children at 4 years, respectively. Serum samples taken at delivery from 71 mothers in the D group and from 34 mothers in the ND group unnderwent study. All parents of the participating infants gave their informed consent. The study was approved by the Human Research Ethics Committee of the Medical Faculty, University of Linköping, Sweden.

Antigens

Insulin. Bovine and human insulin were purchased from Sigma (St. Louis, MO, USA: catalog numbers I-1882 and I-2643, respectively).

GAD65. A baculovirus expression vector pVL 1393 (Invitrogen, San Diego, CA, USA) carrying the human GAD65 gene was used to infect *Spodoptera frugiperda* (ATCC, Rockville, MD, USA) cells in suspension cultures as described (Moody et al 1995). The cell pellets from these cultures were stored at -70ºC 48 to 54 h after infection. For GAD65 purification the cells were lysed by sonication. Lysis buffer (200mM $NaHCO_3$ containing 1 mM pyridoxal phosphate, 1 mM 2-aminoethyl isothiouronium bromide and 1 mM phenylmethylsulfonyl fluoride) and supernatant were cleared from unsolubilized material by centrifugation (12,000 rpm for 10 min at 4ºC). Immunoaffinity purification was performed with monoclonal antibody GAD-6 (Developmental Studies Hybrinoma Bank, Iowa, IA, USA). The purified antibody was coupled to CnBr-activated sephrarose 4B (Pharmacia, Uppsala, Sweden). Supernatant from infected cells and antibody resin were mixed, and the antibody-antigen reaction was carried out at pH 9.2 for at least 16 h by rotating the mixture at 4ºC. The resin was washed twice and transferred to a column that was developed at pH 2.7. The effluent was neutralized, and the precipitated GAD65 pelleted and solubilized in 100 nM/l $NAHCO_3$, pH 9.2. The purity of the preparates was confirmed by SDS-PAGE, followed by staining with Coomassie brilliant blue and Western blot analysis with GAD-6 or polyclonal rabbit anti-GAD65 as primary and horseradish peroxidase-conjugates as secondary antibodies, and ECL-reagent (Amersham, Buckinghamshire, UK) to

visualize the result on X-ray film. The endotoxin content of the antigen preparations was tested by Limulus test, and it fell below the detection level of the test (0.062 EU/ml corresponding to about 2 pg/ml of endotoxin) in all the antigen preparations used.

Tetanus toxoid (TT) from the National Public Health Institute, Helsinki, Finland and *tuberculin purified protein derivate (PPD)* from Statens Serum Institut, Copenhagen, Denmark, served as control antigens.

sICAM-1 (catalog number BMS201) and *sL-selectin* ELISA kits (BMS206) were purchased from Bender MedSystems, Vienna, Austria.

Assay for measurement of soluble ICAM-1 and L-selectin (I)

Plasma samples from each age group (0, 3, 6, 9, 12 and 24 months of age) were run in the same plate to assess soluble ICAM-1 and L-selectin levels. These concentrations were measured by specific enzyme-linked immunosorbent assay (ELISA) kits purchased from Bender Medsystems, Vienna, Austria. Briefly, diluted plasma samples (1:20 for soluble ICAM-1 assay and 1:200 for soluble L-selectin assay) were added as duplicates to the polystyrene microtiter plates, which were coated with a specific monoclonal antibody. A specific monoclonal horseradish peroxidase-conjugated second antibody was added to the wells, and the plates were incubated for 1 hour in the ICAM-1 assay and for 2 hours in the L-selectin assay at room temperature. After this first incubation the wells were washed three times. After incubating the plates with a combination of substrate 1 (tetramethyl-benzidine) and substrate 2 (0.02 % buffered hydrogen peroxide), the process was stopped with sulfuric acid, and the absorbances were measured at 450 nm wavelength by Multiskan MS (Labsystems, Helsinki, Finland). A dilution series of sICAM-1 or sL-selectin standards were run in each plate, and a standard curve was calculated. The levels of soluble adhesion molecules as ng/ml were calculated from the standard curve.

T-cell proliferation assay (II, IV)

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized peripheral blood by Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation. The PBMCs were suspended in RPMI-1640 (Gibco, Paisley, Scotland) containing 5% pooled human AB+ serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) and 2 mmol/l L-glutamine. They were cultured at 1 x 10^5 cells (200 µl) per well in quadruplicates on a U-bottomed microwell plate (Nunc, Røskilde, Denmark) with BI or HI 100 µg/ml (IV), GAD65 10 µg/ml (II), TT 8 µg/ml (II, IV) or PPD 10 µg/ml (IV). Eight

replicates for the baseline value (cells with medium alone) were included in our T-cell assay. After a 5-day incubation, 1 μCi tritiated thymidine (Amersham Life Science, Little Chalfont, Buckinghamshire, UK) was added, and the cultures were harvested 16 h later for thymidine incorporation measurement. Proliferation response in Study IV was expressed as stimulation index (SI) = median counts per minute (cpm) in the presence of antigen divided by median cpm without antigen, and as Δ cpm = median cpm without antigen subtracted from median cpm in the presence of antigen. In Study II, proliferation response was expressed as median (Δ) cpm ± SD. The samples were analyzed blindly and in sequential order, with samples of different individuals and different time points included in the same assay in the prospective Study IV.

Depletion of α4β7-expressing lymphocyte population from PBMCs (II)

After isolation of PBMCs by density centrifugation, they were suspended at a cell concentration of 8 x 10^6 cells/ml to the culture medium. Monoclonal antibodies to α4β7-integrin (anti-ACT-1) (Lazarovits et al 1984) were added to the cell suspension with a final ratio of 0.5 μg/ml of protein per 10^6 cells. After a 30-min incubation, the cells were washed, and magnetic beads coated with monoclonal antibodies to mouse immunoglobulin (Dynal, Oslo, Norway) were added. Lymphocytes bound to the magnetic particles were separated in the magnetic particle concentrator (Dynal MPC-E), and the supernatant corresponding to the α4β7-depleted population of PBMCs (α4β7^{low}) was collected. This α4β7-depleted population of PBMCs was used in the proliferation assay described above on the same plate as the whole PBMC population from the same individual.

Depletion of CD8 lymphocytes from the PBMC population (II)

Immunomagnetic depletion of CD8+ lymphocytes from the PBMC population was performed in four patients with newly diagnosed type 1 diabetes by use of magnetic bead-conjugated monoclonal antibodies to CD8 (Dynabeads M-450, Dynal) according to the manufacturer`s protocol. The CD8-depleted population of PBMCs was used in this proliferation assay on the same plate as the whole PBMC population from the same individual.

Flow-cytometry analysis of lymphocyte surface antigens (II)

For cell staining, Ficoll-isolated PBMCs and α4β7-depleted PBMCs were preincubated with goat IgG to block non-specific antibody binding. For two-color analysis, cells were consecutively stained with anti-ACT-1 (0.3 μg/10^5 cell), goat anti-mouse Fab conjugated to

fluorescein isothiocyanate, normal mouse IgG, and phycoerythrin (PE)-conjugated monoclonal antibodies to CD4 and CD8 (Becton Dickinson, San Jose, CA, USA). Incubations were performed at room temperature for 15 min, and cells were washed after incubation with both anti-ACT-1 and PE-conjugated antibodies. The lymphocyte gate was set with CD14/CD45 antibodies (Leucogate, Becton Dickinson). CD14 monoclonal antibodies were used to confirm the presence of monocytes in the cultures.

Immunoblotting of BI in study formulas (III)

The presence of insulin in the CM formulas, Enfamil® and Nutramigen®, was studied by immunoblotting in non-reducing conditions. The ten-fold concentrate of CM formula, compared with the dilution according to the manufacturer`s protocol, was electrophoresed in 16% polyacrylamide. The proteins were transferred to a pure nitrocellulose membrane (Trans-Blot®Transfer Medium, Bio-Rad Laboratories, Hercules, CA, USA), and the membrane was blocked with 2% human serum albumin (HSA) in phosphate-buffered saline (PBS). The membrane strips were incubated with polyclonal guinea-pig anti-porcine insulin antiserum (Dako, Carpinteria, CA, USA) diluted 1:1,000 in 0.2% HSA 0.05% Tween 20 in PBS. The second antibody was biotinylated goat anti-rabbit IgG (Dako). Alkaline phosphatase-streptavidin complex (Zymed, San Francisco, CA, USA) was added and the reaction was developed with an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories). BI (0.5 μg/lane) was run as a positive control protein in the immunoblotting experiment.

Radioimmunoassay (RIA) for detection of insulin in CM products (III)

The amount of immunoreactive insulin recognized by insulin antibodies in CM products was quantified by a commercial solid-phase ^{125}I RIA designed for the quantitative measurement of insulin in serum (Coat-A-Count®Insulin, Diagnostic Products Corp., Los Angeles, CA, USA).

Enzyme immunoassay (EIA) for IgG antibodies to BI and HI (III, IV, V)

Polystyrene plates (Combiplate® Enhanced binding, Labsystems, Helsinki, Finland) were coated with BI or HI 1 μg/ well (Sigma); 1% human serum albumin (HSA) in phosphate-buffered saline (PBS) was used for residual coating, and 0.05% Tween-20 in PBS as a washing buffer. The samples were diluted 1:20 in PBS containing 0.2% HSA, 0.05% Tween. Alkaline phosphatase-conjugated rabbit anti-human IgG-antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used as the secondary antibody and P-nitrophenyl phosphate (Sigma) as

a substrate. Absorbance was measured by an optical reader at 405 nm, and the results were expressed as optical density units (OD). The samples were run blindly, each child's sequential samples on the same plate. As quality controls, in Study III two positive samples from patients with newly diagnosed type 1 diabetes and two negative samples from healthy children, and in Studies IV and V a pool of five known positive and negative samples were run on each plate. The mean intra-assay variation of the method in Study III was 6.2% and interassay variation 11.5%; the respective values were 12 and 20% in Studies IV and V. For inhibition assays, 0.1, 1.0, 10, and 100 μl/ml BI , HI or B-chain of BI (Sigma) were incubated with the plasma sample of two patients with newly diagnosed type 1 diabetes for 2 h at room temperature before analysis of the sample in the EIA for antibodies to BI in Study III. Inhibition assays in Studies IV and V were performed in serum samples taken at 6 months of age. 200 μg/ml (IV) or 1,000 μg/ml BI (V) were incubated with the serum sample for 2 h at room temperature before analysis of the sample in the EIA for antibodies to BI. The same serum sample without the inhibitor but treated otherwise in a similar manner was always run on the same plate.

IAA radioligand assay (IV)

IAA were analyzed by a micro-assay as previously described (Williams et al 1997) at Oulu Research laboratory, University of Oulu, Finland. The cut-off limit for IAA-positivity was set at the 99th percentile (≥ 1.56 relative units, RU) in 373 non-diabetic Finnish infants and children.

EIA for IgG to antibodies to BLG (IV)

IgG antibodies to BLG were measured as previously described (Savilahti et al 1993) in the Research laboratory of the Hospital for Children and Adolescents, University of Helsinki. The levels of antibodies were expressed as percentages of the standard having a very high titer of BLG antibodies.

HLA typing (IV)

HLA-DQB1 typing was performed in Turku Immunology Center, University of Turku, Finland, by a technique developed for screening of type 1 diabetes susceptibility based on the presence of HLA-DQB1 alleles associated with a significant risk for (HLA-DQB1*0302,*02) or with protection against (HLA-DQB1*0301, *0602, *0603) this disease (Sjöroos et al 1995). In the second pilot of TRIGR, this screening was used, and infants who were at increased genetic risk were included in the study (HLA-DQB1*02/*0302, *0302/x or *02/y genotypes, where x stands for alleles other than *02, *0602 or *0603 and y for alleles other than *0302, *0602 or *0603). To analyze the effect of HLA genotype on insulin immunization, these children were divided into three groups by their risk genotypes.

Statistical analysis

The difference in the levels of soluble adhesion molecules between the groups was analyzed by Mann-Whitney U-test and by regression analysis for repeated measurements. The correlation between the levels of sICAM-1 and sL-selectin was tested by Spearman correlation test (I). This correlation test was used in all studies in which correlations between different parameters were calculated. In Studies III and V, the difference in the levels of insulin antibodies was analyzed by Mann-Whitney U-test. In Study V, the Wilcoxon signed-rank test was used to compare the levels of antibodies during follow-up in a particular group. In Study IV, differences between the groups were analyzed by Kruskal-Wallis H-test or by Mann-Whitney U-test with the Bonferonni correction for multiple comparisons. Differences during the follow-up within a specific group were evaluated by the Wilcoxon signed-rank test (IV).

RESULTS

Soluble adhesion molecules in infants (I)

Levels of sICAM-1 were higher throughout the study in infants who received CM-based formula than in infants fed HC-based formula ($p = 0.05$, regression analysis up to 12 months) (Figure 4). On the other hand, the median levels of sICAM-1 in cord blood did not differ between the groups, being 146 (range 135-222) ng/ml in the CM group and 130 (119-169) ng/ml in the HC group. Levels of sL-selectin did not differ between the study groups at any age. The median level of sL-selectin in the cord blood samples was 658 (range 550-950) ng/ml. Median levels of sICAM-1 and sL-selectin are shown in Table 3. No correlation existed between sICAM-1 and sL-selectin levels ($r = 0.168$, ns).

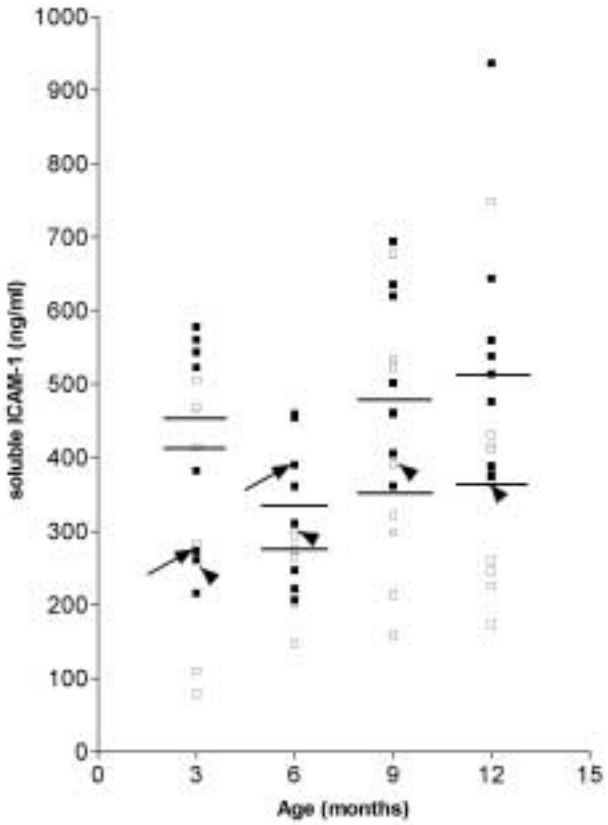

Figure 4. Levels of sICAM-1 in infants receiving cow´s milk (CM)-based formula (■) and in infants receiving hydrolyzed casein (HC)-based formula (□) at 3, 6, 9, and 12 months of age. Medians of two groups shown by horizontal lines, upper medians representing CM and lower medians HC group. Levels of sICAM-1 of the individual diagnosed with CM allergy at 7 months (*arrow*) and of the one who progressed to manifest type 1 diabetes at 14 months of age (*arrowheads*).

Table 3. Median levels (range) (ng/ml) of sICAM-1 and sL-selectin in children exposed to cow´s milk-based formula (upper row) or hydrolyzed casein-based formula (lower row).

	3 months	**6 months**	**9 months**	**12 months**	**24 months**
sICAM-1	453 (216-578)	332 (206-460)	482 (361-694)	514 (374-934)	458 (284-704)
	415 (80-546)	275 (147-397)	357 (158-678)	365 (174-749)	316 (234-543)
	p=0.34	p=0.15	p=0.17	p=0.04	p=0.03
sL-selectin	2300 (1700-5600)	2100 (1700-5100)	2640 (1440-9600)	2500 (1720-4600)	
	3200 (1120-5600)	2200 (1240-2880)	3800 (1400-11000)	2360 (1540-4080)	
	p=0.89	p=0.63	p=0.92	p=0.35	

Cellular response after depletion of α4β7-expressing PBMCs (II)

Treatment with monoclonal antibody to α4β7-integrin resulted in depletion of the PBMC population with high α4β7 expression. This decrease was more pronounced in the CD8+ than in the CD4+ population. Changes in the α4β7-expressing CD4+ and CD8+ lymphocyte populations are shown in a representative case in Fig. 5. Monocytes for antigen presentation were present in the PBMC population before and after the depletion (data not shown). Cellular response to GAD65 and TT in the α4β7-depleted population as measured by proliferation was compared to the same responses in the whole PBMC population in six children with newly diagnosed type 1 diabetes and in one boy at high risk for the disease. After depletion a marked decrease in the proliferative response to GAD65 was detected in three patients (cases 2, 3, and 6) and in the subject with elevated ICAs (case 5), these decreases being 93, 60, 45 and 84%, respectively (Fig. 6a). In contrast, marked increase in the proliferation response to TT was detected in four patients (cases 1, 2, 4, and 6) at 154, 79, 368, and 160%, respectively (Fig. 6b). A decrease of 37% in the cellular response to GAD65 after depletion was seen in the one APD-I patient with type 1 diabetes, whereas the two nondiabetic APD-I patients showed no change in responsiveness (Fig. 6c). An increase in the proliferation to TT was found in all patients with APD-I (Fig. 6d). In the healthy individuals without GAD-reactivity, the proliferation to GAD65 after depletion remained low, but the proliferation response to TT increased in the same manner as in patients with type 1 diabetes or APD-I. In the wells without an antigen, the proliferation did not change significantly, with the median cpm being 552 (SD 514) in the whole PBMC population and 320 (SD 271) in the α4β7-depleted population.

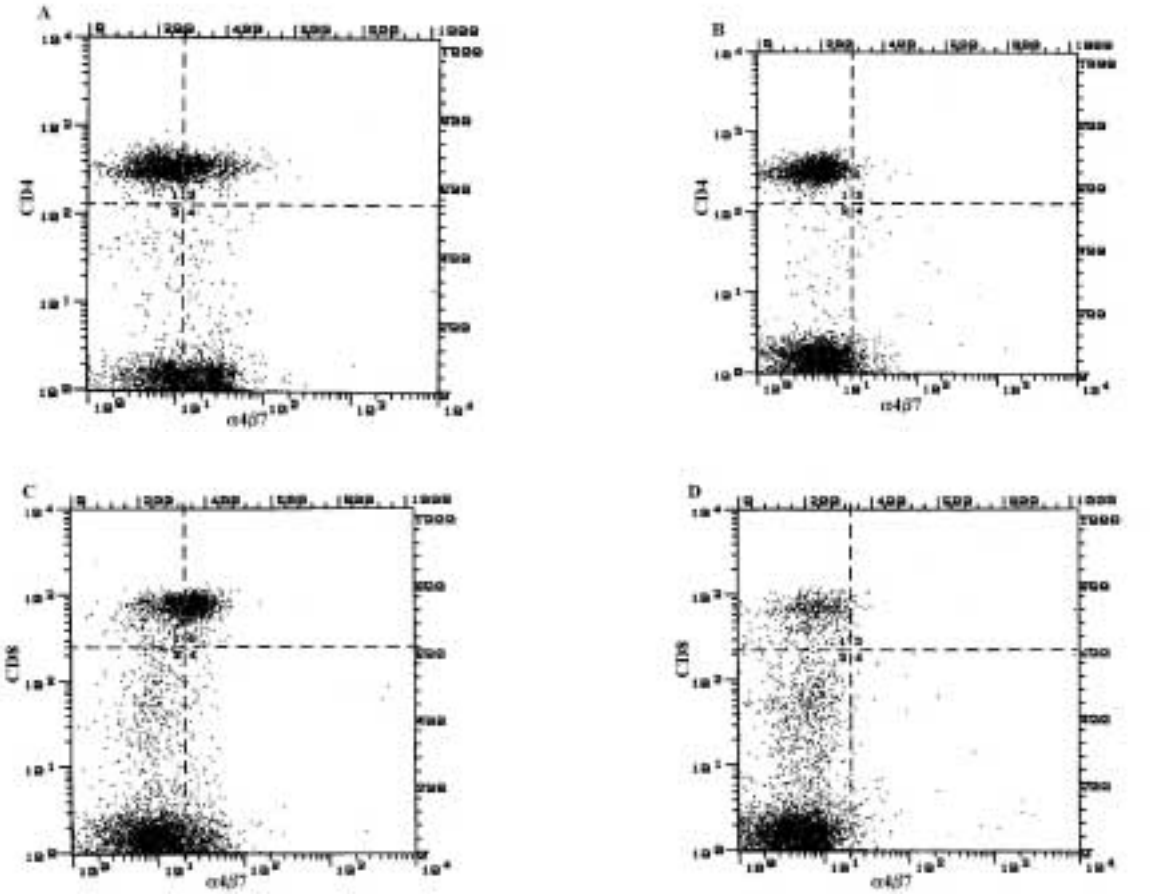

Figure 5. Expression of α4β7 integrin on the CD4+ and CD8+ lymphocyte populations in peripheral blood mononuclear cells (PBMCs) (A, C) and in PBMCs after depletion with antibodies against α4β7 integrin (B, D) in a representative case with type 1 diabetes.

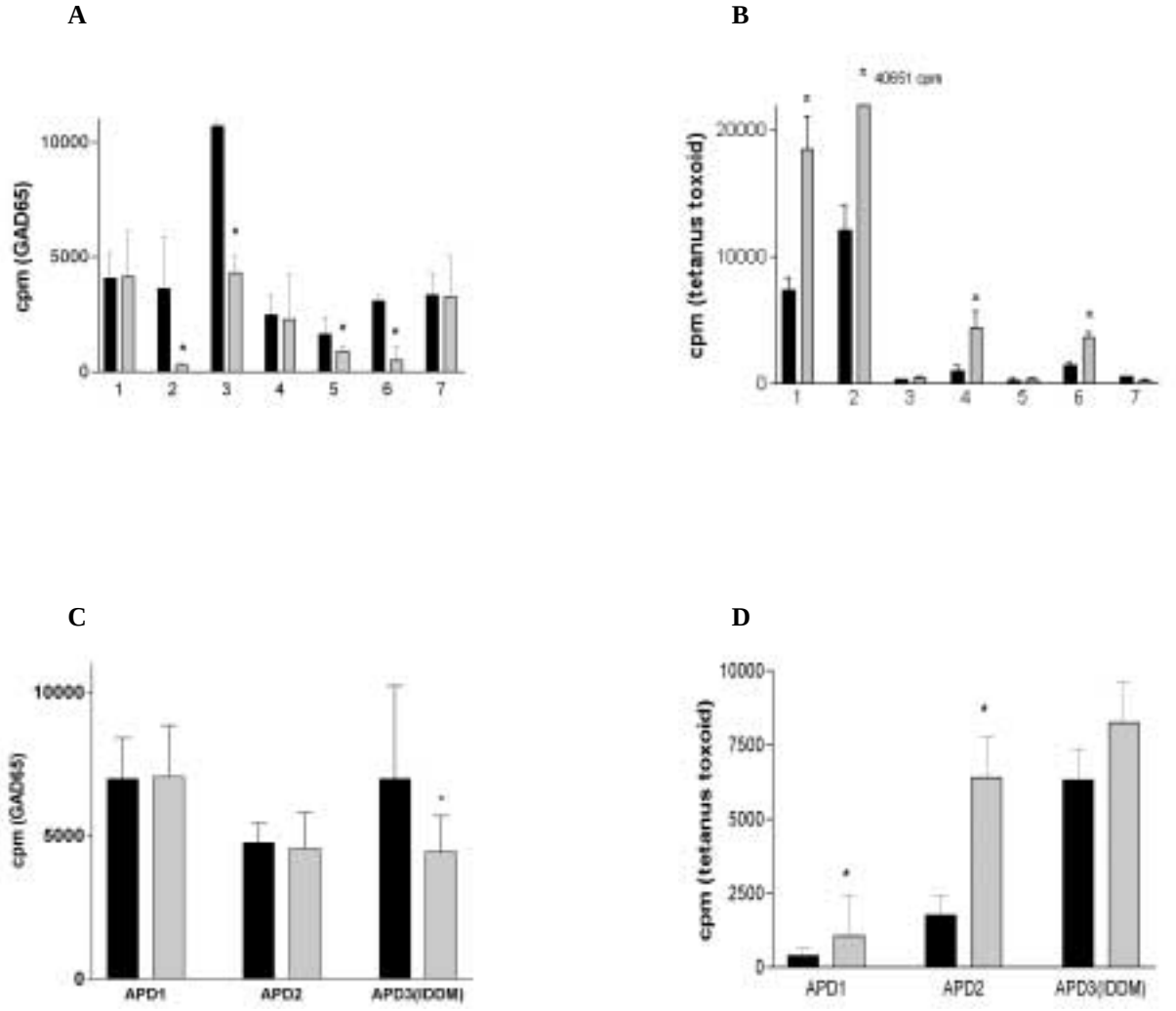

Figure 6. Proliferative response of peripheral blood mononuclear cells (PBMCs) (*black bars*) and α4β7-depleted PBMCs (*gray bars*) to GAD65 (A and C) and to tetanus toxoid (B and D) in newly diagnosed diabetic patients (horizontal axis, cases 1-4, and 6 and 7) and the one prediabetic individual (case 5) (A and B) and in patients with autoimmune polyendocrine disease type I (patients APD1-3) (C and D). Data expressed as median counts per minute (cpm) ± SD calculated from quadruplicate wells. Any change of more than 30% in proliferation is marked with an asterisk. IDDM, insulin-dependent diabetes mellitus.

Cellular response to GAD65 after depletion of CD8+ lymphocytes (II)

After CD8 depletion, the cellular response to GAD65 increased in all four cases of type 1 diabetes (mean increase 47%, range 20-100) (data not shown).

Presence of insulin in study formulas (III)

BI was confirmed to be present by immunoblotting in Enfamil®, but not in Nutramigen®, which contains only peptides less than 1,200 MW. The amount of immunoreactive insulin by RIA in Enfamil® was 5 mU/l. Immunoreactive peptides of insulin were detected by RIA in Nutramigen®, as well (8 mU/l). The amount of insulin in native CM varied from 35 to 42 mU/l in the four samples studied.

Insulin-binding antibodies to BI and HI (III, IV, V)

Insulin-binding antibodies in children at risk for type 1 diabetes (1st pilot study of TRIGR) (III)

Insulin-binding antibodies were studied from 6 months of age on in Study III, because any confounding effect of maternal IAA could be excluded, except in one case. At the age of 6 months, those children who received CM formula had higher levels of IgG antibodies to BI (median 0.480) than did children who received either HC formula (median 0.185, P = 0.04) or those exclusively breast fed (median 0.160, P = 0.04) (Fig. 7A). At the age of 9 months, children in the CM group had higher antibody levels to BI than did children in the HC group (Fig.7B), but after the end of the dietary manipulation at 12 and 24 months of age, the difference between the CM and HC groups had disappeared. Levels of IgG antibodies to HI did not differ between the two study groups at any age (Table 4). Levels of antibodies to BI and HI by EIA correlated, however (r = 0.546; P < 0.0001). Levels of antibodies to BI at the age of 6 months in the CM group did not correlate with the age at the start of the formula feeding. At the age of 6 months, three children in the HC group had IAA. One developed type 1 diabetes at the age of 14 months and another had maternal-transferred IgG antibodies (Fig. 7A). At the age of 9 months, only the child who later manifested type 1 diabetes had IAA (Fig 7B). His IgG antibody levels to BI and HI at the ages of 6, 9 and 12 months were 0.539 and 0.457, 0.390 and 0.374, 0.605 and 0.332 OD units, respectively. Data on IAA measurements have been published elsewhere (Martikainen et al 1996). In inhibition experiments, HI and BI inhibited the binding of IgG antibodies from patients with type 1 diabetes to solid-phase HI or BI in the same manner, but the B-chain of BI showed no inhibition (Fig. 8)

Table 4. Median (range) levels of IgG-antibodies to bovine insulin (BI) and human insulin (HI) as optical density in children exposed to cow´s milk-based formula (upper row) or hydrolyzed casein-based formula (lower row).

	6 months	**9 months**	**12 months**	**24 months**
BI-IgG	0.480 (0.213-0.656)	0.403 (0.213-0.841)	0.364 (0.184-0.722)	0.471 (0.130-1.235)
	0.185 (0.112-0.539)	0.230 (0.105-0.414)	0.308 (0.131-0.893)	0.304 (0.089-1.495)
	p=0.04	p=0.02	p=0.81	p=0.51
HI-IgG	0.258 (0.130-0.672)	0.316 (0.143- >2.0)	0.386 (0.095-1.890)	0.463 (0.242- >2.0)
	0.194 (0.110-0.621)	0.261 (0.144-0.374)	0.332 (0.125-1.116)	0.588 (0.114-0.981)
	p=0.64	p=0.15	p=0.78	p=0.89

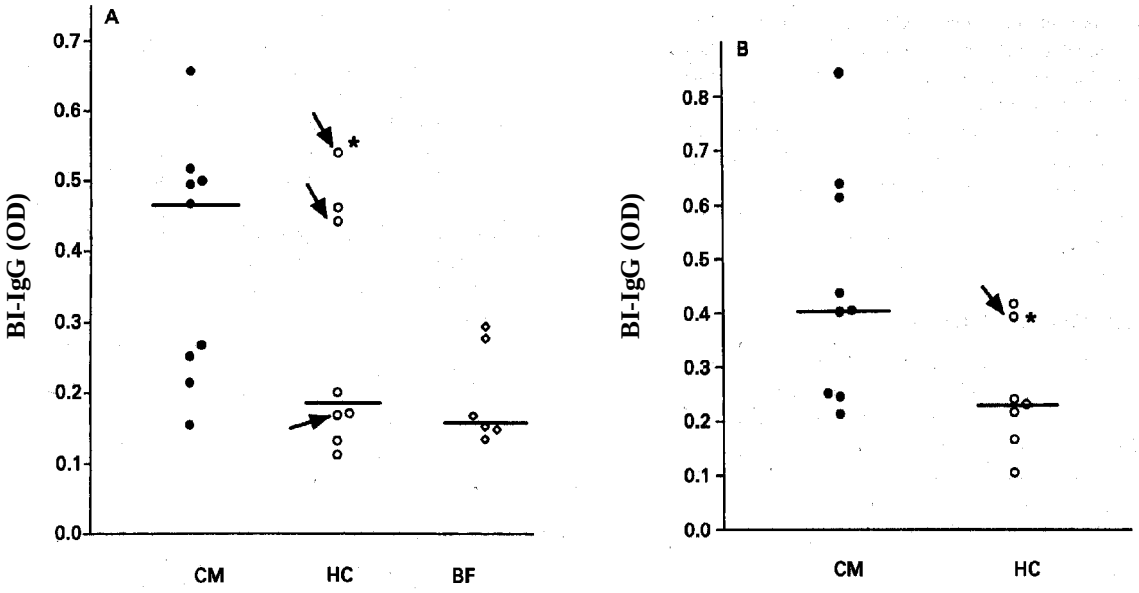

Figure 7. Levels of IgG antibodies to bovine insulin (BI-IgG) at age 6 months (A) and at age 9 months (B) in children receiving either cow´s milk (CM)-based formula or hydrolyzed casein (HC)-based formula and in those fully breast fed (BF). Medians of each group shown by horizontal lines. Arrows are children with IAA. The child who developed type 1 diabetes at 14 months has an asterisk. OD, optical density.

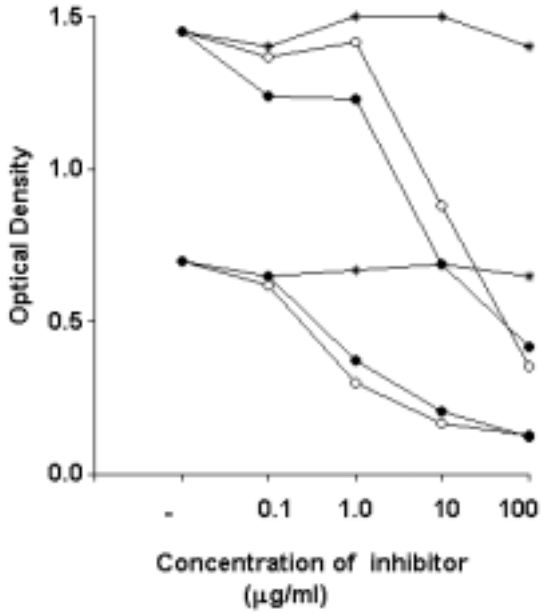

Figure 8. The inhibition of IgG antibody binding to solid-phase bovine insulin (BI) by liquid-phase BI (❍), human insulin (●) or by B-chain of BI (✳) incubated at different concentrations (x-axis) with plasma samples from two patients with newly diagnosed type 1 diabetes.

Insulin-binding antibodies in children at increased genetic risk for type 1 diabetes (2nd pilot study of TRIGR)(IV)

At 3 months of age, median levels of IgG antibodies to BI and HI were highest in infants exposed to CM formula as compared to infants exposed to HC formula or those fully BF, but after this age, no significant differences could be detected among the groups (Table 5). However, in a comparison only of infants exposed to the study formula before 3 months of age, median levels of antibodies to BI at 6 months of age were higher in the CM group (0.272) than in the HC group (0.149, P = 0.038) (previously unpublished data). IgG antibodies to BI and to HI correlated in all groups (P < 0.001; data not shown). At 6 months of age, a trend toward an inverse correlation was detected between the age of introduction of the formula and levels of IgG antibodies to BI in the CM group (r = –0.28, P = 0.055), but not in the HC group (r = –0.18, P = 0.34). Levels of insulin-binding antibodies increased from 3 to 6 months of age in children exposed to CM formula before 3 months of age (median BI-IgG 0.210 vs 0.253, P = 0.001, Wilcoxon`s signed-rank test), but did not increase in children who started the same formula between 3 and 6 months of age (0.183 vs 0.199, p=0.36). In the HC group, no significant changes were detected during this period (data not shown). In this series (IV), four cases converted to positivity for IAA (Fig. 9); all were in the CM group; one, however, did not receive

the study formula at all but was exposed to ordinary CM formula at the age of 7 months after the intervention period (Fig. 9B). In inhibition experiments, soluble BI (200 μg/ml) inhibited the binding of IgG antibodies to solid-phase BI at 6 months most efficiently in the CM group. The median percentages of inhibition were 34%, 19%, and 19% in the CM, HC, and BF groups, respectively (P = 0.003, Kruskal-Wallis H-test; CM vs HC, P = 0.01/P = 0.03, Mann-Whitney U-test/Bonferonni correction; CM vs BF, P = 0.003/P = 0.009).

Table 5. IgG antibody levels to bovine insulin (BI) and human insulin (HI) in the cow´s milk (CM) and hydrolyzed casein (HC) groups and in those fully breast fed (BF)

		CM	HC	BF	P
3 mo	BI	0.210 (0.022-0.541)	0.138 (0.033-0.588)	0.151 (0.028-0.736)	*0.01*
	HI	0.221 (0.055-0.845)	0.147 (0.062-0.635)	0.150 (0.029-0.500)	*0.01*
	n	31	21	54	
6 mo	BI	0.246 (0.066-1.076)	0.192 (0.061-1.644)	0.261 (0.045-1.819)	*0.10*
	HI	0.323 (0.033-0.884)	0.231 (0.050-1.694)	0.275 (0.073-1.811)	*0.12*
	n	48	32	24	
9 mo	BI	0.332 (0.072-0.936)	0.238 (0.072 to >3.0)		*0.08*
	HI	0.311 (0.079-1.643)	0.242 (0.072 to >3.0)		*0.12*
	n	49	45		
12 mo	BI	0.303 (0.052-1.197)	0.235 (0.043-1.358)		*0.14*
	HI	0.306 (0.070-1.075)	0.290 (0.062-2.668)		*0.48*
	n	50	47		
18 mo	BI	0.289 (0.065-1.555)	0.233 (0.033-1.547)		*0.62*
	HI	0.303 (0.062-1.742)	0.280 (0.072-1.657)		*0.36*
	n	40	40		
24 mo	BI	0.331 (0.061-1.234)	0.267 (0.011-1.488)		*0.57*
	HI	0.418 (0.069-1.285)	0.281 (0.053-1.637)		*0.66*
	n	35	34		

Data are optical density units (median and range). The P-values of Kruskal-Wallis-H test are shown. P-values determined by Mann-Whitney U-test (Bonferonni correction) are as follows: at 3 months of age for BI-IgG CM vs HC, P = 0.01 (P = 0.03); CM vs BF, P = 0.007 (P = 0.02); and for HI-IgG CM vs HC, P = 0.012 (P = 0.036); CM vs BF, P = 0.009 (P = 0.027). At 6 months of age for BI-IgG CM vs HC, P = 0.058 (P = 0.17); CM vs BF, P = 0.74 (P = 1.0); and for HI-IgG CM vs HC, P = 0.10 (P = 0.30); CM vs BF, P = 0.33 (P = 0.99).

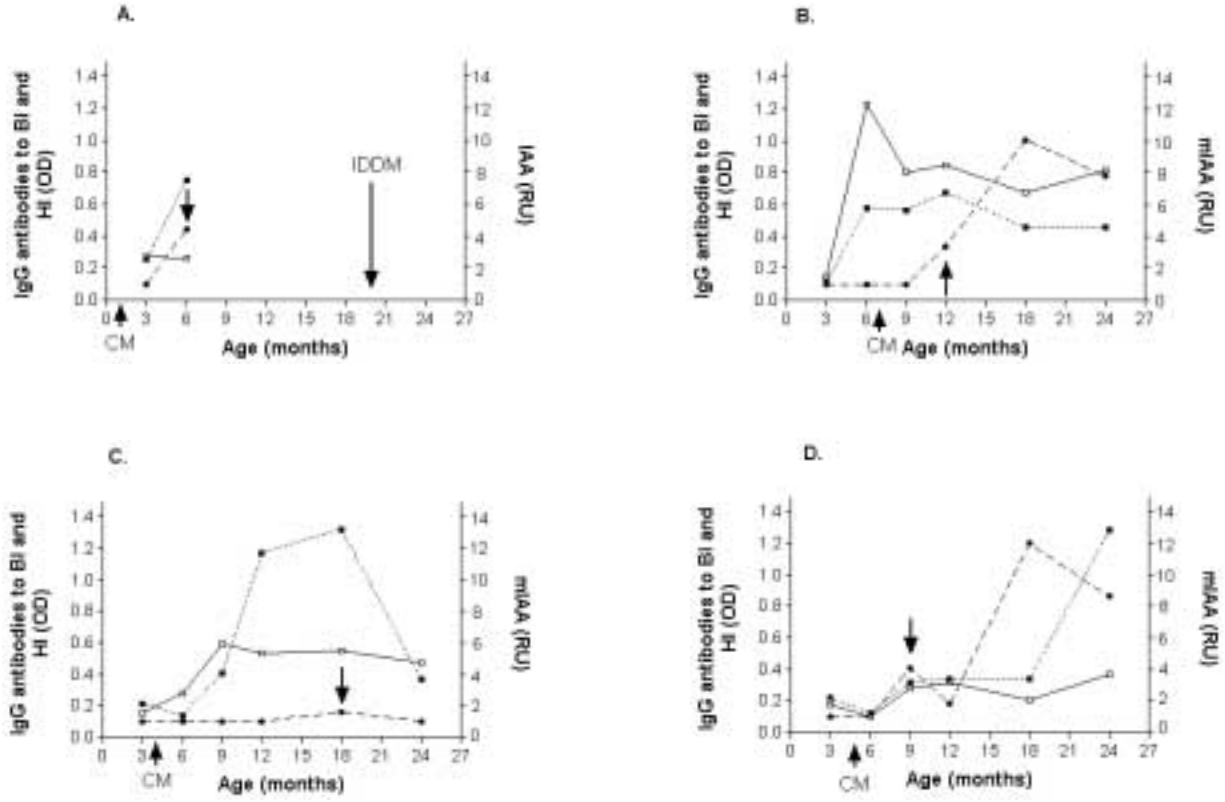

Figure 9. Development of IgG antibodies to bovine insulin (BI) (■) and human insulin (HI) (□) in the four infants with IAA (●). Arrows designate the first positive IAA. The arrowheads below the x-axis designate the start of cow´s milk (CM) formula. RU, relative units; OD, optical density; IDDM, insulin-dependent diabetes mellitus.

Insulin-binding antibodies in children at risk for allergy (V)

Levels of IgG antibodies to BI and HI were low at birth and at 3 months of age in both groups (Table 6 and Fig. 10). Because these values were at the background level of the assay, the differences between the groups were not considered biologically significant. Levels increased after 3 months of age in both groups. No difference in median levels of insulin-binding antibodies was seen between the two groups at 6 months of age, (i.e., during the time when all the mothers consumed CM products) or at 12 months of age, (i.e., after infants were exposed to CM products in their diet). At 18 months of age, IgG antibodies to both BI and HI were lower in infants whose mothers were on an unrestricted diet (ND) than in infants whose mothers followed the CM-free diet (D) during the first 3 months of lactation (median levels 0.287 vs 0.500, $P < 0.0001$ for BI-IgG; 0.249 vs 0.414, $P < 0.003$ for HI-IgG, respectively). At 4 years, the antibody levels no longer differed between the groups (Table 6). An increase in levels of IgG antibodies to BI was observed from 3 to 6 months of age in infants in the D group (0.152 vs 0.202, $P < 0.0001$, Wilcoxon`s signed- rank test), after the cessation of mothers´ dietary manipulation. No significant increase was seen in infants in the ND group from 3 to 6 months of

age (0.123 vs 0.160, P = 0.11). A peak in the levels of IgG antibodies to BI and HI was reached at 12 months of age in the ND group, and at 18 months of age in the D group. The IgG antibodies to BI and HI correlated at all time-points (r = 0.53, P < 0.0001 at birth; r = 0.62, P < 0.0001 at 3 months; r = 0.75, P < 0.0001 at 6 months; r = 0.56, P < 0.0001 at 12 months; r = 0.69, P < 0.0001 at 18 months; and r = 0.58, P < 0.0001 at 4 years). The binding of IgG antibodies to solid-phase BI at a concentration of 1,000 μg/ml liquid phase BI was inhibited to a similar degree in both groups. Median percentages of inhibition at 6 months of age were 47 and 40.5 % (ns) in the D and ND groups, respectively.

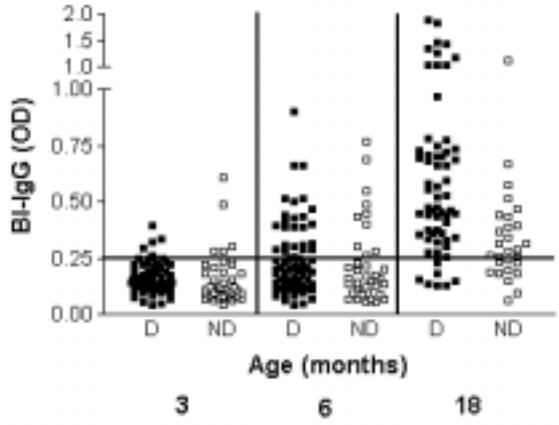

Fig. 10. IgG antibodies to bovine insulin (BI) at 3 months (P < 0.05), at 6 months (P = 0.25), and at 18 months (P < 0.0001) in infants whose mothers avoided CM during the first 3 months of lactation (D) and in infants of mothers on an unrestricted diet during lactation (ND). The lower level of detection shown by the horizontal line.

Table 6. Median levels of IgG antibodies to bovine insulin (BI-IgG) and human insulin (HI-IgG) expressed as optical density units in infants whose mothers avoided CM during the first 3 months of lactation (D), and in infants of mothers on an unrestricted diet during lactation (ND).

		BI-IgG			HI-IgG		
		D	ND		D	ND	
	0 months	0.219	0.213	ns	0.195	0.163	p<0.05
Stop of maternal dietary elimination →	3 months	0.150	0.114	p<0.05	0.169	0.176	ns
Start of CM formulas →	6 months	0.202	0.159	ns	0.246	0.191	ns
	12 months	0.318	0.303	ns	0.351	0.304	ns
	18 months	0.500	0.287	p<0.0001	0.414	0.249	p<0.003
	4 years	0.226	0.221	ns	0.226	0.225	ns
	Mother	0.174	0.174	ns	0.212	0.208	ns

Cellular immune responses to insulin (IV)

At 3 months of age, T-cell reactivity to BI differed between the three groups (P = 0.04, Kruskal-Wallis H-test), with the SI being highest in the CM group (Fig. 11A), whereas no difference in cellular responses to HI was detected between the groups (Table 7). Infants in the CM group had enhanced T-cell responses to BI when compared with BF infants (p=0.015 Mann-Whitney U-test; with Bonferonni correction P = 0.045), but no difference was observed between infants in the CM and HC groups. Reactivity to BI decreased during the intervention period from 3 to 6 months in the CM group (median SI 2.2 vs 1.3; P = 0.006, Wilcoxon`s signed-rank test), and at 6 months of age cellular immune responses to insulin did not differ between the groups. Nor at 9 months of age, when all infants had been exposed to ordinary CM formula for at least 1 month, did cellular responses to insulin differ (Fig. 11B, Table 7). T-cell responses to BI and HI correlated at all ages in the CM group (data not shown). T-cell responsiveness to HI above SI of 2 was observed in the CM group in seven of 21 children aged 9 months, whereas earlier, the reactivity to HI was low (at 3 months of age none of 14 infants had a SI>2; at 6 months of age, one of 19 infants had a SI>2). At 9 months of age, the same individuals with cellular responses to BI also showed T-cell reactivity to HI (Fig. 12). T-cell responses and IgG antibodies to BI correlated in the CM group at 6 (r = 0.47, P = 0.05) and at 24 months of age (r = 0.72, P = 0.02), but not at other time-points (data not shown). No correlation was detectable in HC group at any age (data not shown). Reactivity to BI did not differ between defined HLA-risk genotypes at any age (data not shown). No differences existed among the groups in the SIs to the control antigens tuberculin PPD or TT (Table 7).

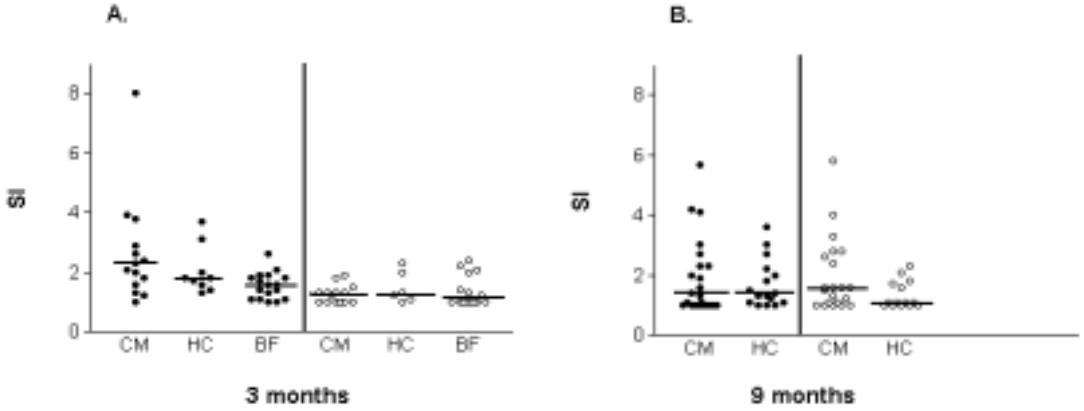

Fig. 11. T-cell response to bovine insulin (BI) (●) and to human insulin (HI) (❍) expressed as stimulation index (SI) in infants receiving cow´s milk (CM)-based formula or hydrolyzed casein (HC)-based formula and in those fully breast fed (BF) during the intervention at 3 months (A) and after the intervention at 9 months (B). Medians are shown by horizontal lines. For SI to BI, P = 0.04 and for SI to HI, P = 0.66 with the Kruskal-Wallis H-test at 3 months. At 3 months with Mann-Whitney U-test (Bonferonni correction) for SI to BI CM vs HC, P = 0.39 (P = 1.0) and CM vs BF, P = 0.015 (P = 0.045). At 9 months SI to BI, P = 0.86 and for SI to HI, P = 0.15.

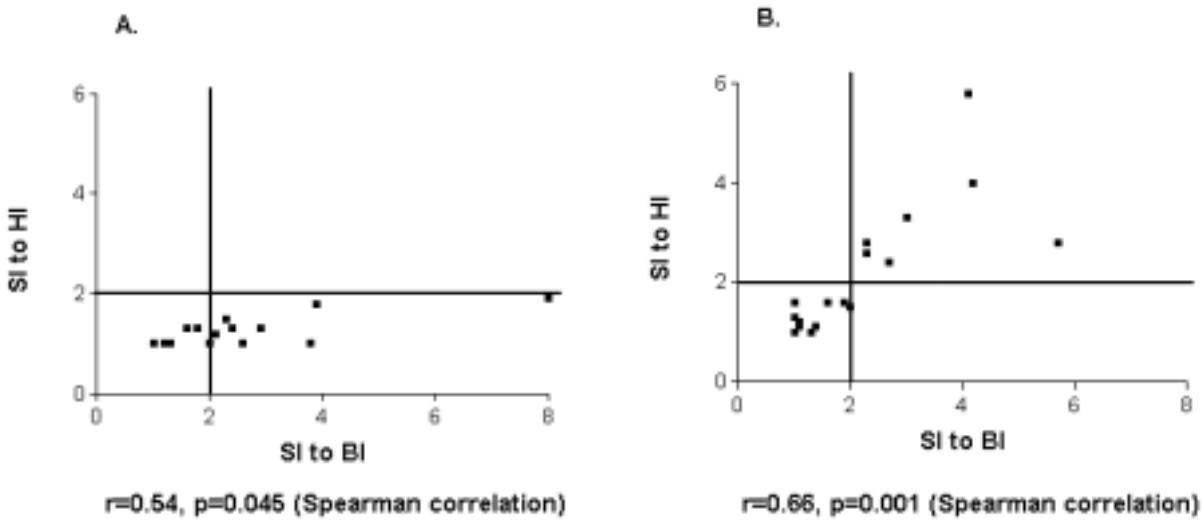

Fig.12. Correlation of T-cell responses to bovine insulin (BI) and human insulin (HI) expressed as stimulation index (SI) at 3 months (A) and at 9 months (B) in the cow´s milk-based formula group.

Table 7. T-cell responses to bovine insulin (BI) and human insulin (HI), tuberculin PPD and tetanus toxoid (TT) as Δ cpm and SIs in the feeding groups

		cpm	Median Δ cpm				Median SI			
		background	PPD	TT	BI	HI	PPD	TT	BI	HI
3 Months	CM n=14	696	9,436		1,039	120	13.1		2.2	1.3
	HC n= 9	554	16,366		352	136	24.0		1.8	1.3
	BF n=17	578	2,824		205	71	10.2		1.6	1.2
	All	P = 0.44	P = 0.19		P = 0.008	P = 0.66	P = 0.39		P = 0.04	P = 0.66
	CM vs HC				P= 0.12				P = 0.39	
	CM vs BF				P = 0.005				P = 0.015	
6 Months	CM n=20	659		6,392	179	3		11.4	1.4	1.0
	HC n=11	714		8,564	198	93		11.4	1.2	1.2
	BF n=10	547		7,298	174	41		20.0	1.5	1.2
	All	P = 0.22		P = 0.78	P = 0.49	P = 0.30		P = 0.54	P = 0.96	P = 0.73
9 Months	CM n=22	1,125		11,622	453	458		14.5	1.4	1.6
	HC n=16	813		11,079	307	36		12.4	1.4	1.1
	CM vs HC	P = 0.36		P = 0.60	P = 0.55	P = 0.08		P = 0.96	P = 0.86	P = 0.15
12 Months	CM n=21	446		7,145	286	318		13.3	1.6	1.3
	HC n=17	607		6,819	306	47		15.0	1.5	1.2
	CM vs HC	P = 0.21		P = 0.99	P = 0.74	P = 0.33		P = 0.52	P = 0.47	P = 0.33
18 Months	CM n=18	1,087		7,401	276	265		11.8	1.4	1.4
	HC n=15	706		9,810	569	470		10.5	1.9	1.6
	CM vs HC	P = 0.63		P = 0.47	P = 0.43	P = 0.86		P = 0.33	P = 0.33	P = 0.22
24 Months	CM n=11	573		11,251	407	145		13.7	1.6	1.2
	HC n=10	774		11,096	306	415		14.0	1.4	1.5
	CM vs HC	P = 0.62		P = 0.78	P = 0.48	P = 0.40		P = 0.27	P = 0.23	P = 0.84

Effect of maternal type 1 diabetes on immunization to dietary insulin (IV)

Cellular immune responses to BI at 9 and 24 months of age and to HI at 9, 12, and 24 months of age were lower in children with a diabetic mother than in children with a diabetic father or a sibling in the CM group (at 9 months, median SI to BI 1.1 vs 2.3; P = 0.05 and SI to HI 1.3 vs 2.5; P = 0.06; at 12 months, median SI to HI 1.2 vs 1.7, P = 0.014; at 24 months, median SI to BI 1.3 vs 1.9, P = 0.081 and SI to HI 1.1 vs 1.5, P = 0.009, Mann-Whitney U-test) (Fig. 13). No effect of maternal type 1 diabetes on cellular immunization to dietary insulin was observed at other time-points (data not shown). No effect of maternal diabetes on cellular immune responses to control antigens, PPD and TT was detectable in the same group of children (data not shown, previously unpublished data).

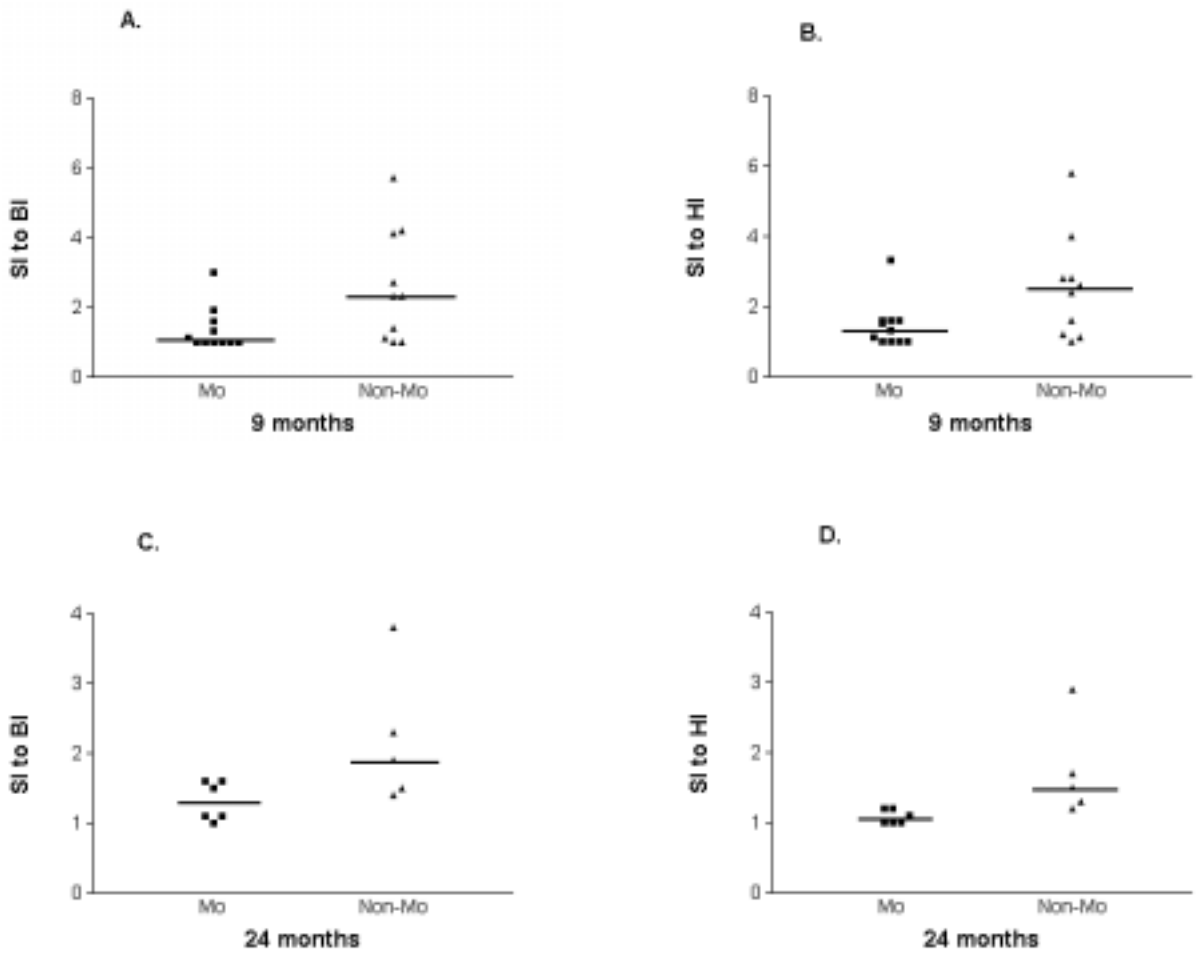

Fig. 13. T-cell responses to bovine insulin (BI) (A and C) and human insulin (HI) (B and D) in infants with a diabetic mothers (Mo) and a non-diabetic mother (Non-Mo) at 9 months (A and B) and at 24 months (C and D) in the cow´s milk-based formula group. For median SI to BI 1.1 vs 2.3, P = 0.05, and for SI to HI 1.3 vs 2.5, P = 0.06 at 9 months. For SI to BI 1.3 vs 1.9, P = 0.081, and for SI to HI 1.1 vs 1.5, P = 0.009 at 24 months. Medians shown by horizontal lines.

Antibody responses to insulin were compared between children with a diabetic mother and children with a diabetic father or a sibling only from 9 months of age on, from which time maternal antibodies are no longer detectable in the serum of the majority of infants (Martikainen et al 1996, Hämäläinen et al 2000). IgG antibodies to BI at 24 months were lower in offspring of diabetic mothers than in those with a diabetic father or a sibling in the CM group (median 0.275 vs 0.483, P = 0.05, Mann-Whitney U-test) (Fig. 14). No significant differences were observed at other time- points (data not shown). Levels of insulin-binding antibodies in children at 9, 12, 18 or 24 months of age did not correlate with maternal IAA levels in samples taken at delivery (data not shown). No effect of maternal type 1 diabetes was seen on antibodies to BLG in the same group of children (data not shown).

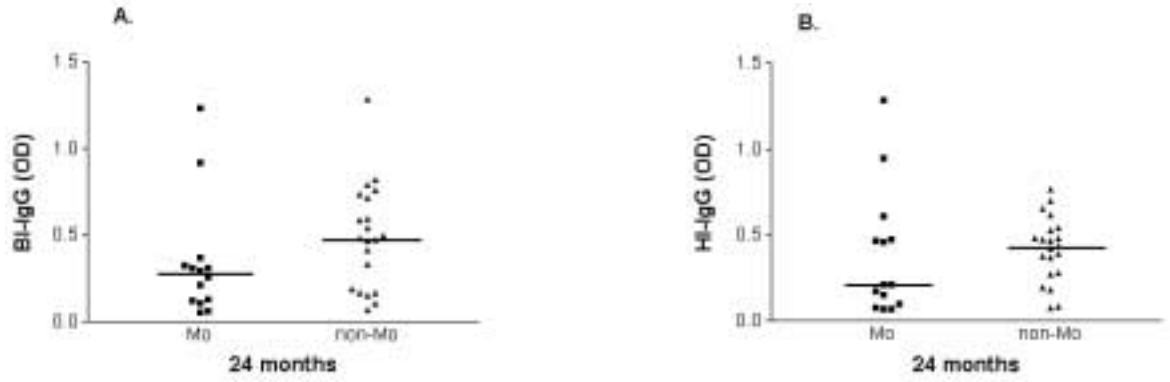

Fig. 14. IgG antibodies to bovine insulin (BI) (A) and to human insulin (HI) (B) in children with a diabetic mother (Mo) or a non-diabetic mother (Non-Mo) at 24 months in the cow´s milk-based formula group. For BI-IgG 0.275 vs 0.483, P = 0.05, and for HI-IgG 0.211 vs 0.433, P = 0.21. Medians shown by horizontal lines.

Relation of BLG antibodies to CM exposure and to insulin-binding antibodies (IV)

Infants exposed to CM formula had the highest levels of BLG-IgG at 3 and 6 months of age as compared to infants exposed to HC formula or those fully BF, but elevated levels of IgG-antibodies to BLG were also detected in some cases in the BF group at 6 months of age. IgG antibodies to BI correlated with IgG antibodies to BLG at 6 months in the BF group (r = 0.65, P = 0.001), but not in the other groups. Median levels of IgG antibodies to BLG at 3 months of age were 77, <10, and <10% (P = 0.0001, Kruskal-Wallis H-test); at 6 months of age were 57.5 , <10, and 20.4% (P = 0.0001) in the CM, HC, and BF groups; and at 9 months of age were 68.7 vs 38.8% (P = 0.02, Mann-Whitney U-test); at 12 months were 80.6 vs 63.0% (P = 0.45); at 18 months 39.8 vs 44.1% (P = 0.61); and at 24 months of age 58.6% vs 58.6% (P = 0.57) in the CM and HC groups, respectively.

DISCUSSION

Immune activation in infancy by CM exposure

Our study (I) showed that levels of sICAM-1 were higher up to 2 years of age in children exposed to CM formula early on compared with levels in those fed HC formula for the first 9 months of life. This finding suggests immune activation provoked by early feeding of intact CM proteins. Early exposure to intact CM proteins has been shown to result in enhanced cellular and humoral immune responses to these proteins even for many years, although a reduction in cellular responses (Vaarala et al 1995) and a plateau/decrease in antibody responses is reached at some point during childhood (Kemeny et al 1991, Jenmalm and Björksten 1998), suggesting development of oral tolerance. One study showed that immune activation, measured as soluble IL-2R and IFN-γ, increased during infancy and this was associated with weaning (Cummins et al 1994). The proportion of activated peripheral lymphocytes was low, and GALT was suggested as the source of this noncirculatory immune activity. In another study, circulating cells secreting specific IgA to CM antigens were detected at 3 months of age in healthy infants fed CM formula despite low levels of IgA serum antibodies, indicating activation of GALT by CM exposure (Kaila et al 1994). Early CM exposure may result in increased intestinal permeability of antigens, as shown in experimental animals and infants (Weaver et al 1987, Kuitunen et al 1994), and may lead to increased immune responses to oral antigens in general. The impairment in mucosal barrier function in suckling rats is suggested to be due to a local hypersensitivity reaction to CM antigens in the gut, irrespective of the possible protective effect of simultaneous breast feeding (Arvola et al 1994). Accordingly, our finding of higher levels of sICAM-1 in the CM group may reflect a more general immune activation in the GALT due to early CM exposure.

On the other hand, infants who received HC formula had still lower sICAM-1 levels at 12 and 24 months of age, after the cessation of dietary intervention and the start of exposure to intact CM proteins, suggesting suppression of immune responses in this group. Lower cellular and humoral immune responses to CM proteins in these infants who received HC formula has indeed been shown (Vaarala et al 1995). Formulas based on hydrolyzed CM proteins have greatly reduced immunogenicity, and are usually successful in treatment of CM allergy (reviewed by Halken and Høst 1997). In one study, infants fed for the first 3 months of life the same casein hydrolysate as in our study, and who then received CM- or soy-based formulas had lower antibody titers to CM and soy proteins up to 12 months of age than did those infants fed

CM- or soy-based formulas since birth; this also showed the effect of early feeding on subsequent immune responses (Eastham et al 1978).

Levels of sICAM-1 and sL-selectin were higher in all our infants than are reported values in healthy adults, and these levels were also elevated when compared to cord blood concentrations in the same infants. Our results suggest that maturation of the immune system in infants exposed to new antigens provokes an elevation of these adhesion molecules in plasma. In adults, comparable elevated levels of circulating adhesion molecules have been reported in various disease states, implying immune activation (Gearing and Walter 1993). Our finding of difference between the groups only in sICAM-1 but not in sL-selectin may relate to the fact that the LFA-1/ICAM-1 pair plays an important role in lymphocyte activation by providing costimulatory signals (van Seventer et al 1990). LFA-1 and ICAM-1 have been shown to be involved in immune responses requiring cell-cell contact, and antibodies to these have been shown to inhibit cytotoxic cell function and/or immunoglobulin production in man and in animals (Fischer et al 1986, Boyd et al 1988). Soluble ICAM-1 may also have a physiologic immunomodulatory function resulting in downregulation of immune responses (Roep et al 1994, Martin et al 1998).

Role of the gut immune system in type 1 diabetes

We showed in our study (II) that in children with newly diagnosed type 1 diabetes, the cellular response of PBMCs to GAD65 decreased markedly after depletion of $\alpha4\beta7$-expressing cells. In contrast, reactivity to the parenteral antigen TT increased, indicating that these two antigen-specific cell populations have distinct homing properties. Our finding that GAD-reactive peripheral T-cells in patients with new-onset type 1 diabetes belong to the gut-associated $\alpha4\beta7$-positive population suggests involvement of the gut immune system in the pathogenesis of type 1 diabetes. This lymphocyte antigen, termed Act-1, was at first shown to be an activation marker both in vivo and in vitro. Anti-Act-1 was identified in murine hybrinoma cells immunized with a human TT-reactive T- lymphoblast line. Act-1 was then shown to be present in markedly greater amounts on activated human T- and B-lymphocytes than on resting peripheral blood lymphocytes, and Act-1 expression on these lymphocytes could be promoted by phytohemagglutinin (PHA), TT, or alloantigens in vitro (Lazarovits et al 1984). In vivo evidence exists of the association of this homing receptor with the gut immune system. In humans, circulating CD4+ cells with a memory for intestinal rotavirus have been shown to

express mucosal homing receptor α4β7 after natural rotavirus infection (Rott et al 1997), and in CM allergy, BLG-reactive PBMCs express this integrin (Eigenmann et al 1998). These findings emphasize that this receptor really is involved in circulation of lymphocytes to the GALT in man as well. That we could detect a difference in α4β7 expression in PBMCs reacting to GAD65 and TT suggests that GAD-reactive T-cells have homing properties to the GALT. A T-cell line propagated from islet-infiltrating lymphocytes of a child who died shortly after diagnosis of type 1 diabetes was α4-positive and showed preferential binding to gut mucosal endothelium in addition to the endothelium of diabetic pancreas (Hänninen et al 1993b). In addition, a PBMC-derived cell line from this child adhered almost as strongly to the gut mucosal endothelium as the pancreatic cell line, suggesting that lymphocytes from both lines were initially activated in the GALT (exposure to dietary or microbial antigens) and that subsequently homing of some cells into the pancreas occurred. Although our results did not indicate whether priming of GAD-specific lymphocytes occurred in the gut or in the pancreas, these results imply that the T-cell reportoire reactive against a pancreatic islet cell antigen in type 1 diabetes seems to recirculate via the gut and is thus exposed there to local cytokines. Because the cytokine milieu in the microenvironment plays a fundamental role in determining the functional phenotype of antigen-reactive cells (reviewed by De Carli et al 1994), changes in the cytokine profile of the gut as a result of various stimuli may result in induction of autoaggressive lymphocyte clones capable of infiltrating the pancreas.

Furthermore, the experiments in the NOD mouse emphasize a role for the gut immune system in autoimmune diabetes and especially in the initiation phase. The gut-associated homing receptor α4β7 was shown to predominate in islet-infiltrating lymphocytes in early insulitis, and the mucosal counter receptor MAdCAM-1 became expressed on islet endothelium at the time when the first lymphocytes infiltrated the pancreas, predominating in the islets of young NOD mice (<12 weeks) (Hänninen et al 1993a and 1996a). Anti-MadCAM-1 treatment was able to inhibit the development of spontaneous diabetes when started at 3 weeks of age but not when started at 10 weeks. In adoptive transfer experiments, anti-MAdCAM-1 treatment of recipient mice delayed the onset and reduced the incidence of diabetes when MLN cells from 6-week-old donors were used, but not when spleen cells or MLN cells from overtly diabetic mouse were used as donor cells (Hänninen et al 1998). In addition, lymphocytes capable of transferring diabetes are prevalent in MLNs of young NOD mice (6 weeks of age) and are more aggressive in inducing the disease than are pancreatic lymph node cells from the same animals at the same

age (Hänninen et al 1996b). These findings in the animal model of type 1 diabetes point to primary events in the GALT leading to autoimmune diabetes.

In type 1 diabetes, GAD is a target of both humoral and cellular autoimmunity. This role of GAD as an autoantigen is further supported by the evidence that GAD-reactive T-cell lines from a GAD-immunized NOD mouse which self-developed diabetes, could transfer insulitis and diabetes to a NOD/SCID mouse (Zekzer et al 1998). These lines were specific to the GAD peptide 524-543, an epitope previously identified as dominant in the early phases of the disease (Kaufman et al 1993). β-cell-specific suppression of GAD expression in anti-sense GAD transgenic NOD mice has been shown to block the generation of diabetogenic T-cells and to prevent diabetes, indicating that GAD expression on β-cells is a requirement for development of diabetes in such mice (Yoon et al 1999). In addition, modulating the autoimmune response to GAD in NOD mice inhibits ongoing autoimmune diabetes and prolongs islet graft survival (Tian et al 1996b, Tisch et al 1998). GAD has thus been suggested as one of the primary antigens in the autoimmune reaction against β-cells.

Depletion with anti-Act-1 antibody resulted in a PBMC population devoid of $\alpha4\beta7^{high}$-expressing cells. GAD65 reactivity was not, however, totally abolished in our diabetic patients when α4β7-expressing lymphocytes were depleted. In three of our 6 patients the reactivity to GAD65 did not decrease after depletion of these cells. This indicated that the cell population showing low expression of α4β7-integrin included GAD65-reactive cells. In NOD mice, the role of mucosa-associated ($\beta7^{high}$) lymphocytes in the development of insulitis is proposed to be important, especially in the early phases of insulitis (Hänninen et al 1996a). Thus, it was not unexpected in our study that at the time of diagnosis of type 1 diabetes, obviously after long-lasting insulitis, the GAD-reactivity was not totally restricted to the α4β7-expressing lymphocytes. At the site of chronic inflammation, several endothelial adhesion molecules are induced and lymphocytes expressing different homing receptors are recruited. We found a decrease of 45% in the proliferative response of PMBCs to GAD65 in the prediabetic child after depletion of α4β7-expressing lymphocytes suggesting that the α4β7-expressing lymphocytes are already primed against GAD before manifestation of type 1 diabetes. Indeed, this child has later manifested with the disease. Studies on prediabetic children in cohort studies would provide more information as to the role of gut-associated lymphocytes in initiation of an immune response against β-cells in human type 1 diabetes.

Because cellular immunity to GAD65 is associated with but not restricted to type 1 diabetes (Ellis and Atkinson 1996), in addition to the specificity of T-cells against an autoantigen, the sites of priming and expansion of the antigen-specific lymphocytes may also be important in development of organ-specific autoimmune diseases. Although the group of patients with APD-I in the present study was small, the GAD-reactive lymphocytes from the one patient with type 1 diabetes expressed the α4β7-integrin, whereas GAD-reactivity in PBMCs of the two other APD-I patients without type 1 diabetes did not show high expression of this gut-specific homing receptor. This finding supports the evidence that GAD-reactive lymphocytes in non-diabetes-related conditions such as stiff-man syndrome or APD-I may not be primed in the gut and may thus not have homing properties to gut-associated sites; perhaps for that reason they do not infiltrate the pancreas (Wagner et al 1994, Schloot et al 1999, Klemetti et al 2000, Lohmann et al 2000). The difference in epitope recognition of GAD65 by peripheral blood T-cells in patients with stiff-man syndrome compared with that of T-cells from patients with type 1 diabetes may imply that nature of the antigen or mode of presentation, i.e., priming differs between these two diseases (gut/pancreas in type 1 diabetes vs CNS in stiff-man syndrome). Additionally, immunoregulation and genetic background may modify disease outcome (Schloot et al 1999, Lohmann et al 2000).

A higher expression of α4β7-integrin has been found on CD8+ than on CD4+ T-cells (Erle et al 1994). Because depletion with anti-Act-1 resulted in a more pronounced decrease in the CD8+ than in the CD4+ population, we studied, in four patients with type 1 diabetes, the effect of depletion of CD8+ cells by immunomagnetic separation on the proliferation response to GAD65. The depletion of CD8+ cells did not, however, reduce the response to GAD but enhanced TT-reactivity, suggesting that our main finding is related to α4β7-integrin-expressing CD4+ cells. Because of the limited blood volume of the patients, this analysis was performed on a different subgroup of diabetic patients.

Immunization to dietary insulin

We demonstrated that CM formulas contain BI and that in some children at risk for type 1 diabetes, exposure to these formulas results in immunization to BI both at cellular and humoral level. The induction of insulin-specific immune responses by BI is based on a difference in three amino acids between BI and HI. When BI was used for treatment of diabetes, it was found to be highly immunogenic (Kurtz et al 1980), whereas porcine insulin with one amino acid difference

from HI is less immunogenic although inducing some antibody response in individuals undergoing therapy (Heding et al 1984). In animals it has been shown that even one amino acid change in a protein makes a difference in antigenic recognition and may radically alter the immunogenicity of the protein (Ohashi et al 1991, Ottesen et al 1994, Homann et al 1999b). A one-amino acid change in the B-chain of the insulin molecule abolished the ability of insulin to confer oral tolerance and protection from diabetes in NOD mice and in a virus-induced transgenic mouse model of type 1 diabetes (Homann et al 1999b).

At 3 months of age, our infants exposed to CM formula had higher T-cell responses and levels of antibodies to BI than did BF infants. T-cell responses and antibodies to BI occurred also in some infants who received HC formula. It is possible that some infants in the HC group developed immunity to insulin peptides present in the formula. In this respect, prevention of early immunization to insulin is not totally avoided by the use of HC formula, although peptides can be considered less immunogenic than whole proteins. The small number of infants in the T-cell analyses may also explain the fact that no difference was observed in T-cell responses to BI between the CM and HC groups at 3 months of age, since a difference in the levels of insulin-binding antibodies was demonstrated between the larger groups at this age. Nor was any difference observed in cellular responses to insulin between the CM and HC groups at 6 months of age. This result may have been influenced by the decrease in T-cell responses to BI from 3 to 6 months of age in the CM group, and may suggest that in the majority of healthy children, continuous use of the CM formula resulted in tolerization. The correlation between insulin-specific T-cell and antibody responses at 6 and 24 months of age in the CM group but not in the HC group suggests that the differing nature of insulin in these two formulas (whole protein vs. peptide) influences the development of insulin-specific immune responses. A difference in insulin-specific responses between the groups was also seen in immune responses to HI. T-cell reactivity to BI had already emerged at 3 months of age in infants in the CM group, and only later (by 9 months of age) was this reactivity observed to mount a cellular response to HI as well. No such development could be detected in the HC group. Indeed, at 9 months of age cellular responses to HI tended to be higher in infants in the CM group than in infants in the HC group. The elevated levels of antibodies to HI observed in infants in the CM group at 3 months of age were most likely due to cross-reactive BI antibodies. Because the number of children in T-cell analyses was small, we are cautious in drawing any conclusions as to the association between T-cell responses to insulin and HLA risk alleles.

Our observations raise the issue whether oral exposure to foreign insulin plays a role in the autoimmune process leading to type 1 diabetes. Although oral antigens have been shown in many animal studies to induce tolerance and protect from autoimmune disease (Weiner 1997), deleterious effects of feeding autoantigens have also been described. In a transgenic mouse model in which ovalbumin (OVA) was expressed on β-cells, feeding of OVA induced cytotoxic lymphocyte (CTL) immunity and islet cell destruction (Blanas et al 1996). On the other hand, CTL induction via oral OVA was also achieved in normal inbred nontransgenic mice, implying that this response is a part of the normal immune system. Autoimmune arthritis in susceptible mice could be induced by oral administration of type II collagen, and co-administration of *E. coli* lipopolysaccharide (LPS) induced an even more chronic arthritis that progressed spontaneously without further administration of collagen or LPS (Terato et al 1996). Oral insulin may not only induce tolerance but also in some situations lead to exacerbation of the development of diabetes. In BB rats, oral insulin with a bacterial adjuvant induced exacerbation of the disease (Bellman et al 1998). In addition, in cotransfer experiments, acceleration of the disease has been shown by Th1-cells from mice fed insulin peptides (Maron et al 1999) and by CD8+ cells from mice fed insulin (Bergerot et al 1994) or insulin-cholera toxin B (Ploix et al 1999) when cotransferred with diabetogenic cells. Oral insulin in patients with recently diagnosed type 1 diabetes was not beneficial for residual β-cell function, but in fact a tendency toward a more pronounced C-peptide decrease at 9 and 12 months was noted in patients < 15 years old in the oral insulin treatment group vs the placebo group (Pozzilli et al 2000). A similar negative effect of oral insulin in type 1 diabetes after 3 months of treatment was also shown in another study (Chaillous et al 2000). All these findings point to the dual nature of oral antigen administration, tolerance vs immunity, in which many factors influence the outcome and suggest caution in regard to this route antigen delivery (reviewed by Vaarala 1999, Hänninen 2000).

Although immunization to dietary insulin occurs in early infancy, only a minority of children included in our TRIGR pilot studies are likely to develop type 1 diabetes. However, it is possible that in some genetically susceptible children, a continuous small-dose exposure to the BI present in CM formula at an early age may lead to loss of tolerance to insulin, as seen in those children in our studies (III, IV) who developed IAA, and in children with multiple autoantibodies described in our other prospective study (Vaarala et al 1999). The initiation of insulin-specific T-cells by dietary insulin in the gut immune system may carry a risk for an autoimmune process leading to β-cell destruction and type 1 diabetes. In this process, the factors

that lead to the activation of insulin-primed T-cells are unknown, but they may be associated with regulation of the gut immune system (Vaarala 1999).

We observed that some children who were fully BF at 6 months of age had insulin-binding antibodies, but no detectable T-cell responsiveness to BI. The immunization to insulin by breast feeding may be caused by small amounts of BI present in breast milk (when the maternal diet contains CM) or alternatively by HI present in maternal milk. Results from our Study V suggest that it is the presence of BI in breast milk that explains these antibodies seen in BF children. Antibodies to BLG also appeared in some fully BF children at 6 months of age, which further supports the view that small amounts of BLG derived from the maternal CM-containing diet and present in breast milk at low concentrations, i.e., 5-33 μg/l (Jakobson et al 1985), may immunize some children (Jakobson and Lindberg 1979). The finding that responses to BLG and to BI correlated in the BF group at 6 months of age provides additional evidence that the immune responses detected may be due to the transfer of dietary antigens into breast milk. The infant who developed IAA during the follow-up and who already had elevated levels of insulin-binding antibodies by 6 months of age during full breast feeding (Fig. 9B) may be an example of immunization to low-dose antigens.

Exposure to BI in the CM formula seemed to result in an immunization to insulin which was distinguishable from that seen in the BF- and HC-fed infants at 3 months of age, but not later on. However, at 6 months of age, when in the subanalysis only infants exposed to the study formulas before 3 months of age were included, a difference existed in insulin-binding antibodies between infants in the CM and HC groups. This finding further emphasizes the effect of early CM exposure on insulin immunization. Exposure to the CM study formula (regardless of whether started before or after 3 months of age) was observed in the levels of antibodies to BLG even at 9 months of age, at which time children in the CM group still maintained higher antibody levels than did children in the HC group who were exposed to intact CM proteins after 6 months of age. This difference in responses to BI and BLG may be due to the modifying effect of the dose of the dietary immunogen. The insulin content in native CM varies from 1 to 10 μg/l (Aranda et al 1991), about 1000-fold less than the concentration of BLG. In a study by Björkstén et al (1996) small doses of mite antigen well below the suggested sensitization threshold level of 2 μg/g dust induced mite-specific T-cell responses in young children. This finding is in accordance with our present finding of T-cell responses to low doses of dietary BI.

Inhibition of insulin-binding antibodies by liquid-phase insulin showed substantial variation. The highest affinity to liquid-phase insulin appeared in antibodies induced by CM formula. Overall, the affinity of these dietary insulin-induced antibodies seemed to be low. This is expected in an early immune response, whereas a more mature response represented by autoimmunity to insulin is of higher affinity (Tikhomirov and Thomas 1997). Although IAA are frequently seen in patients with type 1 diabetes, only low levels of peripheral T-cell reactivity to insulin have been observed in individuals at high risk of type 1 diabetes (Keller 1990) and in newly diagnosed patients before treatment with exogenous insulin (McCuish et al 1975). This may be explained by sequestration of insulin-specific T-cells in the pancreas, since Wegmann et al (1994a) showed that the majority of islet-infiltrating lymphocytes are insulin-reactive in NOD mice, but that peripheral T-cells do not proliferate to insulin. The observation that insulin-reactive T-cell clones isolated from NOD mice were able to transfer the disease to healthy mice (Daniel et al 1995) and trigger β-cell specific destruction of MHC-unrelated islet xenografts residing in NOD SCID recipients (Crawford et al 1997) suggests that insulin may play a role in the autoimmune process leading to diabetes. In fact, insulin has been shown to be capable of producing autoimmune diabetes in experimental animals (Grodsky et al 1966, LeCompte et al 1966).

The role of insulin in the pathogenesis of type 1 diabetes is supported by data showing that insulin has a dual nature in the disease process, either pathogenic or tolerogenic. A recent finding shows that a cloned Th1 insulin-reactive cell line isolated from the pancreatic lymph nodes of NOD mice could reduce insulitis and totally block development of spontaneous diabetes in NOD mice, as well as the adoptive transfer of diabetes into irradiated NOD mice following the injection of splenocytes from diabetic mice (Zekzer et al 1997). In animals, insulin admnistered by various routes (sc, iv, orally or nasally) has been shown to act as a tolerogen, reducing the development of insulitis and the incidence of diabetes. In subjects at risk for type 1 diabetes, sc insulin alone or combined with iv administration of insulin has been shown to delay the progression to the disease (Keller et al 1993, Füchtenbusch et al 1998). The above data, like our finding of insulin tolerization in the offspring of diabetic mothers shows that modulation of immune responses to insulin has a major effect on the autoimmune process. On the other hand, it has been shown that highly pathogenic CD8+ T-cells from insulitis lesion in NOD mouse recognize the same insulin B-chain epitope (Wong et al 1999) as do previously isolated pathogenic CD4+ T-cells (Daniel et al 1995). These CD8+ cells were derived from the islet infiltrates of a 7-week-old female NOD mouse, pointing to a role for insulin in the early phases

of the disease. Likewise, the insulin-reactive CD4+ lymphocytes with a Th1-like phenotype are also found in early islet infiltrates, i.e., as early as in 4-week-old NOD mice (Wegmann et al 1994b). In addition, IAA expression by NOD mice at 8 weeks of age was strongly associated with early development of diabetes, which occurred by 16 to 18 weeks age (NOD mice IAA-positive at 8 weeks: 83% diabetic by 18 weeks versus 11% of those IAA-negative at 8 weeks) (Yu et al 2000). In man, IAA are also the first autoantibodies to appear in birth/infant cohort studies, even as early as 6 months of age (Ziegler et al 1999, Yu et al 2000, Åkerblom HK, personal communication). IAA seem to be a marker for an early onset of type 1 diabetes, since in the DAISY study of five infants with persistent IAA before 1 year of age, four have progressed to diabetes (all before 3.5 years of age) and the fifth was less than 2 years old in 2000 (Yu et al 2000). In all, the data suggest that in type 1 diabetes the primary autoantigen may be insulin. Our findings concerning the immunization to dietary insulin and the development of insulin-binding antibodies in young children may explain the early-appearing IAA and may imply that BI is the primary trigger of the autoimmune cascade leading to type 1 diabetes. Supporting our findings and hypothesis is the discovery that diabetes-related IAA are crossreactive with BI, whereas diabetes non-related IAA are specific to HI (Potter and Wilkin 2000).

Effect of maternal type 1 diabetes on immunization to dietary insulin

Development of immune responses to dietary insulin in the present study (IV) was modified by maternal type 1 diabetes. On the other hand that maternal diabetes/insulin therapy had no effect on immune responses to control antigens showed that the tolerization effect was antigen-specific. A lower risk for type 1 diabetes has been reported in children of mothers with type 1 diabetes than in children of fathers with the disease (Warram et al 1984, Tuomilehto et al 1992). It has been hypothesized that this may be due to induction of immune tolerance to β-cell antigens presented in utero by exposure to maternal diabetes, but the immunologic mechanisms are unknown. We found that the immune responses to insulin were lower in children with a diabetic mother than in those with a non-diabetic mother. This phenomenon was observed after the age of 6 months when maternal antibodies have been cleared from the infant´s sera, and accordingly the responses did not show a correlation with maternal IAA levels. These lower immune responses to insulin in offspring of diabetic mothers may be due to tolerization to insulin by maternal insulin therapy through transplacental transfer of insulin-antibody-complexes in utero (Gergely and Sarmay 1996). Interestingly, it has been reported that children exposed to maternal immunotherapy (directed against an allergen) during pregnancy had lower

levels of these allergen-specific IgG antibodies than did their siblings who were not exposed to maternal immunotherapy while in utero (Glovsky et al 1991); this supports our findings regarding tolerization to insulin. Indeed, in experimental animals, maternal antigenic stimulation during pregnancy has been shown to reduce the antibody response in the offspring to the same antigen after oral or parenteral challenge (Peri and Rothberg 1981, Davis and Gill 1975, Yamaguchi et al 1983). In mice maternal immunization (sc) with human gammaglobulin after delivery resulted in absorption by the offspring of this antigen from colostrum, leading to a complete, antigen-specific tolerant state (Halsey and Benjamin 1976). Results from our study V show that a similar type of mechanism may be involved in humans as well, since a maternal CM-containing diet during lactation resulted in lower levels of insulin-binding antibodies in their offspring than in children not exposed to CM proteins through maternal milk. Based on our findings, it seems that exposure of offspring to maternal diabetes and insulin therapy results in tolerization to insulin. This may be the mechanism which decreases the risk for type 1 diabetes in children with a diabetic mother and may explain the epidemiological findings of lower risk for diabetes in offspring of diabetic mothers vs. offspring of diabetic fathers. The importance of insulin tolerization is supported by the observation that the risk for type 1 diabetes in infants born before maternal onset of diabetes was higher than in those born after disease onset, and who had consequently been exposed to maternal diabetes and insulin therapy in utero as well as in infancy (Warram et al 1991).

It is possible that the tolerization to insulin observed in infants with a diabetic mother may also have had an impact on our results. In the present studies (III, IV), levels of insulin-binding antibodies in infants exposed to the CM formula were lower than those observed in our other study (Vaarala et al 1999), in which none of the infants included had a mother with type 1 diabetes. Two other differences between the 1999 study and the present studies may also explain the lower levels seen in the latter ones. All infants in our other study carried the HLADQB1*0302 allele, which is in linkage equilibrium with the DR4 allele linked to high levels of IAA in diabetic patients. In study IV, infants having the DQB1*0201 allele without protective alleles were also included; and in Study III infants were selected not by HLA criteria but by family history of type 1 diabetes, meaning that infants with a variety of HLA genotypes participated. A third difference between the studies is that the majority of children in the 1999 study were exposed to a liquid CM formula, whereas the study formulas in the present trials (III, IV) were powdered ones. As the proteins are less denaturated in liquid formulas, they thus may elicit stronger immune responses.

Effect of maternal diet during lactation on development of insulin-binding antibodies in children at risk for allergy

The IgG antibody levels to BI were lower at 18 months of age in children whose mothers had been on an unrestricted diet during the first 3 months of lactation than in infants whose mothers had followed a diet free of CM products during this time (V). An increase in the IgG antibody levels to insulin was observed between the ages of 3 and 6 months in infants whose mothers began to use CM products after the elimination diet of 3 months. This indicates that exposure to small amounts of CM proteins through breast milk during the first 3 months of life is associated with less antibody production later in life when CM proteins are introduced into the diet. Our findings may reflect an early tolerization to small amounts of dietary BI present in breast milk. This effect may be dose-dependent, since exposure to CM proteins present in infant formulas during the first 3 months of life resulted in increased levels of IgG antibodies to BLG (Jenmalm and Björkstén 1998). Alternatively, our finding may reflect differences in antigen processing when exposed to dietary antigens in breast milk or in intact CM formula. Indeed, the form of dietary antigens in breast milk is suggested to favor the induction of tolerance in the offspring (Machtinger and Moss 1986). Contradictory findings on the role played by breast feeding in the outcome of immune responses in the infant may be explained by the combined effect of infant feeding, maternal food-antigen-specific antibodies, maternal diet, and genetic factors (Wright et al 1999). In addition, non-antigen-specific factors in breast milk may be important in tolerance induction such as the levels of TGF-β (Saarinen et al 1999, Kalliomäki et al 1999).

Our finding is in accordance with animal studies showing that a maternal antigen-containing diet during pregnancy and/or lactation results in lower levels of dietary antibodies in the offspring when exposed orally to the same antigen after weaning (Pathriana et al 1981, Gibney and Gallagher 1982). The effect of maternal diet on immune tolerance to parenteral antigen challenge was demonstrated in young animals which had been exposed to the antigen during gestation and/or during lactation (Nicklin and Miller 1987). Telemo et al (1987) also demonstrated that fetal exposure of guinea pigs to dietary CM proteins through maternal CM consumption resulted in lower levels of IgG antibodies to BLG in these offspring upon immunization with milk whey-protein concentrate than in offspring of mothers on a milk-free diet; in that experiment, the offspring were not nursed. In humans, a maternal hypoallergic diet during pregnancy altered neither the incidence of atopy, nor levels of total serum IgE in the offspring (Fält-Magnusson and Kjellman 1987). In the same cohort as we studied here, a maternal hypoallergic diet during lactation had no effect on emergence of positive skin-prick

tests to allergens nor on allergen-specific IgE antibodies later in life, but a decrease in the incidence of atopic eczema was observed (Hattevig et al 1989 and 1990, Sigurs et al 1992). Our results on the appearance of IgG antibodies to BI indicate that elimination of food antigens from the diet of a nursing mother may modulate the development of food-antigen-specific IgG antibodies in the child. The previous results on IgA antibody responses to BLG in the same cohort are in accordance with our findings. The number of children with a positive IgA response to BLG at 18 months of age was lower among children whose mothers followed an unrestricted diet during the first 3 months of lactation than in children whose mothers avoided CM during this period. However, levels of IgG antibodies to BLG and OVA did not differ between the groups (Hattevig et al 1990). That amount of these latter antigens in the diet is much higher than that of BI may have had an effect on the results.

No difference was observed between the groups in the present study at 3 months of age, i.e., during the period of maternal dietary manipulation. Despite the fact that the systemic immune response was undetectable at 3 months of age, the early antigen exposure through breast milk seemed to have had an effect on the kinetics of the IgG antibody responses to insulin later in life. The peak in levels of insulin-binding antibodies appeared earlier in children whose mothers were on a normal diet during the first 3 months of lactation than in children whose mothers adhered to the CM-free diet during this period (i.e., 12 vs 18 months of age). The difference in the levels of insulin-binding antibodies had disappeared by 4 years of age. It is possible that this difference between the groups reflects only a difference in the timing of tolerization. However, qualitative differences in immune responses to dietary BI may exist, despite the fact that no difference in the levels of the antibodies was evident later. The timing of tolerization may influence the characteristics of an immune response reactivated later in life by a stimulus such as parenteral immunization. Based on our findings, exposure to dietary insulin through breast milk may account for a significant component of tolerance to this diabetes-associated autoantigen.

GENERAL DISCUSSION

Methological aspects

T-cell assay

The role of T-cells in the pathogenesis of type 1 diabetes is well acknowledged. Although cellular reactivity to a variety of islet cell antigens has been described in patients with type 1 diabetes and in subjects at risk for the disease, at this stage prediction of type 1 diabetes can be achieved only by autoantibody, not by T-cell measurements. The existence of several discrepancies between observations on cellular immunity to diabetes-related antigens has initiated a discussion about the standardization of T-cell assays. To improve methodological aspects of T-cell assays, the first T-cell workshop was carried out in the year 1997. In this workshop a series of candidate autoantigens and some control antigens were blindly distributed to 26 laborotories world-wide for the analysis of cellular immunity in 10 patients with newly diagnosed type 1 diabetes and in as many control subjects. The results of this workshop were discussed at the third Immunology of Diabetes Society conference held in Chicago in June 1998 (Roep 1999). All centers were able to measure reproducibly T-cell reactivity to tetanus toxoid. However, only a few laboratories could distinguish diabetic patients from controls with regard to proliferative responses to individual islet cell antigens, and generally no differences between the diabetics and controls were seen, indicating the difficulty in analyzing T-cell autoimmunity. Indeed, the reason why T-cell responses to autoantigens seem to be more difficult to measure than those against non-self proteins may be due to the relatively low precursor frequencies of autoreactive cells or regulatory immune responses directed at controlling autoreactivity. Another problem in analyzing cellular reactivity in type 1 diabetes is that peripheral T-cell reactivity may not be the best representative of the autoimmune reaction prevailing in the pancreas, the gut or both. Problems identified that can be more readily approached included the quality of recombinant autoantigen preparations and the need for HLA-matching, the latter issue already indicated previously (Petrovsky and Harrison 1995).

In our study, the quality of our GAD preparations was ascertained as described, and the endotoxin content was found to be under the detection limit by Limulus test (0.062 EU/ml corresponding to about 2 pg/ml of endotoxin). In addition, T-cell reactivity to a control protein produced by a similar protocol as GAD and to a lysate of baculovirus-infected Sf9 cells was previously found to be low or absent (Klemetti et al 2000). Because our subjects in Study IV did not differ in their HLA-status, this should not have affected our results regarding cellular

responses to insulin. Although T-cell responses to insulin were low, we could detect a difference between the study groups. It is possible that primary immunization (here to insulin) is easy to detect, because of the possibility to compare exposed infants with naive, non-exposed ones.

EIA for insulin-binding antibodies

In type 1 diabetes research, the detection of insulin-binding antibodies has been a controversial issue. The main purpose for the development of insulin antibody assays has been to improve their predictive value for this disease. The frequent occurrence of insulin-binding antibodies in healthy children has, however, limited the use of these antibodies alone as a risk marker for type 1 diabetes. For predictive studies, a competitive RIA for IAA is the best method among the various options for IAA measurement. Insulin-binding antibodies detected by EIA differ from the IAA detected by RIA notably because of differences in the affinity of the antibodies (Greenbaum et al 1992). Because our interest was focused on the possibility that BI in CM induces antibodies in infants, i.e., the primary immunization to insulin and not the autoantibody response, we studied the occurrence of IgG antibodies to BI and HI by EIA. In inhibition studies performed on samples from diabetic subjects and from infants exposed to CM formula, the binding of antibodies to solid-phase BI or HI was readily inhibited by BI and HI in the aqueous phase – not by B-chain of BI – (see also Vaarala et al 1999), whereas a lower inhibition was noted in insulin-binding antibodies produced by exposure to HC formula or breast milk. That the antibody response was best inhibited in diabetic patients is not suprising, because these indivuals also have autoimmune reactivity to insulin and possible antibody responsiveness to exogenous insulin therapy. These findings confirm the fact that the specificity of these antibodies detected by our assay was against insulin, and not against insulin chains. That we were able to achieve the same results with two insulin molecules from different sources (BI purified from bovine pancreas and recombinant HI) supports the view that these antibodies were insulin-specific.

The gut immune system and autoimmunity/allergy

Our findings that GAD-reactive cells in newly diagnosed type 1 diabetic patients belong to the gut-associated $\alpha 4\beta 7^{high}$ population and that insulin-specific T-cell responses are primed in the gut by dietary insulin suggest involvement of the gut immune system in human type 1 diabetes, as well. These insulin-specific cells, if turned into autoaggressive, have the capacity to circulate to the pancreas, which seems to be a prerequisite for antigen-specific cells to be able to induce an organ-specific autoimmune disease. In humans sc/iv insulin acts as a tolerogen delaying the

onset of type 1 diabetes (Keller et al 1993, Füchtenbusch et al 1998), whereas oral insulin may be harmful for β-cell function (Chaillous et al 2000, Pozzilli et al 2000). Likewise, evidence has shown in experimental animals that immunization with foreign insulin in complete Freund´s adjuvant generated autoreactive cytotoxic CD8+ lymphocytes (CTLs) (Ma et al 2000) as well as CD4+ T-cells (Whiteley et al 1988) without development of insulitis and diabetes, even after boosting with insulin. The authors speculated, in fact, that one mechanism of failure to produce diabetes by this insulin immunization procedure could be the lack of essential signals required to target the CTLs to the pancreas. This may be the case, because in NOD mice the gut-associated lymphocytes as well as the mucosal homing receptor MAdCAM-1 are required for the development of a β-cell lesion (Hänninen et al 1996a and 1998). In addition, in NOD mice, a higher frequency of γδ TCR-bearing T-cells in the islet infiltrates than in the periphery suggests involvement of the GALT in autoimmune diabetes as well (Goldrath et al 1995).

That a susceptibility to the induction of an autoimmune reaction in the gut in type 1 diabetes may exist is based on the evidence of several defects in the mucosal barrier and the GALT in patients with type 1 diabetes. First, proteolysis may be altered in these patients, causing increased macromolecular transport and disturbance in oral tolerance induction (Lorini et al 1990, Hanson et al 1993). Disturbance in oral tolerance has, in fact, been suggested in type 1 diabetes because enhanced humoral and cellular immune responses to CM proteins exist in such patients (Vaarala 1999). Secondly, IgA deficiency is more common in these patients (Alaswad and Brosnan 2000) and if combined with sIgA deficiency, it may affect antigen handling by the gut, resulting in increased permeability. Increased permeability has been shown both in patients with type 1 diabetes (Carratú et al 1999, Kuitunen M, personal communication) and in BB rats (Meddings et al 1999). The integrity of the intrinsic barrier in individuals progressing to type 1 diabetes is unknown, but at least an immune activation of the jejunum occurs in these patients (Savilahti et al 1999). It is an interesting possibility that because intestinal epithelium is able to support T-cell development and maturation without thymic influence, IELs may contain autoreactive T-cells (Poussier and Julius 1994). Usually autoreactive cells are negatively selected during intrathymic T-cell maturation. In addition, it can be speculated that since γδ TCR+ IELs are associated with oral tolerance, and because as few as 100 clones may account for all the TCRs expressed by these cells – leading to only a limited range of local antigens inducing expansion of a few T-cell clones – individuals at risk for type 1 diabetes may carry “autoimmune-prone” TCRs in the gut (e.g. reactivity to BI). On the other hand, other CM

antigens cause food hypersensitivity in individuals with “allergy-prone” TCR clones, and a similar rule may apply to gluten-specific T-cells in celiac disease vs. food allergy. Last, the described impairment in yield, phenotype, and function of monocyte-derived dendritic cells (DCs) in humans at risk for type 1 diabetes, especially the impairment in costimulation through CD28 on T-cells due to a lower proportion of DCs expressing costimulatory molecules (B7-1 and B7-2) (Takahashi et al 1998), plus a decreased number and function of normally IL-4 secreting NK-T-cells in patients with type 1 diabetes (Wilson et al 1998) may be the reason for the shift to Th1 reactivity and disturbance in low-dose oral tolerance induction in the gut (Weiner 1997, Liu et al 1999) leading to a cell-mediated attack on β-cells and thence to type 1 diabetes.

It is of note that all three major autoantigens in type 1 diabetes are related to the gut. Our finding that dietary insulin primes insulin-specific T-cells in the gut provides evidence that insulin immunity is initiated there. On the other hand, GAD (Williamson et al 1995) and IA-2 (Li et al 1997) are expressed in the stomach/intestinum, making it possible that the gut may be a target of autoimmunity in type 1 diabetes, just as it is a target in celiac disease. The high prevalence of antibodies to tissue transglutaminase C in patients with newly diagnosed type 1 diabetes without celiac disease may suggest this (Lampasona et 1999). Based on our findings, GAD-reactivity resides in the gut-associated lymphocyte population, but we could not differentiate whether the priming antigen was pancreatic or intestinal GAD or even the Coxsackie B virus. Evidence that something pathological may be occurring in the gut of patients with type 1 diabetes is suggested at least in diabetic patients who have microscopic colitis. Patients with this syndrome carry an overall increased risk for autoimmune diseases (Koskela 2001). A recent study has shown that mononuclear cell inflammation of the small intestine, often accompanied by partial or subtotal villous atrophy, is frequent in patients with this syndrome, and the authors suggest that the small intestinal histopathology may be caused by immunological gluten sensitivity (Fine et al 2000). In addition, in patients with celiac disease, diabetes-associated autoantibodies (ICA, IAA and GADA) disappear just as do antiendomysium antibodies during a gluten-free diet (Ventura et al 2000). Interestingly, of the diabetes-associated autoantibodies, IAA and GADA were the first to disappear, making it conceivable that the antibody responses to these antigens could be elicited or boosted in the gut. Disregarding the question as to whether gluten intake can cause diabetes, it is clear that the intestinal damage in celiac disease may predispose to diabetes-related autoimmunity in the gut.

The cascade of autoimmune process initiated in the gut may be a consequence of many influencing factors, may be the combined effect of dietary factors, (viral) infections, and the gut flora. Interestingly, in one diabetes-susceptible rat strain, it was shown that the antigenic stimulus for lymphocyte activation came from the gut – either from dietary or from gut-flora antigens (Fowell and Mason 1993). Recent birth cohort studies provide more evidence for the association of the gut immune system and type 1 diabetes, reporting a relationship between enterovirus (Lönnrot et al 2000b) and rotavirus infections (Honeyman et al 2000) and type 1 diabetes. Further, a recent study following subjects at risk for type 1 diabetes re-emphasizes the importance of enterovirus infections at least in accelerating the progress of the disease (Luppi et al 2000). Enterovirus and rotavirus antigens share a molecular similarity with GAD (Atkinson et al 1994) and with IA-2 (Honeyman et al 1998). It is therefore possible that immune responses to a viral agent in the gut result in breakage of tolerance to these autoantigens and in that way may lead to type 1 diabetes. Alternatively, (viral) infections in the gut may affect the cytokine milieu and provide a bystander effect, for example on BI-reactive T-cells which may then turn into autoaggressive cells (Tough et al 1996). Interestingly, in animal models, oral administration of bacterial adjuvants has influenced the cytokine gene expression in the gut and the development of diabetes (Hartmann et al 1997, Bellmann et al 1998). Another possibility is that gastrointestinal infections may lead to exacerbation of insulin immunity, because many such infections affect gut permeability and thus enhance absorption of macromolecules (Jalonen et al 1991).

Changes in the gut microflora and fewer childhood infections may in part explain the rising incidence both of type 1 diabetes and of allergies (Kolb and Elliott 1994, Wold 1998). It has been reported that asthma and allergic rhinitis are inversely associated with exposure to foodborne and orofecal microbes but not to infections (viral) transmitted otherwise (Matricardi et al 2000). This suggests a regulatory role for the gut immune system and supports the idea that adequate stimulation of the GALT is necessary to avoid atopic sensitization to environmental allergens as well as to avoid initiation of autoimmune reactions (Rook and Stanford 1998). Indeed, oral exposure to bacterial lipopolysaccharins or glycoproteins may be benificial not only for avoidance of allergies (Wold 1998) but also for prevention of diabetes (Sai and Rivereau 1996, Bellman et al 1997). The fact that the gut is the site for primary induction of insulin-reactive T-cells implies that manipulation of the gut immune system may offer prevention of type 1 diabetes as previously suggested (Bellmann et al 1997, Vaarala 1999). In an animal model utilizing Th1-type induction to prevent development of airway eosinophilia, BCG

administration to the nasopharynx resulted in greater efficacy than when given either sc or ip (Erb et al 1998). Manipulation of the gut immune system by probiotics has proven to be efficient at least in food allergy (Majamaa and Isolauri 1997, Isolauri et al 2000). In addition, in one experimental study a preventive effect by oral feeding of *Lactobacillus casei* on autoimmune diabetes was described (Matsuzaki et al 1997). All studies point to the fact that modulation of the gut immune system may offer ways to prevent these diseases.

Primary role for insulin in the pathogenesis of type 1 diabetes

Of the two major autoantigens (insulin and GAD) in type 1 diabetes, insulin may be the primary one, based on the following evidence. First, IAA are the first autoantibodies to appear in children with signs of autoimmunity (Ziegler et al 1998, Naserke et al 1999, Yu et al 2000), and our finding concerning immunization to BI may explain this. Second, HLA-DR-restricted T-cell lines from newly diagnosed type 1 diabetic patients were shown to be specific for insulinoma but lacked reactivity to GAD (Huang et al 1995). Third, transgenic expression of mouse proinsulin on APCs, including those in the thymus, resulted in an almost complete absence of insulitis and prevention of diabetes in such NOD mice (French et al 1997), whereas similar transgenic expression of GAD in the thymus of NOD mice only reduced the disease incidence (Tisch et al 1998), suggesting a pivotal role for insulin in driving β-cell autoimmunity. In attempts to obtain islet-specific T-cell lines from islet infiltrates it was found that the majority are insulin-specific (Wegmann and Eisenbarth 2000). In contrast, none were found to respond to the reported dominant epitopes of GAD65. In fact, GAD65-specific T-cell lines could be established from islet infiltrates only by repeated in vitro stimulation with synthetic peptides of GAD, and the success rate was much lower than with insulin. Whereas insulin-specific clones induced diabetes with high efficiency and a relatively short mean time to diabetes, GAD65 clones were less efficient, with a longer time to diabetes onset, providing evidence that insulin is a more dominant antigen in the pathogenic T-cell response to β-cells than is GAD65. Similarly, experimental autoimmune insulitis could be induced by T-lymphocytes specific for a peptide of proinsulin but not by cells specific for a GAD peptide (Griffin et al 1995) supporting this view of insulin as the primary autoantigen.

CM and type 1 diabetes

The debate around CM and type 1 diabetes continues (Wasmuth and Kolb 2000). Patients with type 1 diabetes have been shown to have had an early exposure to CM formula and to consume higher amounts of CM before the diagnosis, both of which have been reported to increase levels

of CM antibodies. These antibodies are, in fact, elevated in patients with recent-onset type 1 diabetes, and these patients also show enhanced cellular immune responses to CM proteins. The findings may be linked to the gut immune system (the effect of early CM exposure on the immature gut and the possible disturbance in oral tolerance), but not necessarily to the role of CM proteins in the etiology of type 1 diabetes. However, our findings on insulin immunization in early life may point to a more specific role for CM proteins in the pathogenesis of type 1 diabetes. This immunization to foreign insulin which takes place in the gut may be the primary event in the pathogenesis of autoimmune diabetes, and may in some genetically susceptible infants lead to a breakage of tolerance to insulin and to β-cell damage. This view is based on evidence in experimental animals that gut-associated lymphocytes are able to circulate to the pancreas and are the first to invade islets, and that islet-infiltrating lymphocytes show insulin-reactivity very early on. The similarities between IAA and insulin antibodies induced by insulin therapy support the hypothesis that both are induced by insulin presentation to the immune system (Brooks-Worrell et al 1999).

Inconsistencies exist in findings concerning the association between duration of breast feeding/early exposure to CM and type 1 diabetes. Many epidemiological studies show an inverse correlation between duration of breast feeding and risk for type 1 diabetes (Table 2). In addition, the risk is shown to be related to the early exposure to CM protein. However, in three cohort studies, DAISY in the USA (Norris et al 1996), the Australian BABYDIAB (Couper et al 1999), and the German BABYDIAB (Hummel et al 2000) in which children of parents with type 1 diabetes were followed for the occurrence of diabetes-associated autoantibodies, no associations between duration of exclusive breast feeding or early exposure to CM proteins and β-cell autoimmunity were found in general. However, the data from the Australian study showed that CM-based formula was introduced at a mean age of 3.5 months in children who developed at least two autoantibodies compared to 7 months in the autoantibody negative children (Couper at al 1999). In addition, in the German study in offspring with an affected father, the duration of exclusive breast feeding was non-significantly reduced in the antibody positive cohort, whereas in children with mothers of type 1 diabetes no association was detectable between breast feeding patterns and islet cell autoimmunity. Regardless of family history of type 1 diabetes, 40% of children who developed autoantibodies without clinical disease and 40% of children who progressed to diabetes were exposed to CM formula before 3 months of age. A recent finding in a Finnish birth cohort study (DIPP) showed that short-term exclusive breast feeding and early introduction of CM-based formula predisposed young

children at genetic risk for type 1 diabetes to progressive β-cell autoimmunity (Kimpimäki et al 2001); this agrees with our own findings in the TRIGR study, in which, concerning children at genetic risk for type 1 diabetes, early exposure to CM-based formula was associated with seroconversion (Åkerblom HK, personal communication). Accordingly, if CM plays a role in the pathogenesis of type 1 diabetes, differences in genetics may account for the geographical variation in disease risk associated with early CM exposure in different countries.

SUMMARY AND CONCLUSIONS

We showed that in genetically susceptible children priming of insulin-specific immune responses during infancy is caused by early exposure to BI present in CM formulas. It is possible that this insulin immunity may turn autoaggressive in some children and lead to β-cell destruction and type 1 diabetes. In this process, the factors that lead to activation of insulin-primed T-cells are unknown, but may relate to the regulation of the gut immune system. We suggest that this insulin immunization may explain the early-appearing IAA in children who develop autoimmunity. Our findings may also explain the epidemiological association between early CM exposure and the risk for type 1 diabetes. That in patients with type 1 diabetes, gut-associated lymphocytes predominate in GAD-reactivity further emphasizes the role of the gut immune system in human type 1 diabetes and suggests that the focus of research in this field should be on factors relating to the GALT.

Children of diabetic mothers showed decreased immune responses to insulin when compared with children with an affected father or sibling. This suggests tolerization to insulin in those exposed to maternal diabetes/insulin therapy in utero and early infancy. Our results may explain the epidemiological finding of lower risk for type 1 diabetes in offspring of diabetic mothers than of diabetic fathers. Based on our findings we suggest that insulin may be the primary autoantigen in type 1 diabetes.

The functional immaturity of the gut epithelial cells, permeability, and immune system in early infancy make this a susceptible period for induction of altered immune responses to oral antigens detected at least as food allergies. Our findings of enhanced immune responses to insulin and elevated levels of sICAM-1 in infants exposed to CM formula before 3 months of age and of the effect of maternal diet during the first 3 months of lactation on tolerance development in the offspring highlight the importance of the first months of life in tolerance induction. In addition, the route of exposure seems important, i.e., breast milk vs CM formula. Exposure to antigens through breast milk during the first months of life seems to result in tolerance induction, whereas exposure to proteins in CM formula without “maternal filtration” results in immunization. Absence of breast feeding and early exposure to CM proteins may thus have an additive effect on diabetes risk: no protection afforded by breast feeding (also a higher risk of gastrointestinal infections) and an immunizing effect from early CM feeding.

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