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Soluble Adhesion Molecules in Preclinical Type 1 Diabetes

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Abbreviations

CLA	cutaneous lymphocyte antigen
CTLA-4	cytotoxic T lymphocyte antigen 4
DASP	Diabetes Antibody Standardization Programme
DiMe	Childhood Diabetes in Finland study
DIPP	Type 1 Diabetes Prediction and Prevention study
ELISA	enzyme-linked immunosorbent assay
ESL-1	E-selectin ligand 1
FPIR	first-phase insulin response
GADA	antibodies to the 65 kDa isoform of glutamic acid decarboxylase
HEV	high endothelial venule
HLA	human leukocyte antigen
IAA	insulin autoantibodies
IA-2A	antibodies to the protein tyrosine phosphatase-related IA-2 antigen
ICA	islet cell antibodies
ICAM	intercellular adhesion molecule
ICARUS	Islet Cell Antibody Register Users Study
IFN	interferon
IL	interleukin
IVGTT	intravenous glucose tolerance test
JDFU	Juvenile Diabetes Foundation Units
kDa	kilodalton
LFA	lymphocyte function-associated antigen
Mab	monoclonal antibodies
MAdCAM-1	mucosal addressin-cell adhesion molecule 1
MHC	major histocompatibility complex
NOD	non-obese diabetic
PCR	polymerase chain reaction
PECAM-1	platelet endothelial cell adhesion molecule 1
PLN	peripheral lymph node
PNAd	peripheral node addressin
PPV	positive predictive value
PSGL-1	P-selectin glycoprotein ligand 1
RU	relative units
Th1	type 1 helper T cells
Th2	type 2 helper T cells
TNF-α	tumour necrosis factor α
TRIGR	Trial to Reduce IDDM in the Genetically at Risk
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late activation antigen 4

1 Introduction

Type 1 diabetes is a chronic disease characterized by deficient insulin secretion due to the loss of insulin-producing beta cells in the endocrine pancreatic islets. The disease remains subclinical until the number of beta cells is too low to produce the amount of insulin needed to maintain glucose homeostasis, resulting in symptoms of hyperglycaemia, such as polyuria and polydipsia with associated weight loss. Although the disease may manifest itself at any age, it has been estimated that about half of the patients are diagnosed during childhood or adolescence, making diabetes one of the most common severe chronic diseases of childhood in industrialized countries.

The incidence of childhood-onset type 1 diabetes appears to be increasing in developed countries, for unknown reasons. The most rapid increase has been seen among young children. Although the prognosis for a child with diabetes has improved substantially over the last two decades, the manifestation of an incurable lifelong disease with an absolute need for exogenous insulin replacement and the awareness of the potential severe long-term complications do present a challenge to the child's normal physical, mental and social development. The disease places an extra burden on the whole family of the affected child, and also on society at large, putting extra pressure on health care resources. Therefore there is a great interest in understanding how the disease is initiated, in the hope that it could eventually be prevented.

Overt type 1 diabetes is preceded by an asymptomatic preclinical period characterized by the presence of markers of beta-cell autoimmunity which are closely associated with progressive destruction of the insulin-producing beta cells. Although the subjects most likely to develop the disease can be identified, prediction on an individual level is still uncertain and no specific characteristics that could completely discriminate the subjects en route to clinical type 1 diabetes have been identified so far. Several intervention trials aimed at preventing or delaying the development of type 1 diabetes at the stage of asymptomatic autoimmunity have been initiated during the last decade. As these use clinical disease as the endpoint, they have to be large and of long duration. Therefore there is an urgent need to establish additional surrogate markers by which the subjects with the highest risk of developing clinical disease could be identified and by which the effects of intervening in the autoimmune process could be assessed.

The immunological mechanisms underlying human type 1 diabetes are still not fully understood, although there is strong evidence to suggest that autoreactive T cells are major players in the pathogenic process. A crucial aspect of endocrine autoimmunity is why and how lymphocytes migrate from the primary lymphoid tissue to their targets. This migration is controlled in part by selective expression of cell adhesion molecules on the surface of the lymphocytes and vascular endothelial cells. These ”sticky” molecules also participate in various other immune reactions thought to

be involved in the process of autoimmunity. It is now well established that soluble isoforms of the cell adhesion structures can be found in the peripheral circulation. The detection of increased levels of these soluble molecules in various inflammatory conditions has led to the hypothesis that they could serve as markers of disease activity, or that they may play a role in the disease process.

2 Review of the literature

2.1 Adhesion molecules

Cell adhesion molecules are cell surface proteins that are critical for many normal physiological processes; they allow cells to interact and communicate with each other and their environment and, in doing so, regulate a range of cell functions. In the immune system they not only mediate the migration and ”homing” of lymphocytes to specific tissues but they are also involved in many cell-to-cell interactions that control immune responses. Given their widespread distribution, it is not surprising that cell adhesion molecules have been implicated in many diverse pathological processes, such as the initiation and propagation of autoimmune diseases. A large repertoire of adhesion molecules and their combinations may potentially create conditions for selective and closely targeted action at different sites and tissues in the body.

2.1.1 Classification

Adhesion molecules that are important in leukocyte interactions can be classified into three major groups on the basis of their structure and certain common properties: selectins, integrins and members of the immunoglobulin supergene family (*Table 1*). Cell adhesion molecules bind to other cells or matrix components through interaction with appropriate counter-receptors, referred to as ligands. In some cases the ligands are themselves adhesion molecules, as is the case with integrin family whose ligands are members of the immunoglobulin superfamily, and vice versa.

Selectins are glycoproteins, and these may be subclassified according to the cell type on which they were originally identified: L-selectin (leukocytes), P-selectin (platelets/endothelium) and E-selectin (endothelium). Their common structural component is a lectin domain which is critical for ligand-binding to sialyl-Lewis^x -like cell surface carbohydrates. The most important receptors are sialylated, fucosylated glycoproteins P-selectin glycoprotein ligand 1 (PSGL-1), E-selectin ligand 1 (ESL-1) and mucin-like vascular addressins, such as mucosal addressin-cell adhesion molecule 1 (MAdCAM-1) and peripheral node addressin (PNAd). The differential expression of vascular addressins on the high endothelial venules (HEVs) in lymph nodes and Peyer’s patches regulates differential trafficking of lymphocyte subsets. All selectins are constitutively active and rapidly shed on activation. L-selectin (CD62L) is expressed on all leukocytes (reviewed by von Andrian and Mackay 2000), including all naïve lymphocytes and a subpopulation of memory cells which share the same recirculatory pattern as naïve lymphocytes (Sallusto et al. 1999). P-selectin (CD62P) is

found on endothelial cells and activated platelets, and E-selectin (CD62E) is an endothelial selectin, the *in vivo* expression of which is mainly seen on activated endothelium, as in inflammatory disorders of the skin and airway (Groves et al. 1991, Gundel et al. 1991).

Integrin adhesion molecules are cell surface proteins that integrate extracellular information into the cytoskeleton. They are heterodimeric, transmembrane structures composed of non-covalently bound α and β subunits. Integrins are named according to the composition of their constituent α and β protein chains, which are identified by a number or letter. They are expressed on various types of cells, and most cells express different integrins. Integrins bind to a variety of extracellular matrix proteins, e.g. laminin, collagen and receptors of the immunoglobulin superfamily (reviewed by Hynes 1992). $\alpha_4\beta_1$, also known as very late activation antigen 4 (VLA-4), and $\alpha_4\beta_7$ are expressed on most leukocytes except neutrophils. Up-regulated expression of $\alpha_4\beta_1$ is seen on effector and memory cells, while enhanced expression of $\alpha_4\beta_7$ is seen on gut-homing effector and memory cells (von Andrian and Mackay 2000).

Adhesion molecules of the immunoglobulin superfamily includes a variety of cell surface molecules that are classified together because each molecule carries immunoglobulin domains repeated a variable number of times in its extracellular part. The immunoglobulin superfamily includes molecules such as major histocompatibility complex (MHC) class I and II molecules, intercellular adhesion molecules 1, 2 and 3, i.e. ICAM-1 (CD54), ICAM-2 (CD102) and ICAM-3 (CD50), vascular cell adhesion molecule 1 (VCAM-1, CD106), platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31) and MAdCAM-1, in addition to the classical immunoglobulins produced by B lymphocytes. The expression of intercellular adhesion molecules is constitutive and there is hardly any change in expression levels after induction, with the exception of ICAM-1, which is expressed on a variety of cell types, including endothelial cells, where it serves as an important counter-receptor for leukocyte integrins. Exposure to interleukin 1 (IL-1), tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ) at least results in augmented cell surface expression of ICAM-1 (Rothlein et al. 1988, reviewed by Springer 1990 and Carlos and Harlan 1994). The RNA and protein synthesis induced by inflammatory mediators has been shown to be a fairly slow process, as marked changes were not seen until 12 hours after stimulation (Dustin et al. 1986). ICAM-2 is expressed on endothelial cells and platelets, and ICAM-3 on resting leukocytes (Fawcett et al. 1992). MAdCAM-1 expression on high endothelial venules in gut-associated lymphoid tissue mediates the homing of lymphocytes to Peyer's patches and mesenteric lymph nodes (Streeter et al. 1988). The predominant ligand of MAdCAM-1 is the $\alpha_4\beta_7$ integrin, but L-selectin has also been shown to bind this molecule (Berg et al. 1993).The expression of MAdCAM-1 on postcapillary venules of mucosal lymphoid tissue appears to be constitutive, but enhanced expression has been demonstrated on cultured endothelial cells by means of inflammatory mediators such as TNF-α (Sikorski et al. 1993). VCAM-1 expression is seen on all endothelial cells and on certain leukocytes, although its major functional expression sites are HEVs in the lymph nodes (reviewed by Salmi and Jalkanen 1997). PECAM-1 is expressed on the endothelium of the vasculature and on some leukocytes. It plays an important role in monocyte transmigration and probably contributes to additional leukocyte adhesion in general (reviewed by Bianchi et al. 1997).

***Table 1**. Selectins, integrins and members of the immunoglobulin superfamily with a role in T-cell migration,*

Adhesion molecule (alternative name)	Distribution	Regulation of function and expression	Ligands	Role in T-cell migration
Selectins		All selectins are constitutively active	Sialyl-Lewis^x-like sugars	
L-selectin (CD62L)	All naïve lymphocytes and a subpopulation of memory cells	Rapidly shed on activation	PNAd, PSGL-1, MAdCAM-1, E-selectin, GlyCAM-1, CD34	Homing to lymph nodes and Peyer's patches
E-selectin (CD62E)	Endothelial cells	Induced by TNF-α, IL-1, endotoxin	CLA, L-selectin, PSGL-1, ESL-1	Homing of memory and effector cells to skin and sites of inflammation
P-selectin (CD62P)	Endothelial cells, platelets	Stored intracellularly in resting cells, rapid surface translocation stimulated by histamine, for example	PNAd, PSGL-1	Homing of memory or effector (Th1) cells to the site of inflammation
β_2 Integrins				
$\alpha_L\beta_2$ (LFA-1, CD11aCD18)	All leukocytes	Enhanced expression on effector and memory cells	ICAM-1,2,3	Homing of all lymphocytes to lymph nodes, Peyer's patches and sites of inflammation; adhesion to antigen-presenting cells
$\alpha_M\beta_2$ (Mac-1, CD11bCD18)	Myeloid cells, some activated T cells	Rapid up-regulation on activated myeloid cells	ICAM-1, factor X, fibrinogen, C3b	Unknown

$\alpha_x\beta_2$ (p150,95, CD11cCD18)	Dendritic cells	Constitutive expression	Fibrinogen, C3b	Unknown
$\alpha_D\beta_2$ (CD11dCD18)	Monocytes, macrophages, eosinophils	High levels of expression on foam cells in intimal plaques	VCAM-1, ICAM-1 and 3	Unknown
α_4 Integrins				
$\alpha_4\beta_1$ (VLA-4)	Most leukocytes except neutrophils	Enhanced expression on effector and memory cells	fibronectin, VCAM-1, α_4 integrin	Homing of memory and effector cells to inflamed tissue, especially lung
$\alpha_4\beta_7$	Lymphocytes, natural killer cells, mast cells, basophils, monocytes	Enhanced expression on gut-homing effector and memory cells	MAdCAM-1, fibronectin, (VCAM-1)	Homing of all lymphocytes to gut and associated lymphoid tissue
Immunoglobulin superfamily				
ICAM-1 (CD54)	Most types of cells	Up-regulated by lipopolysaccharide and inflammatory cytokines	$\alpha_L\beta_2$ and $\alpha_M\beta_2$ integrins, fibrinogen	Critical endothelial ligand for β_2 integrins
ICAM-2 (CD102)	Endothelial cells, platelets	Constitutive expression, no change in level of expression during inflammation	$\alpha_L\beta_2$ integrin	Unknown
VCAM-1 (CD106)	Endothelial cells, bone marrow stroma, follicular dendritic cells, osteoblasts, mesothelium	Absent on most resting endothelial cells, induced by cytokines	$\alpha_4\beta_1$, $\alpha_4\beta_7$ and $\alpha_D\beta_2$ integrins	Homing of memory and effector cells to inflamed tissue
MAdCAM-1	HEVs in gut-associated lymphoid tissue and sites of chronic inflammation, lamina propria, spleen	Constitutive expression in HEVs, induced in insulitis, thymic hyperplasia, some forms of arthritis	$\alpha_4\beta_7$ integrin, L-selectin	Homing of all lymphocytes to gut and associated lymphoid tissue

C3b complement 3b, CLA cutaneous lymphocyte antigen, GlyCAM-1 glycosylation-dependent cell-adhesion molecule 1, Mac-1 macrophage antigen 1, p150,95 protein with 150 kDa and 95 kDa subunits (modified from von Andrian and Mackay 2000)

2.1.2 Functional aspects

The interactions of the adhesion molecules in adaptive immune responses culminate in three important cellular functions: lymphocyte homing to lymphoid tissues and to inflammatory sites, and co-stimulation of cellular activation. Most adhesion molecules play fairly broad roles in the generation of immune responses. While the mechanisms underlying migration and homing are similar and fairly well characterized, those involved in cellular activation are, as yet, incompletely delineated. Through their expression on lymphoid and non-lymphoid tissues, adhesion molecules may be involved in the initiation and propagation of the disease processes in many organ-specific autoimmune/inflammatory disorders, which from the histopathological point of view are characterized by lymphocytic infiltration in the target tissues. These include type 1 diabetes.

2.1.2.1 Lymphocyte homing

Lymphocyte homing can be perceived as a specific, regulated cascade characterized by four distinct phases, each involving different sets of adhesion molecules. As each step involves a large number of adhesion molecules and endothelial counter-receptors, the number of interactive combinations is high.

The initial contact made by lymphocytes with the endothelial cells is called tethering, and this is followed by a firmer contact, ”rolling”, if there are strong enough interacting ligands at the site. Tethering and rolling are almost exclusively properties of selectins, although α_4 integrins may contribute. Rolling is followed by the activation of integrins mediated by chemokines, which are small cytokine-like chemotactic molecules released from the tissue, resulting in firm adhesion. Subsequently the lymphocytes undergo changes in shape in order to be able to transmigrate through the junctions between the endothelial cells towards the site of highest concentration of the inflammatory chemokines released by the target tissue at a site of inflammation (von Andrian and Mackay 2000).

The first event in an adaptive immune response is homing of naïve T cells to lymph nodes and Peyer´s patches. L-selectin was originally known as a peripheral lymph node (PLN) homing receptor, since blocking of its function prevents the homing of lymphocytes to peripheral lymph nodes. L-selectin also mediates binding to gut-associated lymphoid tissue (Hamann et al. 1991) and to the activated endothelium of extra-lymphoid tissue (Dawson et al. 1992**,** Ley et al. 1993). The preferred site of migration from the blood to the lymphoid tissues is through vessels with the morphology of high endothelial venules, although leukocyte migration may occur across any endothelial cell barrier. HEVs are composed of specialized postcapillary venules of a kind found in all secondary lymphoid tissues or inflamed non-lymphoid tissues that support high levels of lymphocyte import from the blood (reviewed by Girard and Springer 1995). Peripheral node addressins are expressed on HEVs in lymph nodes, while mucosal Peyer’s patch and lactating mammary gland HEVs express MAdCAM-1 (reviewed by Springer 1994, Girard and Springer 1995). L-selectin binds both of these addressins, while binding of $\alpha_4\beta_7$ integrin to MAdCAM-1 is needed to maintain rolling in Peyer’s patches. Gut-homing effector cells expressing high levels of $\alpha_4\beta_7$ integrin attach directly to MAdCAM-1, whereas naïve T cells preferably engage L-selectin first and then MAdCAM-1. Lymphocyte function-associated antigen 1 (LFA-1), which is highly expressed on all normal lymphocytes, is the most important integrin mediating lymphocyte homing, and ICAM-1 and ICAM-2 are the principal ligands involved in LFA-1-mediated firm adhesion of lymphocytes to the endothelium (von Andrian and Mackay 2000).

The best-understood tissue-selective homing pathways are those in the skin and intestine (reviewed by Butcher and Picker 1996 and by Robert and Kupper 1999), but there may be other selective migration streams, such as to the lungs, joints and central nervous system. Antigen-experienced T cells often display a tissue specificity that may improve their chances of re-encountering an antigen, and the differential expression of adhesion molecules can direct different subsets of effector T cells to specific sites. Thus T cells that have been exposed to cutaneous pathogens in skin-draining lymph nodes express cutaneous lymphocyte antigen (CLA) and migrate preferentially to the skin, whereas effector cells that arise in Peyer's patches in response to enteroviral infections are most useful in the gut, expressing considerable levels of $\alpha_4\beta_7$ integrin. The ligands of the latter two molecules are E-selectin, which is present on the skin venule endothelium, and MAdCAM-1, found on the gut lamina propria HEVs, respectively. Effector T cells that recognize antigens in tissues produce cytokines, such as TNF-α, which further activate endothelial cells to express E-selectin, VCAM-1 and ICAM-1. Enhanced expression of ICAM-1 and VCAM-1 on endothelial cells will bind VLA-4 and LFA-1 on effector T cells, recruiting more of these cells into tissues that house the antigen. Monocytes are recruited to the site by binding to E-selectin.

2.1.2.2 Aspects of lymphocyte activation

While migrating through the cortical region of the lymph node to become activated, the naïve T cell recognizes a foreign or a native peptide bound to a MHC molecule and binds transiently to the antigen-presenting cell that it encounters. Cell surface molecules of the immunoglobulin superfamily are important for the interactions of lymphocytes with antigen-presenting dendritic cells: ICAM-1 and 2, LFA-3 and also a dendritic cell receptor DC-SIGN (CD209), a recently discovered lectin, on antigen-presenting cells interact with CD2, LFA-1 and ICAM-3 on naïve T cells. The precise role of each molecule has been difficult to delineate, perhaps due to synergies in the functioning of different cell adhesion molecules. There seems to be enough redundancy in the molecules mediating adhesive T-cell interactions to enable immune responses to occur in the absence of any one of them (Janeway et al. 2001a).

Naïve (pre-antigen encounter) lymphocytes home to lymph nodes through the binding of L-selectin to sulphated carbohydrates, while memory lymphocytes tend to return to the environment in which they first encountered the antigen, which results in preferential trafficking to non-lymphoid tissues and inflammatory lesions. This dichotomy results from the differential expression and activation of lymphocyte cell surface adhesion molecules which occurs during the naïve-memory transition. After having encountered an antigen, naïve T cells differentiate into antigen-specific armed effector T cells, and many lose expression of L-selectin during this process, while expression of $\alpha_4\beta_1$ integrin and LFA-1 are enhanced. These molecules bind to VCAM-1 and ICAM-1 on peripheral vascular endothelial cells at sites of inflammation and this binding helps in the extravasation of the effector T cells. Memory T cells share the surface characteristics of activated armed effector T cells (Janeway et al. 2001b).

The effector T-cell interactions with target cells are initiated by antigen-non-specific cell adhesion molecules. The initial binding of an armed effector T cell to its target, like that of a naïve T cell to an antigen-presenting cell, involves non-specific adhesion molecules. The major initial interaction occurs between LFA-1 on the T cell and ICAM-1 or 2 on the target cell. This allows the T cell to remain in contact with the target cell and to scan its surface for the presence of MHC complexes. Effector T cells that recognize pathogenic antigens in the tissues produce cytokines such as TNF-α, which activates endothelial cells to express E-selectin, VCAM-1 and ICAM-1. These can then act on effector cells to

activate their adhesion molecules, and the presence of interactive adhesion molecules facilitates the recruiting of more effector T cells to the tissue that houses the antigen. At the same time monocytes are recruited to these sites by adhesion to E-selectin. Thus one or a few specific effector T cells encountering an antigen in a tissue can initiate a potent local inflammatory response that recruits both a greater number of specific armed effector cells and many more non-specific inflammatory cells to that site (Janeway et al. 2001b).

In addition to homing and T-cell activation, adhesion molecules are involved in a variety of other immune processes, such as T and B-cell maturation; adhesion molecules such as VCAM-1 and VLA-4 on bone marrow stromal cells have been shown to play a role in B-cell maturation (Jacobsen et al. 1996), although a full understanding of the factors that regulate B-cell differentation has yet to be achieved.

2.1.3 Soluble adhesion molecules

Additional information on cell adhesion cascades and an increased understanding of their complexity have been obtained over the last decade thanks to the demonstration of soluble isoforms of various selectins, members of the immunoglobulin superfamily and a few others in the peripheral circulation in concentrations of several nanograms per millilitre, as measured by commercially available sandwich enzyme-linked immunosorbent assays, i.e. ELISA kits.

The physiological function of these soluble molecules is unknown, but given the importance of cell adhesion molecules in immune responses, the possibility has been raised that the levels of soluble adhesion molecules might be useful monitors of disease activity in various inflammatory disorders. Additionally, since *in vitro* studies have shown that circulating selectins and members of the superfamily of immunoglobulins retain functional activity, it has been proposed that the molecules have physiological effects *in vivo* by competing in cell-cell adhesion or by triggering a response in cells carrying counter-receptors (reviewed by Gearing and Newman 1993 and Spertini 1997). These mechanisms are probably overlapping, especially at sites of local production, where the concentration might reach an adhesion-blocking level. Indeed, elevated levels of these molecules have been frequently, although not conclusively, demonstrated in various inflammatory disorders such as trauma, bacterial infections, cancer and many autoimmune diseases.

There are several points worth bearing in mind when evaluating the clinical usefulness of soluble adhesion molecules, however. It is not known whether the measurable systemic levels reflect increased synthesis or release at the tissue level, since the affinity and the association rate constants of soluble adhesion molecules for ligand-bearing cells remain to be established. Significant proportion of the shed molecules may be unavailable, for instance, due to adhesion to cells that have counter-receptors. In some cases the measurable concentration may reflect decreased clearance, or both, since very little is known about the mechanisms of clearance or the circulating half-life of these molecules. Absolute average values cannot be compared between studies since different, unstandardized ELISA kits can report variable concentrations in the same samples due to differences in antibody specificity and in the standards used. Furthermore, although systemic information is scarce, there are indications that soluble adhesion molecule concentrations in the sera of healthy children may differ from those of normal healthy adults, the information provided by the ELISA kits suggesting age-related expression of cell adhesion molecules (Nash et al. 1996, Paronen et al. 1996, Sack et al. 1998, Ponthieux et al. 2003), whereas no sex-related difference has been observed in childhood (Andrys et al. 2000, Ponthieux et al. 2003).

This chapter will focus on the structure and function of soluble L-selectin and ICAM-1, the soluble forms of which have been measured in this work. A series of other soluble adhesion molecules have been identified, including P and E-selectin, LFA-3, VCAM-1 and ICAM-3.

L-selectin is shed from the surface of leukocytes following their activation (Kishimoto et al. 1989, Griffin et al. 1990, Jung and Dailey 1990), a major portion of the extracellular domain of the molecule being released from the cell surface by enzymatic cleavage (reviewed by Meager et al. 1996).The shed form, i.e. soluble (s)L-selectin, is detectable in serum and other body fluids circulating in at least two isoforms: a smaller one with a molecular weight of 62 kDa and a larger one with a molecular weight of 75-100 kDa, which are thought to represent the same fragment although differently glycosylated and derived from lymphocytes and neutrophils, respectively (Schleiffenbaum et al. 1992, Gearing and Newman 1993). The time-course of L-selectin shedding from activated T lymphocytes is substantially slower than that from neutrophils, as its expression decreases several hours to several days after T-lymphocyte activation, whereas it disappears within minutes of the exposure of neutrophils to activating stimuli (Spertini 1997). The physiological significance of L-selectin release from the cell surface is not understood. Initially, it was proposed that this was required for leukocytes to detach from the endothelial cell surface before entry into tissues (Kishimoto et al. 1989). Alternatively, it was proposed that rapid L-selectin shedding modulates the leukocyte's ability to migrate and enter sites of inflammation (Griffin et al. 1990). Nevertheless, the shed forms of L-selectin are bioactive and both *in vitro* and *in vivo* studies indicate that the increased sL-selectin levels present in certain pathological conditions may adversely affect leukocyte migration (Schleiffenbaum et al. 1992, Gearing and Newman 1993, Tu et al. 2002).

Increased levels of sL-selectin have been observed especially in haematological malignancies (Stucki et al. 1995, Spertini et al. 1997), while smaller increases have been reported in patients with various inflammatory diseases, including type 1 diabetes. Reduced levels have been reported in patients with a high risk of developing acute lung injury in connection with the adult respiratory distress syndrome. This is thought to be due to sequestration of the circulating sL-selectin by binding to activated endothelium in the microvascular beds of pulmonary blood vessels (Donnelly et al. 1994).

The circulating form of ICAM-1 (sICAM-1) is detectable in at least three molecular forms, the smallest having a molecular weight of about 240 kDa, the next 430 kDa and the largest more than 500 kDa (Seth et al. 1991). The proportions of the isoforms seem to vary between individuals. The reason for the variation is not known but may be explained by complex formation between the sICAM-1 molecules or between these and other molecules in the plasma, or different mRNA transcripts, as described by Simmons et al. (1988). sICAM-1 contains most of the extracellular region of the cell-bound ICAM-1, suggesting that it is most likely generated by proteolytic cleavage of cell surface ICAM-1 (Spertini 1997). Since sICAM-1 retains the ability to bind to LFA-1, it could potentially act as a carrier molecule that neutralizes excess ligand and prevents it from binding to cell surface ICAM-1, and accordingly it has been implicated as being able to regulate cell adhesion. Alternatively, sICAM-1 could be the indirect consequence of inflammation or tissue damage, and thus provide useful information as a marker of inflammatory disease. The first suggestion was that mononuclear cells were the cellular source of sICAM-1 (Rothlein et al. 1991), but later on it was shown to be distributed on most types of cells (von Andrian and Mackay 2000). The mean sICAM-1 levels detected in healthy blood donors in various studies range from 100 to 450 µg per litre (Spertini 1997).

Increased circulating sICAM-1 levels have been reported in a series of inflammatory disorders, such as acute trauma, acute asthma, atopic dermatitis, multiple sclerosis with clinically active disease, various rheumatic diseases, active Crohn's disease, vascular diseases, sepsis, episodes of graft rejection and cancer. Increased levels have also been observed in the cerebrospinal fluid of patients with inflammatory neurological diseases and in synovial fluid from patients with rheumatoid arthritis. Bacterial diseases

have been reported in some studies to increase serum sICAM-1 concentrations (Edgar et al. 2002), while other reports have remained negative in this respect (Darai et al. 2002). At least some enteroviruses, respiratory syncytial virus (RSV) and human rhinoviruses have been reported to use ICAM-1 as their receptor (Racaniello 1995, Smyth et al. 1997, reviewed by van Kempen et al. 1999). RSV (Smyth et al. 1997) and cytomegalovirus (Furukawa et al. 1993) have been observed to increase serum levels of sICAM-1, although Kulander et al. (2001) were unable to detect elevated serum sICAM-1 concentrations in 35 adult patients with evidence of viral infection, in contrast to patients with bacterial infections.

2.2 Type 1 diabetes

2.2.1 Epidemiology of the clinical disease

The incidence of type 1 diabetes varies widely around the world, with annual rates in children younger than 15 years of age ranging from less than 1 per 100 000 children in China to 45 in Finland (Karvonen et al. 2000, Tuomilehto et al. 1999), i.e. the global variation in its incidence is more than 400-fold, and up to tenfold even within Europe (EURODIAB ACE Study Group 2000). Most populations in Asia and South America have a very low incidence, while the highest rates are found in northern and north-western Europe, North America and New Zealand (Karvonen et al. 2000, EURODIAB ACE Study Group 2000). In most populations the incidence is highest among children aged 10-14 years (Karvonen et al. 2000).

The incidence of childhood diabetes has increased steadily in most populations over the past 30-50 years, particularly in countries with a low initial incidence (Onkamo et al. 1999, Gale 2002). The EURODIAB collaborative study, a registry consortium involving 44 centres in most European countries and Israel, indicates an annual rate of increase of 3-4%, but in some central and eastern European countries the increase is far more rapid.

Although an increase has been seen in all age groups in Europe (EURODIAB ACE Study Group 2000) it has been extremely rapid over the past 10-20 years in the under fives (Tuomilehto et al. 1999, Gardner et al. 1997, EURODIAB ACE Study Group 2000). Thus, rather than a general increase in all age categories, a shift towards younger age at diagnosis has been suggested (Pundziute-Lyckå et al. 2002, Weets et al. 2002). The overall sex ratio in children diagnosed under the age of 15 years is roughly equal (Karvonen et al. 2000, Pundziute-Lyckå et al. 2002), while populations with a very high incidence of type 1 diabetes show a male excess, especially among those diagnosed after puberty (Tuomilehto et al. 1992, reviewed by Gale and Gillespie 2001).

2.2.2 Aetiology and pathogenesis

2.2.2.1 Genetic susceptibility

Family and twin studies have shown that type 1 diabetes has a genetic basis, but the inheritance of the disease does not follow a simple mendelian single-locus pattern. The major genetic determinants reside in the human leukocyte antigen (HLA) gene region within the MHC on the short arm of chromosome 6, as these gene loci are thought to contribute about 45% of its heritability. The function of these genes in the mounting of any immune response is well known, i.e. presentation of antigenic peptides to the T lymphocytes. It is hypothesized that susceptible HLA-DR and DQ molecules bind diabetogenic antigens with low affinity and allow self-reactive T cells from the thymus to escape into the periphery, while protective HLA molecules bind with high affinity, resulting in negative selection of autoreactive T cells in the thymus. This implies that rather than predisposing the subject to autoimmune disease, the HLA genes fail to provide efficient protection (reviewed by Field 2002).

Type 1 diabetes is most closely associated with the DR, DQA1 and DQB1 alleles of the HLA class II loci (*Table 2*). Various DR4 allelic variants modify DQB1-associated susceptibility to type 1 diabetes, whereas protection is conveyed by DQB1*0602, linked to DR2. In Caucasians DQB1*0302 and DQB1*02 and their linked DR specificities DR3 and DR4 provide disease susceptibility, particularly in the heterozygous combination, as the DR3/4 phenotype is present in 30-50% of patients with type 1 diabetes and only 1-6% of the background population (reviewed by Wassmuth and Lernmark 1989 and Deschamps and Khalil 1993). The heterozygous DQB1*0201/0302 genotype is found in 25% of patients and in only 3% of healthy controls. DQB1*0302 alone, without protective alleles such as DQB1*0602 and 0603, is associated with moderate risk (Ilonen et al. 1996), and such a genotype is found in 70% of patients with type 1 diabetes, while the corresponding frequency among healthy controls is 20-30% (Reijonen et al. 1997). Pugliese et al. (1995) reported that DQB1*0602 is present in 0.5% of patients with type 1 diabetes and in 20-25% of the general population. The protective effect of DQB1*0602 is dominant over the susceptibility conferred by the high-risk DQB1 alleles (Baisch et al. 1990, Pugliese et al. 1995). Age at onset may be influenced by DQB1*0602, as this protective allele was absent in patients diagnosed before the age of 10 years, but increased in frequency with increasing age at onset (Kockum et al. 1996).

Another genetic determinant of susceptibility to type 1 diabetes has been confirmed as residing on chromosome 11p15, which contains the insulin gene region, and this polymorphism has been said to explain about 5-10% of the heritability of type 1 diabetes. The mechanism by which this gene region is involved in the disease process has yet to be ascertained, but it has been shown that the protective genotype is characterized by increased expression of insulin in the developing thymus, which could promote immune tolerance of insulin and thereby reduce the predisposition to autoimmunity (Pugliese et al. 1997, Vafiadis et al. 1997).

A locus on chromosome 2 harbouring the gene encoding the cytotoxic T lymphocyte antigen 4 (CTLA-4) has been shown to be associated with susceptibility to autoimmune diseases, including autoimmune thyroiditis and type 1 diabetes (Ueda et al. 2003)

In addition to HLA, the insulin gene region and the CTLA-4 region, about 20 other chromosomal regions have been identified in genome-wide screening as loci potentially associated with a genetic predisposition to type 1 diabetes. It is conceivable that, in addition to genes regulating the immune system, genes related to beta-cell development might also be involved (Field 2002).

Table 2. *Some combinations of DQA1 and DQB1 genes associated with type 1 diabetes susceptibility or protection in Black, Caucasian and Japanese subjects (modified from Thorsby and Rønningen 1993),*

DQA1 allele	DQB1 allele	Position†	DQ serotype	DR serotype‡	Ethnic group
Susceptibility					
*0301	*0302	cis	DQ8	DR4	all
*0501	*0201	cis	DQ2	DR3	all
*0301	*0201	cis/trans			all
*0501	*0302	trans			all
*0301	*0402	trans			all
Protection					
*0102	*0602	cis	DQ6	DR2,DR11	all
*0103	*0603	cis	DQ6	DR6	Black and Caucasian subjects
*0501	*0301	cis	DQ7	DR5	all

† cis (on the same haplotype) or trans (on a different haplotype) position of the DQA1 and DQB1 genes

‡ commonly associating DR serotype

2.2.2.2 Role of environmental factors

Despite extensive research, no environmental agent(s) responsible for triggering/precipitating the pathogenetic process involved in type 1 diabetes have definitively been identified. An apparent seasonal variation in the clinical presentation of type 1 diabetes, with a peak in the autumn, a characteristic often linked to viral infections, was reported for the first time almost 70 years ago (Adams 1926), and viruses have been implicated in the pathogenesis of type 1 diabetes. Other major influences proposed are dietary factors, particularly early infant diet, and toxins. As all the potential major risk factors interact in the gut-associated lymphoid tissue, the role of the gut immune system in the development of type 1 diabetes has received a lot of attention recently (Harrison and Honeyman 1999, reviewed by Vaarala 1999). The young age at diagnosis and the long prodrome, on the other hand, both suggest that for the majority of patients the disease process may have its origins in early life (Leslie and Elliott 1994).

The association between congenital rubella virus infections and the development of human diabetes is well established, although most recent data indicate that the form of diabetes associated with this lacks signs of beta-cell autoimmunity (Viskari et al. 2003). The possible connection between type 1 diabetes and many other viruses, such as mumps, chickenbox, measles, cytomegalovirus, Epstein-Barr virus and rotavirus, is a controversial issue (reviewed by Hyöty and Taylor 2002). Currently the main candidate for involvement in the pathogenesis of human type 1 diabetes is the group of enteroviruses, which comprise more than 60 serotypes including the polioviruses, coxsackie A and B viruses and echoviruses. Infection with various serotypes is common, starting as early as during the first months of life, and the virus frequently causes a viraemia (Hyöty and Taylor 2002). Age is one of the principal host-related factors affecting the incidence and outcome of enterovirus infections, which are more frequently seen in young children. Coxsackie B virus infections in particular have been reported to be more severe in neonates than in older children and adults (Mackay-Scollay et al. 1973**,** Melnick 1996). The timing and the infectiousness of the virus strain may also be critical for diabetogenicity.

There are several lines of evidence to suggest that an enterovirus infection may be involved in inducing the autoimmune process leading to human type 1 diabetes. A recent case report of an infant affected by neonatal autoimmune diabetes associated with a maternal echovirus 6 infection acquired during pregnancy suggests that enteroviral infections may induce beta-cell autoimmunity even *in utero* (Otonkoski et al. 2000). In the prospective Childhood Diabetes in Finland (DiMe) study, enterovirus infections, diagnosed by antibody assays and by the presence of viral RNA in serum, were observed to be more frequent in the time period preceding the appearance of autoantibodies in the initially non-diabetic siblings who became diabetic than in non-diabetic (or antibody-negative) control siblings (Hyöty et al. 1995). A seasonal pattern in the appearance of the first autoantibodies similar to that of enterovirus infections, with peaks in the autumn and winter months, has been reported in Finland (Kimpimäki et al. 2001b). These findings were confirmed in the prospective Type 1 Diabetes Prediction and Prevention (DIPP) study cohort followed from birth, in that enterovirus infections were almost twice as frequent in the subjects who became autoantibody-positive than in sex and HLA-matched control children. Infections were also clustered during the 6 months period preceding the first appearance of autoantibodies (Lönnrot et al. 2000). The German BABYDIAB study, however, did not find any association between serologically verified enterovirus infections and the development of beta-cell autoimmunity in children genetically prone to type 1 diabetes (Füchtenbusch et al. 2001).

Viruses can act by at least two possible mechanisms, either via a direct cytolytic effect or by triggering an autoimmune process leading gradually to beta-cell destruction. The suggested virus-induced autoimmune process could include modification of the surface antigens to immunogenic forms, e.g. by up-regulation of cytokines, MHC class I and/or class II molecules (reviewed by Jun and Yoon 2003) and, as recently suggested, adhesion molecules (Zanone et al. 2003) on the target tissue of the host. Structural similarities between antigenic epitopes on the virus and molecules in the host tissue could lead to the generation of antigen-specific effector T cells and/or antibodies that recognize target cells (molecular mimicry) (Jun and Yoon 2003). An alternative mechanism for initiating autoimmunity is thought to occur through bystander T-cell activation; i.e a local infection induced by an enterovirus may lead to inflammation, tissue damage and the release of islet antigens, resulting in the re-stimulation of resting autoreactive T cells (Horwitz et al. 1998).

Diabetic children have been reported to experience infections during the year preceding diabetes onset more often than control subjects (Blom et al. 1991, Verge et al. 1994), implying that infections may precipitate the on-going beta-cell destruction and reveal the already existing insulin deficiency by increasing the insulin need. Attendance at pre-school day-care, which increases the frequency of most common childhood infections, has been reported to provide protection against type 1 diabetes (EURODIAB Substudy 2 Study Group 2000, Kaila and Taback 2001), and there is some evidence from animal experiments and indirect epidemiological associations in humans to suggest that a lack of immune stimulation due to insufficient exposure to infections early in life might increase the risk of contracting type 1 diabetes (Gale 2002). An inverse association between the number of infections during the first year of life and the risk of diabetes has been reported in Southampton, UK (Gibbon et al. 1997) and in a group of children in Lithuania who developed diabetes after the age of 4 years (Pundziūte-Lyckå et al. 2000). No association between early infections and the risk of type 1 diabetes was observed in a Swedish study (Blom et al. 1991).

The immune system is functionally immature during fetal life and in early infancy, when tolerance to various antigens is induced. Additionally, the gut is more permeable to proteins during the first months of infancy (Jakobsson et al. 1986, Kuitunen et al. 1994). Early infancy is a time of rapid growth and development, in which certain periods may be critical for later health. Early introduction of cow's milk into the infant diet and/or short duration of breast-feeding have been implicated in an increased risk of

type 1 diabetes. The risk of diabetes was shown to decrease in BB (biobreeding) rats and NOD mice when they were fed with synthetic amino acid and casein hydrolysate diets instead of standard intact casein-containing diets (reviewed by Åkerblom and Knip 1998). A diabetogenic effect of cow's milk proteins was seen during a relatively narrow weaning period in BB rats (Daneman et al. 1987). The protective effect of breast milk has been attributed either to an antiviral property of human milk or alternatively to the early introduction of foreign antigens such as cow's milk proteins in association with the early cessation of breast-feeding (Kostraba et al 1993).

Early introduction of supplementary cow's milk-based formula was shown to entail an increased risk of type 1 diabetes (Virtanen et al. 1993, Verge et al. 1994), although two meta-analyses of multiple studies in which diabetes prevalence was associated retrospectively with infant feeding revealed only a marginal increase (reviewed by Davis 2001). The risk attached to early cow's milk exposure or short duration of breast-feeding in relation to the type 1 diabetes has been reported to be dependent on the age at diagnosis, in that the younger the patient is, the higher is the risk (Dahlquist et al. 1991). Moreover, genetic susceptibility appears to increase the risk, so that Kostraba et al. (1993) reported relative risks of 11.3 and 13.1 for children with HLA susceptibility genes who had early exposure to cow's milk or shorter periods of breast-feeding.

Kimpimäki et al. (2001a) showed in a recent prospective study of Finnish genetically susceptible young children that short-term breast-feeding, i.e. less than two months, and the early introduction of cow's milk-based formula predisposes these subjects to progressive signs of beta-cell autoimmunity, i.e. the development of IA-2A or of all four autoantibodies. Prospective studies of high-risk infants in Germany, the United States and Australia have failed to confirm any association between the duration of breast-feeding or early introduction of cow's milk formula and the development of islet cell autoimmunity (Norris et al. 1996, Couper et al. 1999, Hummel et al. 2000). A large-scale intervention study, Trial to Reduce IDDM in the Genetically at Risk (TRIGR), is now running in HLA-susceptible offspring of families with at least one affected member to explore whether the elimination of cow's milk proteins over the first 6-8 months of life results in a reduced risk of beta-cell autoimmunity and progression to clinical diabetes.

Viral infections may interact with exposure to cow's milk proteins, as among genetically susceptible infants exposed to cow's milk before the age of three months, those who had a T-cell proliferation response to enterovirus antigen at the age of three months had higher concentrations of antibodies to bovine insulin at the ages of six and nine months than infants who had no T-cell response to enterovirus antigen (Vaarala et al. 2002).

Plant proteins, i.e. wheat protein gluten and soy, have been shown in some animal experiments to be diabetogenic (Åkerblom and Knip 1998). Dietary supplementation with foods containing gluten before the age of three months has been reported to be associated with a four-fold risk of the appearance of diabetes-associated autoantibodies as compared with children who received only breast milk until the age of three months among the offspring of affected parent(s) who had HLA-conferred susceptibility to type 1 diabetes (Ziegler et al. 2003). Norris et al. (2003) found a four to five-fold risk of developing IA-2 antibodies if the child was exposed to cereals before the age of three months or at the age of seven months or later, the respective HLA-adjusted hazard ratios in children with the highest risk genotypes being 5 and 12. Prenatal and early postnatal vitamin D supplementation is associated with a decreased risk of type 1 diabetes (EURODIAB Substudy 2 Study Group 1999, Hyppönen et al. 2001).

2.2.2.3 Autoimmunity

2.2.2.3.1 Humoral autoimmunity

The phase of active beta-cell autoimmunity is characterized by circulating autoantibodies that react with antigens in the pancreatic islet cells. Although the pathogenesis of type 1 diabetes is obviously T-cell-mediated, epidemiological studies have defined autoimmunity as the presence of autoantibodies, since in contrast to the cellular markers, their measurement is reliable and standardized across laboratories. Several islet cell autoantigens and related antibodies have been identified, but only a few of them are clinically relevant with regard to diabetes prediction. Earlier studies have described the epidemiology of beta-cell autoimmunity by measuring islet cell antibodies (ICA) with the classical immunofluorescence test using pancreatic tissue. This test has been difficult to standardize and has been replaced in most laboratories by a combination of assays for antibodies against biochemically characterized autoantigens such as insulin, glutamic acid decarboxylase (GAD) or ICA512 (IA-2). These tests are quite sensitive and predictive in relatives of patients with type 1 diabetes, and also in the general population. Prediction on the individual level, however, has proven to be a complicated issue, since not all subjects with autoantibodies seem to progress to clinical type 1 diabetes. The prevalence of beta-cell autoimmunity is definitely higher than that of clinical type 1 diabetes (Williams et al. 2002).

ICA were first detected in patients with polyendocrinopathy including type 1 diabetes, suggesting that humoral autoimmunity is involved in the development of the latter (Bottazzo et al.1974). The semiquantitative assay uses frozen sections of human pancreas, and as a consequence it is labour-intensive and difficult to standardize and reproduce. Quantitative radioimmunoassays with high specificity and sensitivity for type 1 diabetes are now available in the case of three major islet autoantigens: insulin, glutamic acid decarboxylase (in particular GAD65) and ICA512/IA-2. GAD65 and IA-2 are the principal autoantigens recognized by antibodies in the ICA test, but ICA also include autoantigens reacting with other, so far unidentified antigens. 70-90% of patients test positive for ICA at diagnosis. The frequency of ICA varies from about 5 to 12% among first-degree relatives of patients with type 1 diabetes, depending on the cut-off point chosen for antibody positivity, and from 0.2 to 4% in the general population.

The frequency of antibodies to GAD65 (GADA) varies from 60 to 85% among patients with newly diagnosed type 1 diabetes, from 3 to 6% among siblings of children with type 1 diabetes and from 0.5 to 3% among children in the general population. The prevalence of antibodies to IA-2 (IA-2A) varies from 50 to 85% in patients with newly diagnosed type 1 diabetes, being 1.5-5% among siblings of children with type 1 diabetes and 0.2-2% in children from the general population. Correspondingly, antibodies to insulin (IAA), the only beta-cell-specific autoantigen so far identified, have been observed with a frequency of 30% among adult patients at diagnosis and up to 80% among children diagnosed before the age of 5 years. An inverse association is observed between both the frequency and the titre of IAA and age (Kulmala et al. 1998, Komulainen et al. 1999, Ziegler et al. 1999). 1.5-7% of siblings of children with type 1 diabetes test positive for IAA and 1-3% of children representing the general population.

2.2.2.3.2 Cellular autoimmunity

There are several lines of evidence to suggest that the cellular arm of the immune system is involved in the destruction of pancreatic beta cells. T-cell-dominated infiltrates are seen in the pancreatic islets of

most patients at the time of diagnosis of type 1 diabetes (Gepts 1965, Hänninen et al. 1992, Imagawa et al. 2001), and they have also been shown to be present in patients with residual beta-cell function, implying continuous insulitis before total destruction (Shimada et al 1999). ”Adoptive” type 1 diabetes has been shown to develop following transplantation with bone marrow undepleted for T cells from a diabetic donor to a related non-diabetic immunocompromised recipient (Lampeter et al. 1993). Immunosuppressive drugs specifically directed against T cells have been reported to delay the disease process (Bougneres et al. 1988).

Several groups have demonstrated the presence of activated T cells that respond to human beta cells (Peakman et al. 1994, Roep et al. 1995) or beta cell-associated proteins (Kallan et al. 1995) in the peripheral circulation of patients with newly diagnosed type 1 diabetes or prediabetic subjects. On the other hand, a high frequency of T-cell responses to diabetes-associated autoantigens has also been described in healthy controls (Schloot et al. 1997, Rharbaoui et al. 1999).

Unlike the situation with autoantibodies, assays of human T-cell function rely on the ability of the *in vitro* systems to mimic *in vivo* situations at the site of inflammation, e.g. the pancreatic islets or draining lymph node, where activated T cells would be expected to be highly concentrated while the corresponding levels in the peripheral circulation are much lower. The reported discrepancies are partly attributable to the obvious limitations in the currently available T-cell assay technology and the quality of the antigen preparations, which has proved to be critical with regard to accurate measurements of circulating autoreactive T cells (reviewed by Roep 2003).

The NOD mouse is a spontaneous animal model for autoimmune diabetes, as all mice of this strain develop humoral autoimmunity to islet cell antigens soon after birth. The infiltration of islets with mononuclear cells starts at about 3-5 weeks of age, beginning from the periphery and progressing to the islet core. Peri-insulitis usually has little beta-cell destruction associated with it and does not cause insulin deficiency until a precipitating event, or a genetic programme, induces islet invasion by macrophage/dendritic cells, CD4 and CD8-positive T cells. The majority of female mice develop overt insulin-dependent diabetes by 13-30 weeks of life (Leiter et al. 1987, reviewed by Lampeter et al. 1989). Peri-insulitis in the NOD mouse is characterized by high levels of type 2 helper T cell (Th2) cytokines, such as IL-4 and IL-10, and low levels of type 1 helper T cell (Th1) cytokines such as IFN-γ (Healey et al. 1995, Kolb 1997). Destructive intrainsulitis is characterized by a dominance of Th1 cytokines such as IFN-γ, IL-2 and IL-12 (Rabinovitch 1994, Fujihira et al. 2000).

Although not demonstrated sequentially, a similar course of events may occur in humans (Shimada et al. 1999). A bias towards Th1-type immune reactivity has been observed in immune cells grown from the blood of patients with type 1 diabetes (Kallmann et al. 1999) and a Th1-dominated cytokine profile has also been verified in subjects at risk for the disease (Hussain et al. 1998, Karlsson et al. 2000).

2.2.3 Expression and role of adhesion molecules during the pathogenesis of type 1 diabetes

2.2.3.1 In human type 1 diabetes

Some of the published work on human type 1 diabetes has compared the expression of certain cell adhesion molecules in recent-onset diabetic pancreata with that in the pancreas of subjects without diabetes. Thus increased ICAM-1 expression was detected in vascular endothelial cells by

immunostaining but not in vessels from uninflamed pancreases (Hänninen et al. 1992), while Itoh et al. (1993) found strong endothelial ICAM-1 expression in two out of nine cases and Somoza et al. (1994) in one recent diabetic pancreas. In addition, VCAM-1 expression was found in morphologically identified dendritic cells scattered around the islets of the diabetic pancreas (Hänninen et al. 1992, Somoza et al. 1994), while the expression of LFA-1 and LFA-3 did not differ from that in control pancreata (Somoza et al. 1994). Although ICAM expression in beta cells has been reported to be an effect induced by cytokines *in vitro* and *in vivo* (Vives et al. 1991), no ICAM-1 expression in human endocrine cells was found within the islets (Hänninen et al. 1992, Itoh et al. 1993, reviewed by Yang et al. 1996).

Aberrations in the gut immune system have been implicated in the pathogenesis of type 1 diabetes (Vaarala 1999). Studies performed on NOD mice and patients with type 1 diabetes show that the islet-infiltrating autoreactive cells express gut-associated homing receptor $\alpha_4\beta_7$ integrin, suggesting the recirculation of lymphocytes between the gut and the pancreas (Yang et al. 1996, Hänninen et al. 1996 and 1998, Paronen et al. 1997). Inflammation and immune activation, such as enhanced expression of HLA-DR and ICAM-1 on the gut epithelium and $\alpha_4\beta_7$ integrin on the T cells in the lamina propria, was demonstrated by immunohistochemistry of the macroscopically normal intestines of patients with type 1 diabetes without signs of coeliac disease compared with control subjects. The findings were not restricted to HLA-DQ2 risk alleles, which are associated with coeliac disease, suggesting that subclinical inflammation of the gut may be an underlying entity in type 1 diabetes (Savilahti et al. 1999, Westerholm-Ormio et al. 2003).

The mean proportions of stimulated monocytes expressing detectable amounts of the adhesion molecules LFA-1 and ICAM-1 have been reported to be lower in 13 patients 2-3 weeks after the diagnosis of type 1 diabetes than in normal controls, a finding thought to indicate trapping of cells expressing these adhesion molecules in the inflamed organ (Martin et al. 1991). This was also shown with two analyses of peripheral blood mononuclear cells in patients with Grave's disease, who similarly had a reduced number of cells expressing adhesion molecules (Guerin et al. 1989, Kabel et al. 1990). Lampeter et al. (1992) reported elevated levels of sICAM-1 (>2 SD of the normal mean observed in 100 healthy blood donors) in four out of 14 patients with newly diagnosed type 1 diabetes, and of sL-selectin in nine out of 14 such patients. Among the six ICA-positive first-degree relatives, increased levels of soluble ICAM-1 were observed in half of the cases and increased sL-selectin in all of them, but ICA-negative relatives with an increased genetic risk of type 1 diabetes (HLA-DR3 and/or DR4) also had increased sICAM-1 and sL-selectin concentrations (Lampeter et al. 1992).

Based on these findings, Lampeter et al. (1992) suggested that the reduced expression of ICAM-1 on the surfaces of monocytes (Martin et al. 1991) and the elevated serum concentration could result from enhanced shedding of ICAM-1, while the increased levels of soluble ICAM-1 and L-selectin could reflect an on-going immune process during which soluble adhesion molecules are released. In line with this finding, Myśliwiec et al. (1999) also reported the highest sICAM-1 levels in 26 prediabetic first-degree relatives, i.e. subjects with ICA 20 JDFU or more, of whom 20 had multiple autoantibodies, either GADA, IA-2 and/or IAA, compared with the levels observed in age-matched healthy controls and patients with clinical type 1 diabetes. Krętowski et al. (2000a) reported increased levels of sL-selectin in patients with newly diagnosed type 1 diabetes and in patients with untreated Graves' disease, and also in first-degree relatives both with and without autoantibodies associated with type 1 diabetes, compared with the levels observed in healthy controls and patients with type 2 diabetes. They also observed that the proportion of memory T lymphocytes expressing L-selectin had decreased in patients with newly diagnosed type 1 diabetes and in relatives with two or more autoantibodies by comparison with healthy controls, and attributed this to alterations in the balance of cytokines produced by helper T cells (Th1/Th2) (Krętowski et al. 2000a). Human memory CD4-positive T cells expressing L-selectin have

been shown to produce mainly IL-4 and IL-5, whereas L-selectin-negative CD4-positive T cells produced mainly IFN-γ. This profile of cytokine expression coincides with that which distinguishes the Th1 and Th2 subsets (Kanegane et al. 1996). An inverse correlation was seen between the ratio of L-selectin-positive and negative helper T lymphocytes and circulating sL-selectin in subjects with type 1 diabetes and autoantibody-positive relatives. High sL-selectin levels correlated with the presence of high-risk diabetes-associated HLA alleles both in family members and in controls. The group suggested that sL-selectin could serve as a late marker of the autoimmune destruction of beta cells (Krętowski et al. 2000a). Increased concentrations of sL-selectin have been observed in 35 children and adolescents with recent-onset type 1 diabetes compared with the levels in patients who had received insulin treatment for 2-12 months, in patients with a disease duration of more than one year and in non-diabetic controls (Kordonouri and Bührer 2000).

Increased levels of sICAM-1, sVCAM-1 and/or sE-selectin have been extensively reported in adult patients with type 1 and 2 diabetes, and it has been implied that these may be involved in the pathogenesis of diabetic vascular disease, since at least ICAM-1 expression in endothelial cells has been observed to be increased under high-glucose conditions (Taki et al. 1996).

2.2.3.2 In vitro

Roep et al. (1994) reported that a monomeric soluble recombinant form of ICAM-1 inhibited specific anti-beta-cell T-cell responses in concentrations similar to those found in first-degree relatives at risk for type 1 diabetes (Lampeter et al. 1992), implying that a naturally circulating elevated concentration of sICAM-1 may down-regulate T-cell inflammation in subjects at risk of developing type 1 diabetes rather than reflecting subclinical insulitis. In such a scenario, increased circulating concentrations of sICAM-1 would represent a defensive mechanism against beta-cell destruction. It was suggested that the mechanism by which the recombinant form of ICAM-1 blocked the antigen-specific T-cell proliferation may depend on the interaction between T cells and antigen-presenting cells (Roep et al. 1994).

2.2.3.3 In murine models

The expression of cell adhesion molecules on the surface of lymphocytes and endothelial cells in the NOD mouse pancreas during the diabetic process has been investigated using semiquantitative immunohistochemistry. The expression of some adhesion molecules is up-regulated in parallel with the development of insulitis, while that of others remains unchanged. A low constitutive expression of MAdCAM-1 is found on the endothelial cells of many vessels in the exocrine pancreas of both NOD and control mice during the first three weeks after birth, but not on the endothelium within the islets. Vessels with the morphology of HEV and endothelial expression of MAdCAM-1 and PNAd were reported to develop within and adjacent to pancreatic islets at the time when T cells enter these islets (Hänninen et al. 1993). MAdCAM-1 seems to be the predominant addressin expressed on endothelial cells in and adjacent to the islets at the early stages of insulitis, while the expression of PNAd increases as insulitis progresses. Most infiltrating lymphocytes express $\alpha_4\beta_7$ integrin at all stages of insulitis and the expression of $\alpha_4\beta_7$ on lymphocytes correlates with high expression of MAdCAM-1 by endothelial cells, suggesting a predominant role for mucosal lymphocyte homing in the development of insulitis. A

minority of the peri-islet and intra-islet lymphocytes present during the early stages of insulitis expressed L-selectin, but this expression intensified on groups of lymphocytes within the inflammatory plaque at more advanced stages of insulitis (Hänninen et al. 1993, reviewed by Yang et al. 1996).

Faveeuw et al. (1994) have demonstrated ICAM-1 expression on endothelial and dendritic cells in infiltrated islets of 14 to 17-week-old NOD mice, while it was absent in healthy areas, suggesting that ICAM-1 is involved in the migration of murine lymphocytes into the islets of Langerhans. The same group also reported the presence of VCAM-1-positive dendritic cells in inflamed islets of NOD mice. Martin et al. (1996) reported that cryostat sections of NOD mouse pancreas islets did not show any expression of ICAM-1, LFA-1, L-selectin or VCAM-1 prior to infiltration by mononuclear cells. ICAM-1 and LFA-1 were first demonstrable with strong peri-insular infiltrates, while L-selectin and VCAM-1 were only seen in islets with mild or strong intra-islet infiltration. The staining pattern for the four cell adhesion molecules was consistent with both T-cell and macrophage infiltration and the distribution of the two cell types in the infiltrate largely overlapped. Adhesion molecule expression was shown to be similar during Th1 and Th2-cell infiltration, suggesting similar adhesion molecule requirements for the two Th subsets (Martin et al. 1996). More recently, the same author suggested on the basis of findings in NOD mice with a disrupted ICAM-1 gene that ICAM-1 has a non-replaceable role in T-cell activation prior to islet inflammation, since in its absence the NOD mice fail to mount any strong autoaggressive Th1 response (Martin et al. 2001). In line with this, Balasa et al. (2001) have suggested a role for ICAM-1 in the generation and/or expansion of islet-specific T cells. Interactions of LFA-1 with ICAM-1 during priming have been shown to generate effector T cells capable of destroying murine beta cells (Camacho et al. 2001).

Administration of 5 μg of recombinant ICAM-1 proteins three times a week for 4.5 months from the age of 35 days onwards has been shown to inhibit insulitis and the onset of autoimmune diabetes in NOD mice (Martin et al. 1998). Similarly, monoclonal antibodies (Mab) directed against ICAM-1 and LFA-1 have been shown to inhibit the development of autoimmune diabetes in mice of this strain (Yagi et al. 1995, Yang et al. 1996). Respectively, treatment with Mab against L-selectin and VLA-4 (Yang et al. 1993) and against β_7 integrin (Yang et al. 1997) and MAdCAM-1 (Hänninen et al. 1998) have been reported to provide protection against the spontaneous occurrence of autoimmune diabetes in NOD mice**.**

2.2.4 Natural course of type 1 diabetes

The natural course of type 1 diabetes includes four fairly distinct stages that can be detected in the majority of patients: 1) preclinical beta-cell autoimmunity with progressive impairment in insulin secretion capacity, 2) manifestation of clinical diabetes, 3) transient remission, and 4) established diabetes.

2.2.4.1 Autoantibody appearance and persistence.

Beta-cell autoimmunity can appear by the age of 6-9 months (Martikainen et al. 1996, Roll et al. 1996, Ziegler et al. 1999), although rarely during the first months of life (Kimpimäki et al. 2002) or as early as *in utero* (Otonkoski et al. 2000). In the DIPP birth cohort study the prevalence of children with

autoantibodies increased steadily with age during the first 7 years (Kupila A, personal communication). There is no predetermined order of emergence of various autoantibodies during the preclinical stage, although IAA seem to be the first or among the first autoantibodies to appear in young subjects (Ziegler et al. 1999, Colman et al. 2000, Kupila et al. 2002), while IA-2A are commonly among the last to appear (Roll et al. 1996, Kupila et al. 2002), indicating rapid progression to clinical disease.

Subclinical beta-cell autoimmunity is present in many more individuals than the number of cases with type 1 diabetes that can be expected. Diabetes-associated autoantibodies can occur transiently, and the reported rate of transient positivity for a single autoantibody has varied between 2 and 14% within a follow-up period of 2-3 years in prospective birth cohort studies (Colman et al. 2000, Yu et al. 2000, Kupila et al. 2002). The disappearance of autoantibodies is mainly restricted to positivity for one low-titre autoantibody, and is rarely seen among the subjects carrying high-risk (DR3/4, DQ2/8) genotypes (Yu et al. 2000, Savola et al. 2001). Any of the four antibodies ICA, IAA, GADA and IA-2A may disappear, but IAA have been observed to do so more often than ICA in unaffected schoolchildren (Lévy-Marchal et al. 1995), and they have also displayed a transient characteristic in young genetically susceptible children (Colman et al. 2000, Kimpimäki et al. 2002). Reports concerning the persistence of IA-2A have been conflicting. Common inverse seroconversions have been reported by Kulmala et al. (2000a) in unaffected schoolchildren and by Colman et al. (2000), while IA-2A proved highly persistent in the DIPP cohort (Kimpimäki et al. 2002). The reason for antibody fluctuations remains to be defined, although viral infections having no correlation to future diabetes have been suggested (Hyöty et al. 1995). Although transient expression of autoantibodies is commonly regarded as a benign sign of self-limited anti-islet autoimmunity, and mostly does not indicate an increased risk of type 1 diabetes, loss of antibody positivity does not invariably indicate the cessation of the process of pancreatic beta-cell destruction (Christie et al. 1997).

2.2.4.2 From the onset of autoimmunity to clinical diagnosis

The duration of preclinical beta-cell autoimmunity is highly variable and can precede the diagnosis by a matter of months or years. Those subjects in whom the disease process is slow may present with type 1 diabetes as adults, so that markers of beta-cell autoimmunity can be detected in 5-30% of adult patients classified as having type 2 diabetes on the basis of their clinical features. The concept “latent autoimmune diabetes of adults” (LADA) has been coined for this slowly progressing form of autoimmune diabetes, which has been estimated to account for about a half of all patients affected by type 1 diabetes (reviewed by Atkinson and Eisenbarth 2001).

In some subjects the process is so slow that they appear to be non-progressors or they may never progress to the clinical disease. No specific characteristics that could completely discriminate between progressors and non-progressors (or slow-progressors) have been identified so far, which supports the heterogeneous nature of beta-cell destruction (Knip et al. 1994, Kulmala et al. 1998, Greenbaum et al. 1999), which is thought to be even more variable in young children (Ziegler et al. 1999). In most subjects with persistent autoantibodies there is progressive impairment in the acute insulin response to intravenous glucose, followed by a deterioration in oral glucose tolerance and fasting hyperglycaemia. The classical symptoms of hyperglycaemia, such as excessive drinking, increasing urinary volumes, weight loss and ketosis, occur late in the natural history of the disease.

The natural history of both preclinical and clinical type 1 diabetes may vary with age. Presentation at a very young age has been reported to associate with a severe metabolic decompensation and a poorly preserved residual beta-cell function at diagnosis (Komulainen et al. 1999). Positivity for multiple autoantibodies also indicates more aggressive beta-cell destruction and accelerated beta-cell failure, so that patients with multiple autoantibodies at diagnosis have been shown to have an increased requirement for exogenous insulin over the second year of clinical disease (Komulainen et al. 1999, Sabbah et al. 1999) and low/non-existent fasting C-peptide levels within 5 years, whereas C-peptide levels remained unchanged during a 12-year follow-up in initially autoantibody-negative subjects (Borg et al. 2002).

Shortly after clinical presentation, most patients undergo a transient fall in their insulin requirement due to improved beta-cell function, reflected by an increase in circulating C-peptide concentrations and increasing insulin sensitivity. This phase is often referred as a "honeymoon" or remission. Mild metabolic derangements, male sex, older age (Knip et al. 1982**,** Örtqvist et al. 1997) and an absence of ICA (Wallensteen et al. 1988, Örtqvist et al. 1997) have been shown to be associated with a more pronounced and longer partial remission. Destruction of the beta cells becomes complete within a few years of diagnosis in most children, especially in those with the HLA-DR3/4 combination (Knip et al. 1986). On the other hand, it has been proposed that the destructive process may be slower in patients who are older at diagnosis and may sometimes not result in total beta-cell destruction (Madsbad et al. 1978**,** Foulis et al. 1986, Shimada et al. 1999), since 15% have been reported to have some beta-cell function preserved more than 15 years after diagnosis (Madsbad et al.1978).

2.2.5 Prediction of type 1 diabetes

Although the pathogenetic process by which the pancreatic islet cells are destroyed is not well understood, several risk factors and immune-related markers are known that accurately identify most of the subjects who will develop the disease. Identification is a prerequisite for any intervention aimed at delaying or preventing manifestation of the disease, and it becomes all the more important the longer the intervention trials and the larger the cohorts are. Three variables are commonly used to express the predictive characteristics of a marker. Sensitivity denotes the number of subjects in a cohort with the disease and with the marker in question divided by the overall number of subjects who develop the disease, whereas specificity is the number of the subjects in a cohort without that marker in question who will not develop the disease divided by the total number of subjects who will not do so. Thus a reciprocal relationship exists between sensitivity and specificity in a given assay. Positive predictive value (PPV) provides information on what proportion of the subjects in a cohort with the marker in question will progress to the disease, and is dependent on the prevalence of the disease in the population tested. Assessment of the risk of developing type 1 diabetes is currently based on the occurrence of autoantibodies and the presence of genetic and metabolic markers. Of all the autoantibody specificities identified, ICA, IAA, GADA and IA-2A are regarded at present as clinically the most useful for predicting the risk of type 1 diabetes in first-degree relatives of affected patients, but their predictive characteristics in the general population, from which about 90% of the newly diagnosed patients are derived, still have to be defined more precisely.

2.2.5.1 First-degree relatives

2.2.5.1.1 Humoral risk markers

ICA positivity is the most sensitive single antibody marker among unaffected family members of children with type 1 diabetes, as it identifies approximately 80% of those who will progress to the clinical disease. Taken alone, ICA above the detection limit are associated with a relatively low disease risk, the PPV varying from 40 to 60% within 9-11 years of follow-up (Kulmala et al. 1998, Yamamoto et al. 1998). The risk of progression is related to the titre (Bingley et al. 1994, Kulmala et al. 1998). In a birth cohort of 1353 offspring of affected parent(s), ICA above the detection limit identified all the children who progressed to clinical disease by 8.5 years and 70% of those who presented with multiple autoantibodies during the follow-up (0.1-8.5 years, median 0.9 years) (Ziegler et al. 1999).

The diagnostic sensitivity of IAA for identifying siblings of diabetic children who will progress to clinical disease is relatively low; 25-58% (Kulmala et al. 1998, Yamamoto et al. 1998), while among younger subjects 92-100% of those who developed the clinical disease by the age of 8.5 years or multiple autoantibodies during the follow-up tested positive for IAA (Ziegler et al. 1999, Colman et al. 2000).

The presence of IA-2A among first-degree relatives indicates a high risk of progression to the clinical disease, the reported PPV ranging from 50 to 80%, with a sensitivity from 56 to 69% (Kulmala et al. 1998, Yamamoto et al. 1998, Decochez et al. 2002). IA-2A were detected in 77-100% of the children who developed the clinical disease by the age of 8.5 years and 68-78% of those who presented with multiple autoantibodies during the follow-up (Ziegler et al. 1999, Colman et al. 2000). More rapid progression to clinical disease has been reported in IA-2A-positive siblings carrying the HLA-DQ2/8 genotype (Kulmala et al. 2000b, Decochez et al. 2002).

Multiple antibodies, i.e. at least two antibodies, confers a risk of disease progression in the range 55-100% in siblings of diabetic patients within a follow-up of 8-13 years, while positivity for a single autoantibody carries a risk of only 2-17% (Kulmala et al. 1998, Yamamoto et al. 1998). The detection of multiple antibodies by the age of 2 years in the offspring of parent(s) with type 1 diabetes was associated with a 50% risk of overt diabetes by five years of age, the cumulative risk in the whole cohort being 1.8% by that age (Ziegler et al. 1999). In another birth cohort study all the children who presented with clinical disease before the age of 4 years tested positive for multiple antibodies before the diagnosis (Colman et al. 2000).

2.2.5.1.2 Genetic markers

While the risk of contracting type 1 diabetes in Finland by 15 years of age is about 0.7%, that conferred by the high-risk genotype, DQB1*0201/*0302, is on average 7% and that conferred by the moderate-risk genotype, DQB1*0302/x (x= other than *0201, *0301 or *0602), is approximately 2.5% (Ilonen et al. 1996). The siblings of affected subjects have been estimated to have an absolute lifetime risk in the range of 6-8%, being 16-20% in HLA-identical siblings, 6-8% in HLA-haploidentical siblings and less than 1% in HLA-non-identical siblings (Platz et al. 1981, reviewed by Eisenbarth 1986, Tarn et al. 1988, Deschamps et al. 1992). The cumulative risk of diabetes is markedly higher among the siblings of probands diagnosed before the age of five years (Gillespie et al. 2002).

Strong genetic susceptibility predisposes children to early-onset beta-cell autoimmunity (Ziegler et al. 1999, Colman et al. 2000) and to presentation with the clinical disease at an early age (Pugliese et al. 1995, Ilonen et al. 1996, Komulainen et al. 1999, Gillespie et al. 2002). Lower frequencies of ICA, GADA, IA-2A and multiple autoantibodies have been observed in unaffected siblings carrying the protective DR2 or DQB1*0602 alleles than in other siblings of children with type 1 diabetes (Kulmala et al. 2000b), although the protective effect of DR2 is not absolute even for the development of clinical disease (Pugliese et al. 1995, Kulmala et al. 2000b).

2.2.5.1.3 Metabolic risk markers

The first-phase insulin response (FPIR) in an intravenous glucose tolerance test (IVGTT), which reflects the discharge of the contents of the releasable pool of insulin-containing granules bound to the plasma membrane of beta cells (Daniel et al. 1999) is currently the best available *in vivo* method for assessing the insulin secretory capacity of these cells. A reduced FPIR has been widely shown to be associated with multiple autoantibody positivity, i.e. with signs of extensive humoral autoimmunity against beta-cell structures, and with a high risk of progression to clinical disease. In the data set on first-degree relatives of diabetic patients with ICA $\geq$ 5 JDFU compiled in the large Islet Cell Antibody Register Users Study (ICARUS), those with a FPIR< 50 mU/l were reported to have an up to 85% risk of progression to clinical diabetes over the next 5 years, the risk for those with a FPIR in the range 50-100 mU/l being 50% and that for subjects with FPIR> 100 mU/l only 17%. The prognostic significance of a low FPIR was shown to be modified by other risk determinants, e.g. its PPV was increased by a high ICA titre and young patient age (Bingley et al. 1996).

HLA identity and the DQB1 high-risk genotype have been observed to be associated with a decreased FPIR in non-diabetic ICA and/or IAA-positive siblings of affected children (Veijola et al. 1995), while carriers of the DQB1*0602 allele are not protected from the autoimmune process, since 29% of the ICA-positive relatives carrying DQB1*0602 were shown to have IAA or low FPIR (Greenbaum et al. 2000).

A decrease in FPIR, the earliest metabolic abnormality during the preclinical disease process, is regarded as a sign of advanced beta-cell destruction, i.e. late prediabetes (Srikanta et al. 1984, Vialettes et al. 1988, Vardi et al. 1991, Herskowitz-Dumont et al. 1993), although the rate of beta-cell destruction and prediction of the time to diagnosis on an individual level still are controversial issues (Robert et al.1991, Knip et al. 1994). A normal FPIR does not exclude the possibility of rapid progression to clinical disease (Robert et al. 1991).

2.2.5.2 The general population

2.2.5.2.1 Humoral risk markers

Reports concerning the predictive value of ICA positivity among children in the general population have been controversial so far. The PPV has been estimated to be in the range 0.5 to 6% within 4-7 years of follow-up (Lévy-Marchal et al. 1995, Knip et al. 1998), while two prospective surveys carried out in Florida and the Netherlands have reported a PPV of 18-50% within 8-11 years of follow-up, implying

that ICA could predict type 1 diabetes equally efficiently as in first-degree relatives (Bruining et al. 1989, LaGasse et al. 2002). The sensitivity was reported to be 57 and 100% in the two studies. The risk appears to increase with rising ICA titres (Karjalainen 1990).

IAA positivity have been reported to yield a sensitivity of 5% and a specificity of 97% in the general population (Hagopian et al. 1995), while the PPV has been observed to be 5% (Landin-Olsson et al. 1992, Bingley et al. 1997). Isolated GADA or IA-2A positivity has been estimated to confer a risk of 5 to 7% for progression to type 1 diabetes over an interval of 10 years among schoolchildren (Bingley et al. 1997).

Multiple autoantibodies, i.e. two or more, by 14 years of age have been reported to confer a PPV of 19-50% over a follow-up period of 5-8 years, with a sensitivity of 100% (Kulmala et al. 2001, LaGasse et al. 2002). Among genetically susceptible young children derived from the general population who persistently tested positive for more than one autoantibody by the age of 2 years and/or progressed to clinical type 1 diabetes by the age of 5 years, the sensitivity of ICA was found to be 80%, that of IAA 93%, that of GADA 60% and that of IA-2A 40%. The respective figures for specificity were 99%, 97%, 100% and 100% (Kimpimäki et al. 2002).

2.2.5.2.2 Genetic risk markers

The predictive value of HLA-DR or DQB1 risk markers in the background population is dependent on the national incidence of type 1 diabetes. Accordingly, the absolute risk attached to the high-risk genotype, DQB1*0201/*0302, is substantially higher in Finland than in Greece, for instance. Rewers et al. (1996) reported that the high-risk genotypes DR3/4 and DQB1*0201/0302 confer a 6.3% risk of developing diabetes by 20 years of age in Colorado, USA. Kulmala et al. (2001) genotyped 600 healthy schoolchildren and found that 7/9 subjects with three or more autoantibodies carried either the DQB1*0302 or the DQB1*02 risk allele while three subjects had a protective genotype. In a study by LaGasse et al. (2002), five out of six progressors were observed to carry HLA-conferred susceptibility to type 1 diabetes.

A cohort identified by typing for HLA-DQB1 alleles to be followed up from birth for the appearance of beta-cell autoimmunity accounted for 10-15% of the total birth cohort and can be estimated to include 65-80% of the future patients who will present with type 1 diabetes before reaching adulthood (Knip 2002).

2.2.5.2.3 Metabolic risk markers

An impaired FPIR has been shown to be associated primarily with positivity for multiple autoantibodies in cohorts representing the general population (Strebelow et al. 1999, Kulmala et al. 2000, Keskinen et al. 2002). In a series of studies on schoolchildren representing non-familial prediabetes, Strebelow et al. (1999) observed a reduced FPIR in 10 out of 17 children tested among 22 with multiple high-titre autoantibodies. Six out of the 22 children progressed to clinical type 1 diabetes within 46 months. A decreased FPIR ($< 5^{th}$ percentile in 129 healthy controls) was seen in four prediabetic children, while two progressors had no IVGTT. A Finnish group has recently shown very low FPIRs shortly after seroconversion to autoantibody positivity in young children with HLA-DQB1-conferred susceptibility to type 1 diabetes, suggesting that the autoimmune-mediated destruction of beta cells may occur early in

the course of prediabetes, at least in young children (Keskinen et al. 2002), as assumed previously by Bingley et al. (1996).

2.2.6 Therapeutic aspects

Strategies for preventing type 1 diabetes can be classified into three categories depending on the stage of disease**.** Primary prevention aims at intervening in exposures that may initiate the disease process before any signs of autoimmunity appear, by identifying the subjects at risk based on a positive family history and/or disease-associated genetic markers, for example. Secondary prevention aims at delaying and possibly suppressing an on-going progressive beta-cell destruction in normoglycaemic subjects with signs of beta-cell autoimmunity. Tertiary prevention is initiated after the clinical manifestation and aims at preventing secondary complications of the disease by preserving or even restoring beta-cell function. Beta-cell regeneration, indeed, may be possible even after the clinical presentation since beta-cell autoimmunity has been shown to remain detectable for years in the majority of the patients affected by type 1 diabetes (Savola et al. 1998b). Preservation of residual beta-cell function is measured in terms of circulating C-peptide levels. Tertiary interventions are usually the first approaches to test the efficiency and safety of an intervention strategy, particularly if the preventive measure may have potential toxic effects.

Several immunosuppressive drugs have been tested for diabetes prevention, e.g. cyclosporin (Bougneres et al. 1988). A disadvantage of these drugs is that they unselectively suppress protective immune functions. The majority of the on-going and planned trials are designed to restore immunological tolerance of beta-cell antigens, e.g. by mucosal administration of insulin. Immunomodulation is yet another way to induce a beta-cell rest; monoclonal antibodies against cell surface proteins or against chemokines interfering with T-cell activation or inducing proliferation of regulatory T cells have been proposed for testing in prevention trials.

2.2.6.1 Primary prevention

The Trial to Reduce IDDM in Genetically at Risk (TRIGR) is an international randomized multicentre intervention trial designed to determine whether the elimination of cow's milk proteins during the first 6-8 months of life will reduce the risk of beta-cell autoimmunity and the incidence of type 1 diabetes in genetically high-risk children. A pilot study indicated that such a nutritional intervention in early infancy could significantly reduce the cumulative incidence of ICA and positivity for at least one autoantibody during observation up to the age of 7 years (Åkerblom et al. in press).

2.2.6.2 Secondary prevention

The elimination of dietary gluten has been tested as an attempt to reduce islet autoimmunity in high-risk subjects. A gluten-free diet pursued for 6-12 months followed by gluten re-exposure in two separate small series of autoantibody-positive subjects did not have any influence on antibody levels (Hummel et al. 2002, Pastore et al. 2003). Interestingly, however, the FPIR increased in 12 out of 14 subjects after the first 6 months of gluten deprivation and decreased in 10 out of 13 subjects during the following 6-

month period of a normal diet, indicating that gluten deprivation may have a beneficial effect on the preservation of beta-cell function in subjects at risk for type 1 diabetes (Pastore et al. 2003).

Oral nicotinamide did not reduce the cumulative incidence of type 1 diabetes at 3 years from 30 to 6 %, i.e. by 80%, in a series comprising very high-risk first-degree relatives in the randomized German Nicotinamide Diabetes Intervention Study (DENIS) (Lampeter et al. 1998), although a smaller protective effect, 56% after an average follow-up of 7 years, was shown in an open population-based survey among schoolchildren in New Zealand (Elliott et al. 1996). The large multinational European Nicotinamide Diabetes Intervention Trial (ENDIT) was a randomized, double-blind, placebo-controlled survey targeting ICA-positive ($\geq$ 20 JDFU) first-degree relatives and aimed at reducing the incidence of type 1 diabetes by 35% over 5 years. The study was recently completed, and the outcome was unfortunately negative: there was no difference what-so-ever between the two groups in terms of progression to clinical diabetes (ENDIT Study Group 2004).

The American Diabetes Prevention Trial-Type 1 Diabetes (DPT-1) is a multicentre two-arm intervention trial, the first part of which has tested parenteral administration of insulin for preventing type 1 diabetes by 50% within 4 years in first and second-degree relatives with $\geq$ICA 10 JDFU and a reduced FPIR ($<10^{th}$ percentile). This part showed no difference in the rate of progression to overt type 1 diabetes between the subjects treated and the control group (DPT-1 Study Group 2002). The second part was an assessment with a randomized, double-blinded design of whether daily oral administration of insulin could reduce the incidence of type 1 diabetes in ICA-positive subjects with a normal FPIR by at least 25% over 4 years. The preliminary results presented at the 2003 American Diabetes Association Scientific Meeting indicated that there was again no difference in the cumulative incidence of type 1 diabetes between the insulin-treated group and the placebo group.

In the DIPP project nasal insulin is being tested in a randomized placebo-controlled trial with autoantibody-positive children having HLA-conferred susceptibility to type 1 diabetes (Kupila et al. 2001). This trial will be completed by the end of 2006.

2.2.6.3 Tertiary prevention

Although the residual beta-cell mass is already small at a time of clinical onset of type 1 diabetes, the trials with immunosuppressive drugs have shown that the course of beta-cell function can still be modified at this phase. Oral insulin initiated at clinical presentation did not prevent the deterioration of beta-cell function in a multicentre randomized double-blind intervention trial (Chaillous et al. 2000), nor in an Italian randomized placebo-controlled trial (Pozzilli et al. 2000). Nicotinamide has been observed to preserve some C-peptide secretion in patients with newly diagnosed type 1 diabetes (Pozzilli 1996). Treatment with a 14-day course of intravenous human anti-CD3 monoclonal antibody resulted in improved preservation of endogenous insulin secretion after a follow-up for 12 months in a randomized open trial (Herold et al. 2002).

3 Aims of the present research

The specific aims of this work were:

I. to define the relationship between circulating concentrations of sICAM-1 and sL-selectin and genetic susceptibility to type 1 diabetes conferred by HLA class II alleles.

II. to define the relationship between type 1 diabetes-associated autoantibodies and circulating levels of sICAM-1 and sL-selectin.

III. to investigate the circulating concentrations of sICAM-1 and sL-selectin in subjects with impaired insulin secretory capacity.

IV. to follow-up the circulating levels of sICAM-1 and sL-selectin during preclinical type 1 diabetes in first-degree relatives of affected children and in young children with genetic susceptibility to type 1 diabetes, and to assess the utility of these molecules as potential surrogate markers of the disease.

4 Subjects and methods

4.1 Subjects

4.1.1 Publications I and II

The subjects referred to in publications I and II were derived from the nationwide Childhood Diabetes in Finland (DiMe) study (Tuomilehto et al. 1992), which is a prospective, population-based family survey initiated at the beginning of September 1986 to investigate the role of genetic, immunological and environmental factors in the development of type 1 diabetes. All children aged 15 years or younger with newly diagnosed type 1 diabetes, their siblings under 20 years of age and their parents were invited to participate. By the end of April 1989, 801 index cases had been diagnosed and 977 unaffected siblings had been recruited for a prospective study. The first blood sample from each sibling was obtained at or close to the diagnosis of the index case and subsequent samples were taken at intervals of 3-6 months over the first 2 years and at intervals of 12 months thereafter for up to 4 years. The siblings were observed up to the end of December 1998, unless type 1 diabetes was diagnosed before that date. Diagnosis was based on clinical symptoms and an increased random blood glucose concentration (>10mmol/l), or elevated fasting blood glucose (>6.7mmol/l), or random blood glucose >10mmol/l on two occasions in the absence of symptoms (WHO Study Group 1985). There were 755 siblings (77.3%) with at least one blood sample available. The first sample for each of the siblings was analysed for islet cell antibodies (ICA), insulin autoantibodies (IAA) and antibodies to the 65 kDa isoform of glutamic acid decarboxylase (GADA) and the protein tyrosine phosphatase-related IA-2 antigen (IA-2A).

The subjects considered in publication I included all 95 siblings whose initial sample was positive for at least one autoantibody out of the four analysed, while the control group comprised 95 sex and age-matched (± 6 months) siblings who remained antibody-negative throughout the follow-up. The mean age of the cases was 9.8 years (range 1.1-18.9) and that of the controls similarly 9.8 years (range 1.4-18.6). The levels of sICAM-1 and sL-selectin were measured in the first blood sample available, these samples having been stored at –20°C or below until analysis. Twenty-nine autoantibody-positive siblings had progressed to clinical type 1 diabetes by the end of 1997.

The subjects discussed in publication II included all 39 siblings who were positive for at least one of the four diabetes-associated autoantibodies on at least one occasion during the 4-year follow-up period and had progressed to clinical type 1 diabetes during prospective observation for a median 10.7 years (range 9.7-12.3 years) between September 1986 and the end of December 1998.Thirty-nine autoantibody-positive siblings who remained non-diabetic, were matched with them for sex, age (± 2 years in 90% of the subjects) and the initial number of autoantibodies (the same number in nine subjects, ± one antibody in 14 subjects, ± two antibodies in 11 subjects and ± three antibodies in five subjects) to serve as a control group. Autoantibodies and soluble adhesion molecules were measured in sequential follow-up samples. There were 19 boys and 20 girls in each group. The median age of the siblings at recruitment was 7.4 years for the progressors (range 1.5-16.4 years) and 7.6 years for the non-

progressors (range 1.1-16.5 years). The median duration of follow-up from the initial blood sampling to diagnosis was 4.4 years (range: 9 days-11.7 years). A subgroup of the initial one comprised 30 sibling-pairs matched for the duration of follow-up in addition to age, sex and the initial number of antibodies. The mean follow-up time for these was 3.0 years (range 0.2-5.7 years) among the progressors and 2.9 years (range 0.2-6.2 years) among the non-progressors. A total of 345 serum samples were analysed for sICAM-1 and 344 samples for sL-selectin, the number of samples per sibling varying from one to nine (median 4.6 for the progressors and 4.5 for the non-progressors).

4.1.2 Publication III

The study of beta-cell autoimmunity in schoolchildren in northern Finland was initiated at the beginning of 1994, when all 7 to 16-year-old schoolchildren (n=4280) living in five municipalities in the province of Oulu were invited to take part (Kulmala et al. 2000a). 3652 non-diabetic schoolchildren were included and were analysed for islet cell antibodies and for antibodies to glutamic acid decarboxylase, of whom 106 (2.9%) tested positive for one or both in their initial sample. These children (the cases), except for two subjects who had progressed to clinical diabetes before the re-examination, were invited to the second part of the study which included blood sampling for autoantibody determinations (ICA, IA-2A, GADA and IAA), an intravenous glucose tolerance test (IVGTT) and more recently analyses for the soluble adhesion molecules sICAM-1 and sL-selectin. In addition, 104 initially autoantibody-negative children matched with the cases for age, sex and place of residence were inivited as controls.

There were 51 boys and 53 girls in the group of autoantibody-positive children and their mean age at the time of the IVGTT was 13.7 years (range 9.4-18.8 years). Two of the antibody-positive subjects had a first-degree relative with type 1 diabetes, an affected father in one case and an affected mother in the other, while none of the control children had an affected family member. The children were observed for progression to clinical diabetes from the time of the second sampling to the end of May 1999, a mean period of 3.4 years (range 3.2-3.6 years), using as a source the Central Drug Register of the Social Insurance Institute, which records more than 99% of all new cases of type 1 diabetes (Reunanen A, personal communication). There were two initially healthy schoolchildren who progressed to manifest diabetes during the observation period: a boy whose sample was taken at the age of 10.7 years, 2.7 years before diagnosis, and a girl whose sample was taken at the age of 10.8 years, 1.4 years before diagnosis. Both of them were positive for multiple autoantibodies, had FPIR values below the 1st percentile and were significantly younger than the non-progressors: 9.1 years vs 11.6 years (p=0.05). The boy carried the DQB1*0301/*0603 genotype and the girl had the DQB1*02/*0602 genotype (Kulmala et al. 2000a).

4.1.3 Publication IV

The Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study was established to refine prediction in the general population and to develop effective strategies to prevent or delay progression to clinical disease. The strategy is based on the identification of HLA-DQB1-conferred genetic susceptibility in infants born at the university hospitals in Turku, Oulu and Tampere and follow-up for the appearance of signs of beta-cell autoimmunity (Kupila et al. 2001). The study also includes an intervention programme targeting children with permanent autoantibody positivity. The objective is to test in a randomized, double-blind, placebo-controlled trial whether daily intra-nasal administration of insulin can prevent or delay the clinical onset of type 1 diabetes.

All the 130 children included in the series in publication IV were carriers of the high-risk genotype DQB1*02/*0302 or the moderate-risk genotype DQB1*0302/x (x= other than *02, *0301 or *0602). To monitor for beta-cell autoimmunity, blood samples were obtained at 3 and 6 months and subsequently at intervals of 3-6 months during the first 2 years and intervals of 6-12 months thereafter, using islet cell antibodies (ICA) as the primary screening test. If autoantibodies were detectable at the age of 3 or 6 months the cord blood sample was also analysed for ICA, IAA, GADA and IA-2A. Samples with decreasing titres of autoantibodies for infants who also had autoantibodies in their cord sample were regarded as maternal and excluded from the analysis. The subjects comprised the 65 children who tested positive for ICA at least once before their fourth birthday (median age 18 months, range 6-36 months) by the end of May 1999 (Kimpimäki et al. 2001a) and 65 control children without diabetes-associated autoantibodies matched with the cases for sex, DQB1 genotype, geographical region and date of birth, as closely as possible, with a median difference of 7 days. The levels of soluble adhesion molecules were quantified in sequential follow-up samples of these subjects with a median duration of the total follow-up period of 24 months (range 13-37 months) for the autoantibody-positive children and 24 months (range 6-36 months) for the control children.

Twenty-one children progressed to clinical type 1 diabetes before the age of 5 years during the observation period, i.e. up to the end of December 2003, thirteen doing so before the age of 3 years and the remainder between 3 and 4.9 years.

4.2 Methods

4.2.1 Autoantibody assays (I, II, III, IV)

Autoantibodies were analysed in the Research Laboratory, Department of Paediatrics, University of Oulu. ICA levels were determined by a standard immunofluorescence method using sections of frozen human blood group 0 pancreas (Bottazzo et al. 1974). All sera with detectable ICA were titrated to end-point dilution, and the results were expressed in Juvenile Diabetes Foundation Units (JDFU). The detection limit was 2.5 JDFU, and all samples positive for ICA initially were retested for confirmation. The sensitivity of the ICA assay was 100% and the specificity 98% in the fourth round of the international workshop on the standardization of the ICA assay (Greenbaum et al. 1992).

Autoantibodies to GAD65 (GADA) were quantified in publications I-III with a radiobinding assay as described by Petersen et al. (1994) and Sabbah et al. (1996), while a slightly modified radiobinding assay was used in publication IV (Savola et al. 1998a). The results were expressed in relative units (RU). The cut-off for GADA was 6.6 RU initially and 5.36 RU in the modified assay, these limits representing the 99th percentile in more than 370 non-diabetic Finnish children and adolescents. Autoantibodies to IA-2 (IA-2A) were analysed with a radiobinding assay described by Bonifacio et al. (1995) and Savola et al. (1998a). The cut-off limit for autoantibody positivity was 0.43 RU (the 99th percentile in 374 non-diabetic children and adolescents). The disease sensitivity of the initial GADA assay was 79% and the specificity 97%, based on 140 samples derived from the 1995 Multiple Autoantibody Workshop (Verge et al. 1998)**,** whereas the sensitivity of the modified GADA assay was 82% and the specificity 98% in the Diabetes Antibody Standardization Programme (DASP) Workshop 2002. The disease sensitivity and specificity of the IA-2A assay were 62 and 100%, respectively, in the DASP 2002 Workshop.

IAA levels were quantified in publications I and II with a radiobinding assay modified from that described by Palmer et al. (1983) and expressed in nU/ml, where 1 nU/ml corresponds to a specific binding of 0.01% of the total counts. A subject was considered to be positive for IAA when specific binding exceeded 54 nU/ml (99th percentile in 105 non-diabetic children and adolescents). The disease sensitivity of our IAA assay was 26% and the specificity 97%, based on 140 samples derived from the 1995 Multiple Autoantibody Workshop (Verge et al. 1998).

In publications III and IV, IAA levels were measured with a radioligand microassay on 96-deep-well plates (Ronkainen et al. 2001), a modification of the radiobinding assay described by Williams et al.(1997), and expressed in relative units based on a standard curve run on each plate using the MultiCalc software (PerkinElmer Life Sciences Wallac, Turku, Finland). A subject was considered positive for IAA when the specific binding exceeded 1.55 RU (99th percentile in 371 non-diabetic Finnish subjects). The disease sensitivity of our microassay was 44% and the specificity 98% in the 2002 DASP Workshop.

4.2.2 Intravenous glucose tolerance test (I, III)

IVGTTs were performed after a preceding fast of 10-16 h by infusing 0.5 g glucose/kg body weight in a 20% solution intravenously over a period of 3 min ± 15 s. The FPIR to glucose was defined as the sum of the 1 and 3-min serum insulin concentrations. Blood glucose levels were quantified by the glucose oxidase method (Hjelm 1966).

For publication I, IVGTTs were performed on 49 autoantibody-positive siblings during the follow-up, with blood samples taken before the infusion (0 min) and 1, 3, 6, 10, 20, 30, 40, 50 and 60 min after its completion. Serum insulin concentrations were measured by radioimmunoassay (Herbert et al. 1965). FPIR levels < 45 mU/l, which correspond to the 3rd percentile of FPIR values measured in healthy control subjects (Srikanta et al. 1984), were considered abnormally low. This limit was defined after correction for assay differences on the basis of an insulin standardization round within the ICARUS survey.

The IVGTTs reported in publication III were performed according to the ICARUS protocol (Bingley et al. 1992). Blood samples were taken 5 and 0 minutes before the infusion and 1, 3, 5 and 10 minutes after it had been completed. Serum insulin concentrations were measured with an ELISA (Andersen et al. 1993) having a sensitivity of 0.5 mU/l and intra-assay and inter-assay coefficients of variation less than 7.5% and 9.3%, respectively. The percentile values for the FPIR in the 103 control children were: 1st percentile 29.5 mU/l, 2.5th percentile 35.0 mU/l, 5th percentile 45.0 mU/l and 10th percentile 52.1 mU/l (Kulmala et al. 2000a).

4.2.3 Soluble adhesion molecule assays (I, II, III, IV)

sICAM-1 and sL-selectin concentrations were analysed with specific ELISA kits according to the manufacturer's instructions (Bender Medsystems Diagnostics GmbH, Vienna, Austria). The assays are based on two monoclonal antibodies directed against different epitopes on the soluble adhesion molecules. Diluted serum samples (1:10 for sICAM-1 and 1:200 for sL-selectin) were added in duplicates to the microtitre wells, which were precoated with murine Mab to human ICAM-1 or L-selectin. A second horseradish peroxidase-conjugated murine Mab was then added to bind a second

epitope on the molecule captured by the first antibody, and the plates were incubated at room temperature for 1 hour in the ICAM-1 assay and for 2 hours in the L-selectin assay. After thorough washing to remove the unreactive component, 3,3,5,5-tetramethylbenzidine was added to the wells to form a coloured end product and the plates were incubated for an additional 15 minutes. A coloured product is formed in proportion to the amount of soluble ICAM-1/L-selectin present in the sample. The reaction was stopped by adding phosphoric acid and the absorbances were measured at a wavelength of 450 nm with a Multiscan MS photometer (Labsystems, Helsinki, Finland). The results were read from a standard curve (prepared from standard dilutions of sICAM-1/sL-selectin). The coefficients of intra-assay and inter-assay variation were less than 5% and less than 8% for sICAM-1 and less than 4% and less than 5%, respectively, for sL-selectin.

When sICAM-1 and sL-selectin levels were determined after aliquots of serum samples had been stored at -20ºC and thawed several times there was no significant loss of their concentrations between 0 and 5 freeze-thaw cycles. Likewise, when aliquots of a serum samples were stored at -20ºC, 2-8ºC, room temperature and +37ºC and sICAM-1 was determined after 24, 48 and 96 hours and sL-selectin after 24 hours there was no significant loss of their immunoreactivity.

4.2.4 HLA typing (I, III)

HLA-DR alleles were typed for publication I using conventional HLA serology, as described by Tuomilehto-Wolf et al. (1989). T and B lymphocytes were isolated by means of a Percoll centrifugation gradient during the first year of the DiMe study and with immunomagnetic beads (Dynabeads, Dynal, Oslo, Norway) coated with monoclonal antibodies against class I or class II antigens from September 1987 onwards. HLA antiserum to recognize all HLA-A, B, C and DR specificities was used in the test panel, according to the 1984 recommendations of the Nomenclature Committee of the WHO.

HLA-DQB1 alleles were defined in publication III by a previously described method based on a polymerase chain reaction (PCR) (Sjöroos et al. 1995). Four sequence-specific probes were used to identify the DQB1 alleles known to be associated with either susceptibility to or protection against type 1 diabetes in the Finnish population: DQB1*0302, DQB1*02, DQB1*0602 or 0603, and DQB1*0301 (Ilonen et al. 1996). All DQB1*02-positive samples were further analysed for the presence of DQA1*05 or DQA1*0201 alleles to differentiate between the DQA1*05-DQB1*02 (DR3) and DQA1*0201-DQB1*02 (DR7) haplotypes (Sjöroos et al. 1998). We used the previously described simplified classification of DQB1 genotypes into high risk: DQB1*02/*0302, moderate risk: DQB1*0302/x (x stands for *0302 or a non-defined allele), low risk: DQB1*0301/*0302, DQB1*02/*0301, DQB1*02/x, DQB1*0302/*0602 or*0603 (x stands for *02 or a non-defined allele) and decreased risk: DQB1 *x/x, DQB1*0301/x, DQB1*02/*0602 or *0603, DQB1*0301/*0602 or *0603 (x stands for a non-defined allele) (Ilonen et al. 1996).

4.2.5 Enterovirus infections (IV)

Enterovirus infections were diagnosed in publication IV from serial serum samples by antibody measurements and detection of viral RNA. IgG and IgA-class antibodies were measured using an indirect enzyme immunoassay (EIA) against purified coxsackie B4 virus, purified echovirus 11 and a synthetic enterovirus peptide antigen (Lönnrot et al. 2000). In addition, IgM and IgA-class antibodies

against a mixture of three enterovirus antigens (coxsackie B3 virus, coxsackie A16 virus and echovirus 11) were analysed using a capture EIA method (Lönnrot et al. 2000). Viral RNA was detected using a highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay that detects practically all enterovirus serotypes and was carried out as previously described (Lönnrot et al. 1999). All positive samples were confirmed as such by repeated RT-PCR and subsequent hybridization assay. A twofold or greater increase in the level of antibodies against an antigen between two consecutive samples and exceeding the cut-off level for seropositivity (15 enzyme immune units), or the presence of enterovirus RNA in serum was considered an indicator of acute infection (Lönnrot et al. 2000).

4.2.6 Infant nutrition (IV)

Data on breast-feeding and the introduction of cow's milk were recorded at every visit for as long as appropriate as described in detail previously (Kimpimäki et al. 2001a). The duration of exclusive and overall breast-feeding and the age at which cow's milk was introduced were assessed for each subject. Exclusive breast-feeding was defined as the period for which the infant did not receive any other food than breast milk, and the introduction of cow's milk formula, cow's milk or other cow's milk products was defined as the age at which feeding with these was initiated. The median duration of exclusive breast-feeding in the cases was 1.8 months [25th and 75th percentiles interquartile range (IQR) 0.5-3.9] and that in the control children 2.5 months [IQR 0.5-3.5]. The median duration of overall breast-feeding in the cases was 6.0 months [IQR 2.9-10.5] and that in control children 6.0 months [IQR 3.0-7.5]. The median age at the introduction of cow's milk was 2.0 months [IQR 0.5-5.0] in the cases and 2.5 months [IQR 0.5-5.2] in the control children.

4.2.7 Data handling and statistical analyses

4.2.7.1 Data handling

The visual inspection of the individual soluble adhesion molecule profiles in the work for publications II and IV was complemented with a summary measure approach to compare the integrated concentrations over time between the groups (Matthews et al. 1990), the total quantity of soluble adhesion molecules being expressed as the area under the curve (AUC). In addition, each sibling discussed in publication II was also analysed for his/her lowest (nadir) and highest (peak) measured concentrations of sICAM-1 and sL-selectin. The siblings were divided into antibody-positive and negative subgroups based on the antibody status in the first available sample when analysing integrated concentrations or when analysing nadir ad peak concentrations during the follow-up.

We also calculated the mean concentrations of soluble adhesion molecules over 3-month periods for each subject in publication IV in relation to initial seroconversion and signs of enterovirus infections (EVI), where initial seroconversion refers to the first appearance of autoantibodies irrespective of the antibody specificity. The levels during such periods were first compared separately among the cases and the controls, and then the concentrations in the cases were compared with those in the controls for each period. If more than one enterovirus infection was detected during the observation period among the cases, the one closest to seroconversion to autoantibody positivity (or the earlier one if two infections

were at equal distance) was chosen. Among the controls the temporally closest enterovirus infection to that in the respective case was chosen. The median age at the detection of an enterovirus infection was 12 months (range 6-24 months) in the autoantibody-positive children and similarly 12 months (range 3-24 months) in the control children.

4.2.7.2 Statistical analyses

The data are presented as means (SD) or medians and interquartile ranges [IQR i.e. the 25^{th} and 75^{th} percentiles]. Normally distributed continuous data were analysed with the t-test or ANOVA. An analysis of covariance was used, after logarithmic transformation in the case of skewed distributions, to adjust for the effect of age on the circulating concentrations of soluble adhesion molecules. In the case of skewed distributions the Mann-Whitney U-test or the Kruskall-Wallis one-way analysis of variance was used for group comparisons. Correlations were tested either with linear regression analysis (r) or with Spearman's non-parametric correlation analysis (r_s). The Bonferroni adjustment for multiple comparisons was used when appropriate. A two-tailed p-value of 0.05 or less was considered statistically significant.
All the statistical analyses were performed using the SPSS statistical software package for Windows (SPSS Inc., Chicago, Illinois, USA).

Table 3. *Subjects and study design in the four publications.*

Publication	Design	Subjects	N	Variables
I	A cross-sectional case-control study within a prospective cohort study	Autoantibody-positive siblings of children with recent-onset type 1 diabetes (cases)	95	HLA-DR phenotype, autoantibodies, FPIR, progression to type 1 diabetes
		Autoantibody-negative siblings of children with recent-onset type 1 diabetes (controls)	95	
II	A longitudinal case-control study within a prospective cohort study	Autoantibody-positive siblings of children with recent-onset type 1 diabetes (cases)	39	Progression to type 1 diabetes
		Autoantibody-positive siblings of children with recent-onset type 1 diabetes (controls)	39	
III	A cross-sectional population-based case-control study	Autoantibody-positive school-children (cases)	104	HLA-DQB1 genotype, autoantibodies, FPIR, progression to type 1 diabetes
		Autoantibody-negative schoolchildren (controls)	104	
IV	A nested longitudinal case-control study within a population-based prospective birth cohort study	Genetically susceptible children who converted to autoantibody positivity during a follow-up from birth (cases)	65	Seroconversion, enterovirus infections, early infant diet, progression to type 1 diabetes
		Genetically susceptible children who remained autoantibody-negative during a follow-up from birth (controls)	65	

5 Results

5.1 Soluble adhesion molecules in relation to sex and age (I, III)

A negative correlation was observed between age and the circulating concentrations of both sICAM-1 ($r=-0.31$, $p<0.001$) and sL-selectin ($r=-0.27$, $p<0.001$) among the control siblings, and the sICAM-1 and sL-selectin concentrations were interrelated ($r=0.46$, $p<0.001$). The sL-selectin level correlated inversely with age ($r_s=-0.36$, $p<0.001$), whereas the sICAM-1 concentrations were not significantly related to age ($r_s=-0.07$, $p=0.49$) among the control schoolchildren. The circulating levels of sICAM-1 and sL-selectin did not correlate among the control schoolchildren ($r_s=-0.02$, $p=0.87$).

The concentrations of soluble adhesion molecules were similar in the boys and girls among both the control siblings and the control schoolchildren (data not shown).

5.2 Relation of soluble adhesion molecules to HLA class II-conferred susceptibility to type 1 diabetes (I, III)

No relationship was observed between sICAM-1 and sL-selectin levels and the degree of HLA identity with the proband (data not shown) or various HLA-DR phenotypes (Fig.1) among the antibody-negative siblings of the children with newly diagnosed type 1 diabetes. There was a trend for lower sICAM-1 and sL-selectin concentrations in siblings with the HLA-DR3/non-DR4 phenotype than in those who were heterozygous for HLA-DR3/4 or carried the DR4/non-DR3 or non-DR3/non-DR4 combinations, but the differences remained non-significant for both adhesion molecules. Siblings with the DR2 allele had soluble adhesion molecule concentrations similar to those observed in the DR2-negative siblings (data not shown).

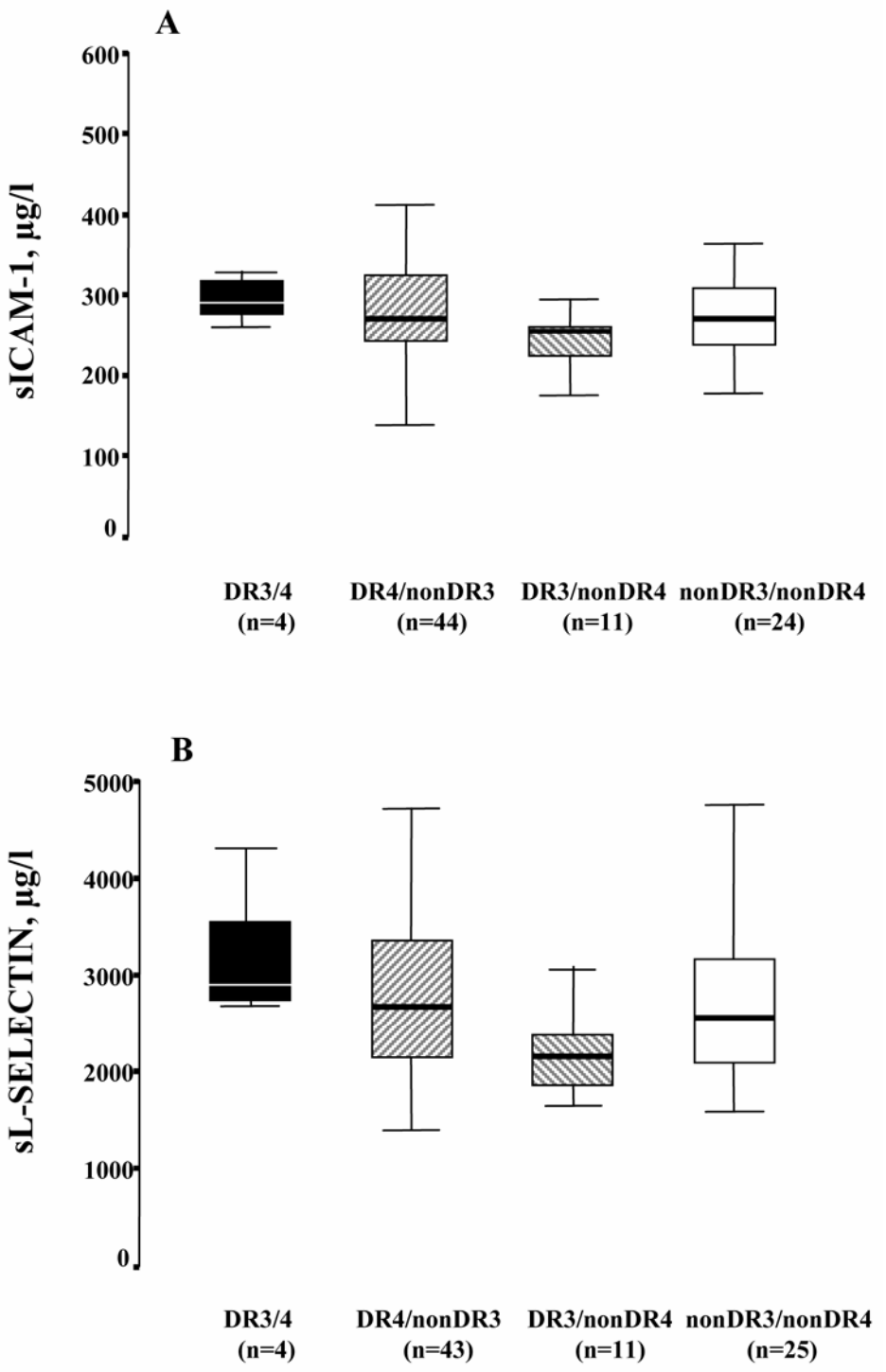

Figure 1. Circulating levels of sICAM-1 (A) and sL-selectin (B) in autoantibody-negative siblings of children with recent-onset type 1 diabetes, by HLA-DR phenotype status. Each box-plot represents the median and the 25th and 75thpercentiles. The error bars represent the smallest and largest values that are not outliers.

There was no association between sICAM-1 levels and the DQB1 risk genotypes among the antibody-negative schoolchildren, but the subjects carrying genotypes conferring a high or moderate risk of type 1 diabetes had lower levels of sL-selectin than those with genotypes associated with a low or decreased risk (p=0.04) (Fig.2). We did not find any significant differences in the levels of soluble adhesion molecules between the autoantibody-negative subjects with or without the alleles indicating

susceptibility to type 1 diabetes, i.e. DQB1*0302 (n=24/79) or DQB1*02 (n=28/75), or between those with or without the alleles indicating protection against type 1 diabetes, i.e. DQB1*0602, *0603 (n=40/63) or DQB1*0301 (n=10/93). Subjects with and without the DQA1*05-DQB1*02 haplotype (n=22/81) had the similar soluble adhesion molecule levels (data not shown).

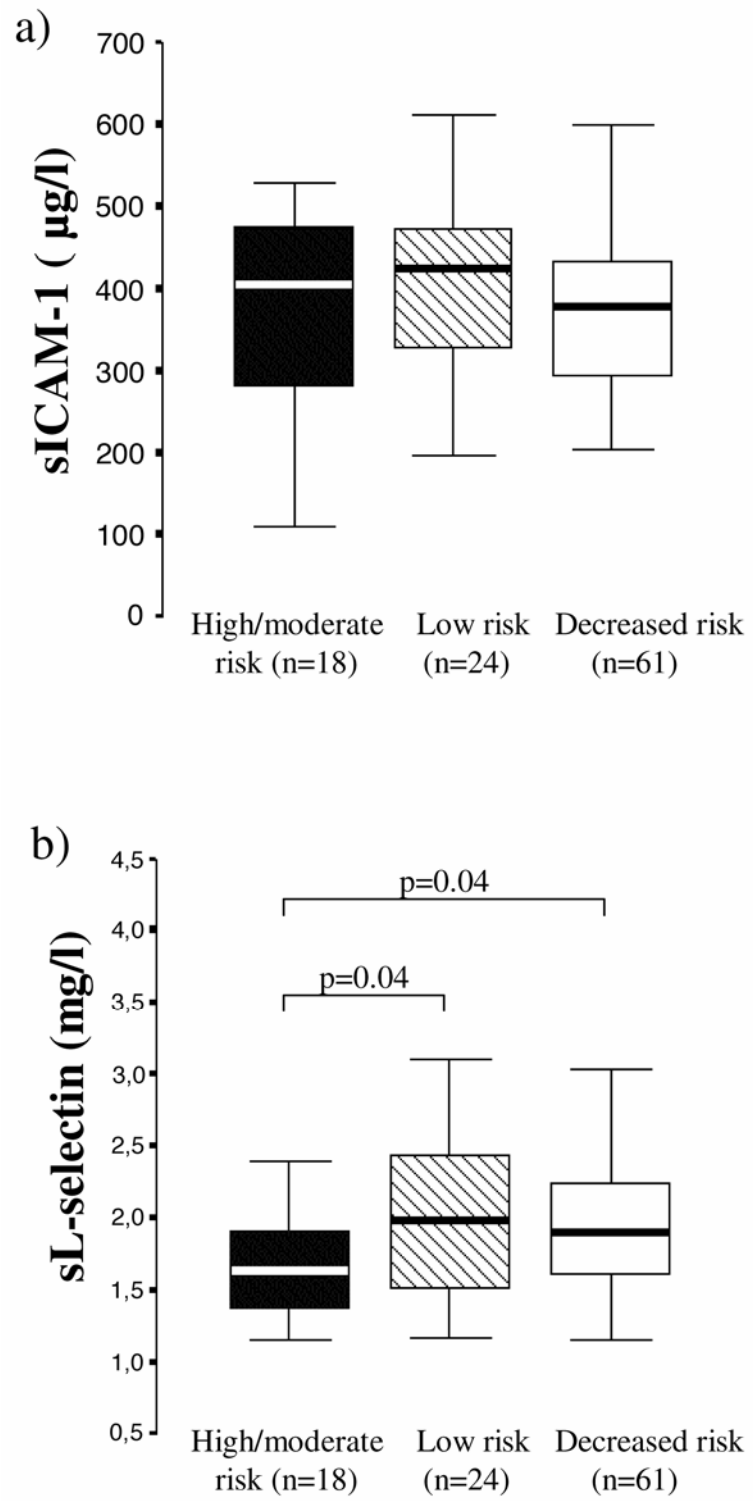

Figure 2. Circulating levels of sICAM-1 (a) and sL-selectin (b) in autoantibody-negative schoolchildren, by HLA-DQB1 genotype status. Each box-plot represents the median and the 25th and 75thpercentiles. The error bars represent the smallest and largest values that are not outliers.

5.3 Relation of soluble adhesion molecules to diabetes-associated autoantibodies (I, II, III)

The autoantibody-positive siblings had a mean serum sICAM-1 concentration of 277 (74;SD) µg/l, which was similar to that seen in the autoantibody-negative siblings [279 (78) µg/l]. There was similarly no significant difference in serum sL-selectin levels between the two groups [2750 (940) µg/l *vs* 2720 (830) µg/l].

The autoantibody-positive siblings with high ICA levels (≥ 20 JDFU) (n= 37) had higher sICAM-1 concentrations than those with low ICA levels, [314 (82) µg/l *vs* 254 (59) µg/l; p<0.001], even after adjustment for age (p=0.001), while the sL-selectin concentrations were of the same magnitude in these two groups [2910 (1010) µg/l *vs* 2650 (880) µg/l; p=0.192]. Siblings testing positive for IA-2A (n=40) had higher sL-selectin and sICAM-1 concentrations than those without detectable IA-2A [sL-selectin 3010 (1020) µg/l *vs* 2570 (830) µg/l; p=0.022, and sICAM-1 296 (77) µg/l *vs* 263 (70) µg/l; p=0.034].

There were no significant differences in circulating concentrations of soluble adhesion molecules between the siblings testing positive for GADA (n=57) and those who were negative for GADA [sICAM-1 288 (79) µg/l *vs* 259 (64) µg/l; p=0.062, and sL-selectin 2750 (990) µg/l *vs* 2750 (860) µg/l; p=0.996] or between the IAA-positive (n=28) and IAA-negative siblings [sICAM-1 260 (68) µg/l *vs* 284 (76) µg/l; p=0.161, and sL-selectin 2660 (930) µg/l *vs* 2790 (940) µg/l; p=0.533].

Siblings testing positive for two or more antibodies (n=48) had significantly higher sICAM-1 levels than those with only one detectable autoantibody specificity or none at all [297 (83) µg/l *vs* 272 (72) µg/l; p<0.05], without any significant age difference between the groups (p=0.124). A more detailed analysis showed increased sICAM-1 concentrations (p=0.006), particularly in those with three or four antibodies, relative to those testing positive for only one antibody, without any significant difference in age between the groups (p=0.056) (Fig.3A). Siblings with three or four autoantibodies had also higher sL-selectin concentrations than those testing positive for two antibodies (p=0.005; Fig.3B).

No difference was observed in the integrated concentrations of soluble adhesion molecules in relation to the initial or maximal number of autoantibodies detected during the follow-up, or when analysed according to the autoantibody status among the siblings of the children affected by type 1 diabetes (data not shown).

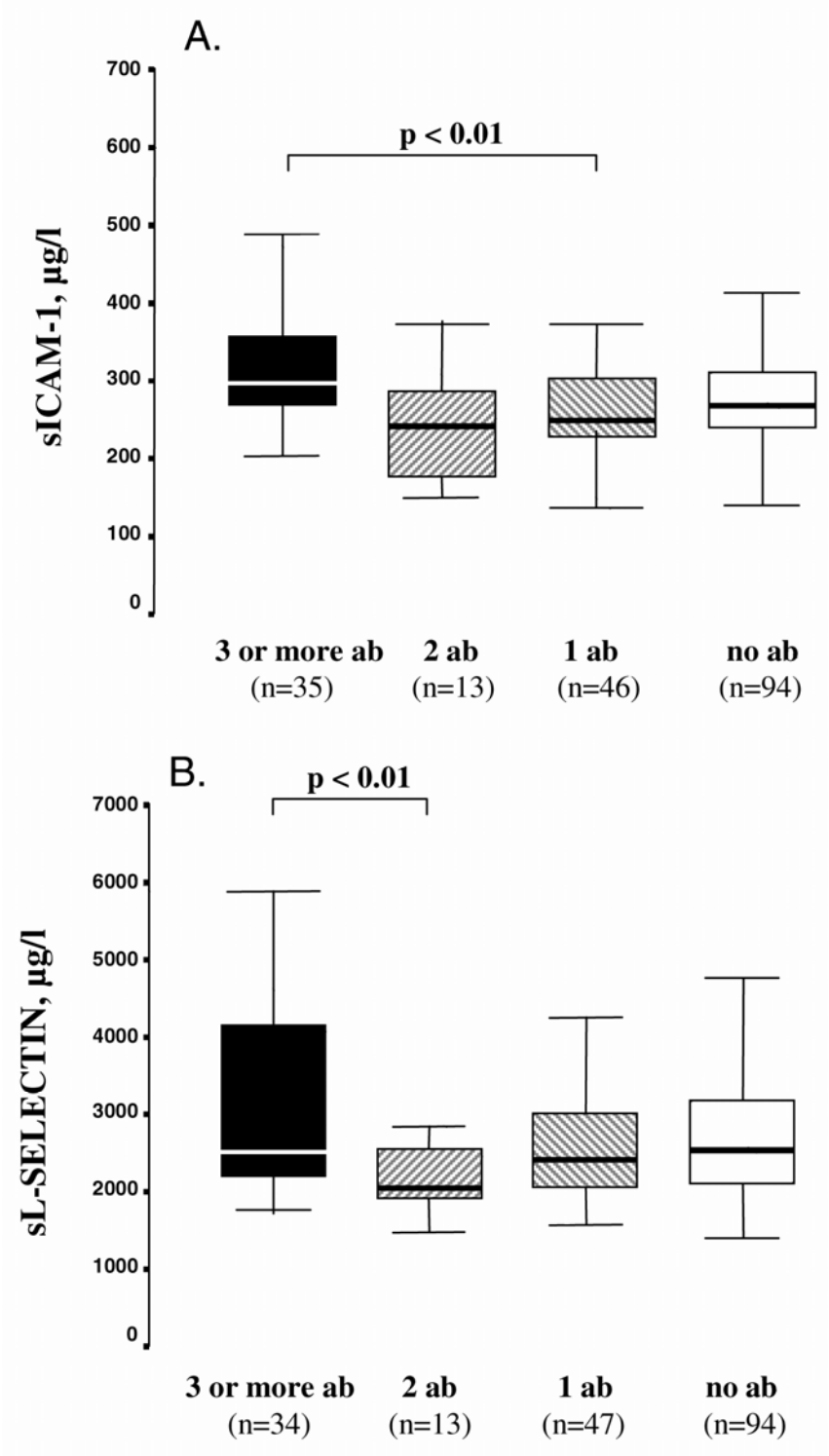

Figure 3. Circulating levels of sICAM-1 (A) and sL-selectin (B), by the number of autoantibodies, in siblings of children with recent-onset type 1 diabetes. Each box-plot represents the median and the 25th and 75th percentiles. The error bars represent the smallest and largest values that are not outliers. Ab indicates antibody.

The levels of soluble adhesion molecules were comparable between the autoantibody-positive and negative initially healthy schoolchildren, and their circulating concentrations were similar in the subjects who were positive for ICA, IA-2A, GADA or IAA and those who tested negative for these autoantibody specificities (*Table 4* and *5*). No significant differences were seen between the subjects who were

positive for any of the biochemically characterized antigens (IA-2A, GADA or IAA) and those who were not, irrespective of ICA status or titre (data not shown). On the other hand, the autoantibody-positive children with an ICA level of 40 JDFU or more tended to have higher levels of sICAM-1 than those with lower ICA titres, although the difference did not reach statistical significance (*Table 4*).

Table 4. *Circulating levels of sICAM-1 (μg/l) in 208 non-diabetic schoolchildren, by diabetes-associated autoantibody status. The data are expressed as medians and interquartile range (IQR, i.e. 25th and 75th percentiles) Ab indicates antibody.*

	Ab-positive	**Ab-negative**	**p-value**
≥ 1 Ab (n=104/104)	370 (310;440)	390 (300;460)	0.84
ICA (n=98/110)	370 (320;450)	390 (300;450)	0.72
ICA ≥ 10 JDFU versus < 10 JDFU (n=84/124)	370 (320;450)	380 (300;450)	0.39
ICA ≥ 20 JDFU versus < 20 JDFU (n=31/177)	370 (320;440)	380 (300;450)	0.89
ICA ≥ 40 JDFU versus < 40 JDFU (n=15/193)	430 (340;460)	380 (300;440)	0.15
IA-2A (n=10/198)	430 (290;470)	370 (310;450)	0.46
GADA (n=17/191)	390 (290;440)	380 (310;450)	0.81
IAA (n=7/201)	350 (340;520)	380 (310;450)	0.49

Table 5. *Circulating levels of sL-selectin (mg/l) in 208 non-diabetic schoolchildren, by diabetes-associated autoantibody status. The data are expressed as medians and (interquartile range (IQR, i.e. 25th and 75th percentiles). Ab indicates antibody.*

	Ab-positive	**Ab-negative**	**p-value**
≥ 1 Ab (n=104/104)	1.79 (1.44;2.16)	1.86 (1.53;2.26)	0.32
ICA (n=98/110)	1.78 (1.44;2.17)	1.86 (1.55;2.24)	0.34
ICA ≥ 10 JDFU versus < 10 JDFU (n=84/124)	1.79 (1.44;2.20)	1.86 (1.53;2.16)	0.65
ICA ≥ 20 JDFU versus < 20 JDFU (n=31/177)	1.80 (1.45;2.17)	1.84 (1.48;2.22)	0.93
ICA ≥ 40 JDFU versus < 40 JDFU (n=15/193)	1.77 (1.44;1.96)	1.86 (1.48;2.22)	0.50
IA-2A (n=10/198)	1.90 (1.70;2.10)	1.83 (1.47;2.19)	0.71
GADA (n=17/191)	1.90 (1.64;2.11)	1.84 (1.47;2.24)	0.92
IAA (n=7/201)	1.46 (1.16;2.04)	1.86 (1.49;2.20)	0.17

There was a positive association between sICAM-1 concentrations and IA-2A titres in the IA-2A-positive children (r_s=0.62, p=0.05), but no relationship was seen between sL-selectin concentrations and IA-2A or ICA titres, nor between the levels of soluble adhesion molecules and GADA or IAA titres (data not shown). There were no differences in the levels of sICAM-1 or sL-selectin in relation to the number of detectable autoantibodies (Fig.4).

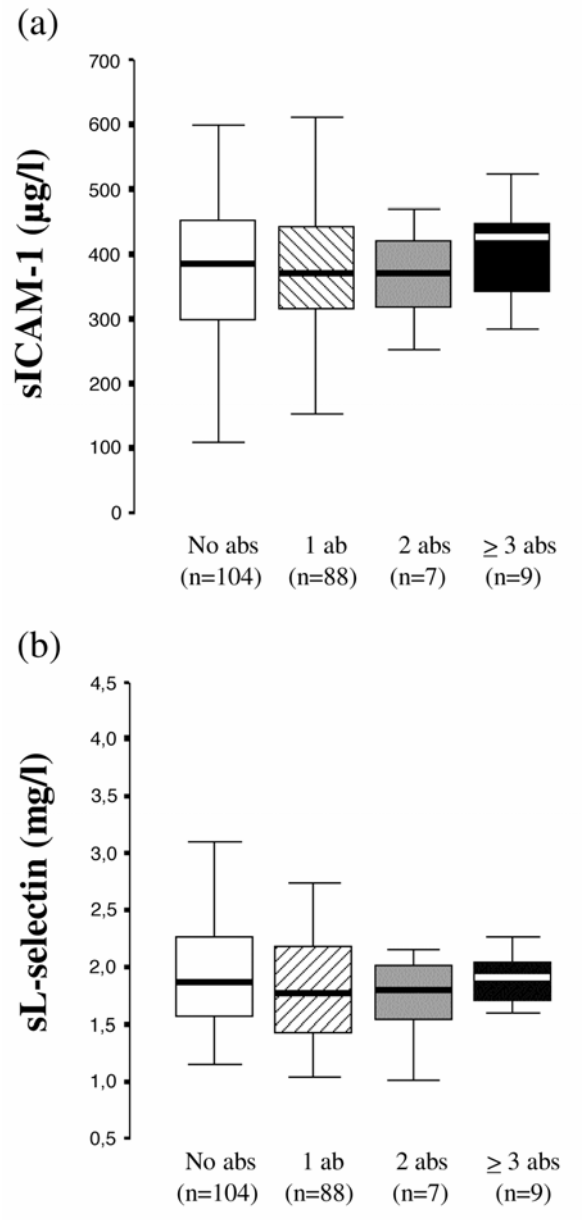

Figure 4. Circulating levels of sICAM-1 (a) and sL-selectin (b), by the number of autoantibodies, in 208 schoolchildren. Each box-plot represents the median and the 25th and 75thper centiles. The error bars represent the smallest and largest values that are not outliers. Ab indicates antibody.

5.4 Relation of soluble adhesion molecules to insulin secretory capacity (I, III)

There were no significant differences in the mean circulating concentrations of sICAM-1 and sL-selectin in the first sample taken at the beginning of the follow-up between the siblings with a reduced FPIR in their first IVGTT and those with a normal FPIR [sICAM-1 316 (65) µg/l (n=13) *vs* 282 (91) µg/l (n=35); p=0.223, and sL-selectin 2760 (780) µg/l (n=12) *vs* 2770 (1040) µg/l (n=36); p=0.980].

No relationship was observed between median sICAM-1 levels and FPIR values in the autoantibody-positive schoolchildren (data not shown), but those who had FPIR values under the 5th percentile (n=11) had significantly higher median sL-selectin levels than those with normal FPIR values: 2.27 mg/l [IQR 1.69-2.71] mg/l *vs* 1.78 mg/l [1.44-2.14] mg/l; (p=0.026), and the same held true for those with FPIR values under the 10th percentile (n=17): 2.16 mg/l [1.73-2.46] mg/l *vs* 1.75 mg/l [1.43-2.08] mg/l; (p=0.009) (Fig.5).

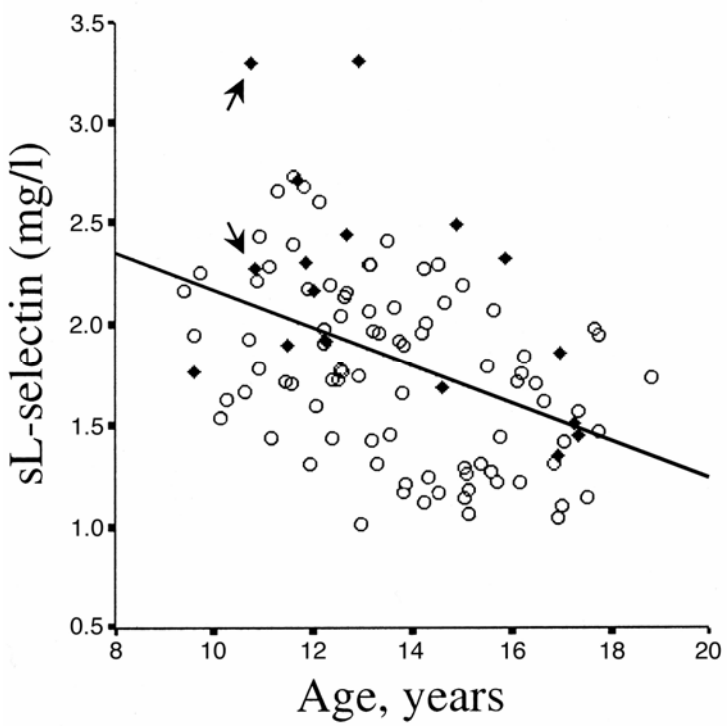

Figure 5. Circulating levels of sL-selectin in autoantibody-positive schoolchildren with a decreased FPIR (filled squares), i.e. <52.1 mU/l, the 10th percentile in the controls, compared with those with a normal FPIR (open circles). The regression line denotes the correlation between sL-selectin and age (sL-selectin=3.05-0.086 x age, r=0.40, p<0.001). The subjects with a decreased FPIR had significantly higher sL-selectin concentrations than those with a normal FPIR (p=0.009). The arrows mark the two children who subsequently progressed to overt type 1 diabetes.

5.5 Follow-up of soluble adhesion molecules during the preclinical phase of type 1 diabetes (I, II, III, IV)

5.5.1 Siblings of affected children (I, II)

Those siblings who progressed to clinical type 1 diabetes during the observation had higher sICAM-1 levels than the non-progressors in the first sample taken at the beginning of the follow-up, the mean (SD) levels being 313 (66) µg/l *vs* 260 (72) µg/l; p<0.001. This difference remained significant after adjustment for age (p=0.03). No significant difference in sL-selectin concentrations was seen between the progressors and non-progressors [2880 (880) µg/l *vs* 2700 (960) µg/l; p=0.40].

No conspicuous general pattern in sICAM-1 or sL-selectin concentrations was seen during the follow-up among the progressors or non-progressors (Fig.6). Considerable fluctuations in the concentrations were observed in both groups. The peak levels of both adhesion molecules and the time points at which the levels peaked during the follow-up varied individually among both groups.

No significant difference in total integrated concentrations of sICAM-1 existed between the progressors and non-progressors, but AUC_{ICAM-1} was significantly higher in the progressors 6-48 months before the diagnosis of type 1 diabetes (p=0.035) (*Table 6*). The difference in the levels of sICAM-1 between the groups was most conspicuous 18-24 months before diagnosis (p=0.015) (Fig.6). The integrated concentrations of sL-selectin over the total observation period were comparable in both groups (data not shown).

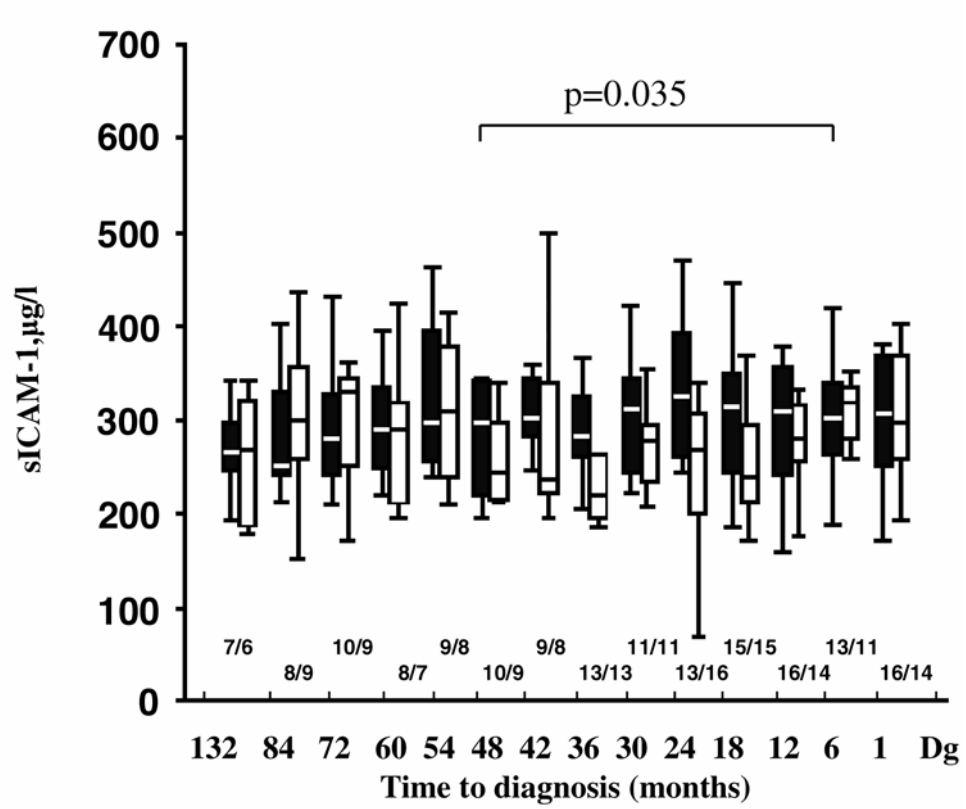

Figure 6. Circulating concentrations of soluble ICAM-1 in 39 autoantibody-positive progressors vs non-progressors with preclinical type 1 diabetes. Each box-plot represents the median and the 25th and 75th percentiles. The bars represent the minimum and maximum values except for the outliers. The figures above the x-axis refer to the number of siblings available in the two groups for each time interval. Solid boxes represent the progressors and open boxes the non-progressors.

Nadir concentrations of sICAM-1 during the follow-up were significantly higher in the progressors among the ICA-positive siblings and tended to be higher in the progressors among the siblings positive for any of the other autoantibody specificities. Similarly, the progressors among the siblings with two or more autoantibodies initially or during the follow-up had higher nadir concentrations of sICAM-1 than the non-progressors (*Table 6*). We did not find any associations between sL-selectin nadir or peak concentrations and the various autoantibody specificities among the siblings. (data not shown).

Table 6. *Circulating concentrations of sICAM-1 in siblings (n=4-39) of affected children who progressed to clinical type 1 diabetes during observation for a median period of 10 year and in unaffected siblings (n=11-39).Ab indicates antibody.*

	Progressors	**Non-progressors**	**p-value**
Total AUC (μg months/l)	10464; 7214, 13177 (n=30)	9110; 5807, 12370 (n=30)	0.287
AUC 48-6 months before diagnosis (μg months/l)	7634; 4420, 9316 (n=24)	4527; 2890, 7435 (n=24)	0.035
Nadir concentration (μg/l)	256; 212, 303 (n=39)	213; 185, 274 (n=39)	0.058
- ICA-positive	262; 218, 303 (n=35)	212; 180,268 (n=28)	0.009
- ICA-negative	211; 197, 329 (n=4)	258; 199, 358 (n=11)	0.794
- GADA-positive	247; 210, 300 (n=34)	210; 189, 265 (n=21)	0.066
- GADA-negative	263; 227, 338 (n=5)	239; 183, 287 (n=18)	0.205
- IAA-positive	250; 211, 304 (n=30)	212; 178, 262 (n=26)	0.071
- IAA-negative	256; 214, 312 (n=9)	239; 194, 288 (n=13)	0.404
- IA-2A-positive	243; 210, 299 (n=33)	210; 181, 257 (n=13)	0.059
- IA-2A-negative	283; 216, 329 (n=6)	239; 191, 284 (n=26)	0.148
- ≥ 2 abs in the 1. sample	262; 210, 299 (n=29)	207; 180, 252 (n=12)	0.039
- ≥ 2 abs during the follow-up	249; 212, 302 (n=36)	207; 177, 239 (n=20)	0.003
Peak concentration (μg/l)	355; 280, 432 (n=39)	347; 290, 403 (n=39)	0.374
- ICA-positive	355; 319, 434 (n=35)	344; 298, 395 (n=28)	0.162
- ICA-negative	303; 206, 366 (n=4)	361; 280, 406 (n=11)	0.267
- GADA-positive	348; 277, 434 (n=34)	345; 298, 404 (n=21)	0.591
- GADA-negative	361; 299, 394 (n=5)	355; 251, 380 (n=18)	0.628
- IAA-positive	360; 336, 434 (n=30)	350; 282, 405 (n=26)	0.158
- IAA-negative	280; 249, 367 (n=9)	343; 293, 371 (n=13)	0.217
- IA-2A-positive	346; 265, 428 (n=33)	329; 307, 409 (n=13)	0.583
- IA-2A-negative	367; 341, 434 (n=6)	350; 285, 371 (n=26)	0.201
- ≥ 2 abs in the 1. sample	346; 261, 421 (n=29)	346; 316, 405 (n=12)	1.000
- ≥ 2 abs during the follow-up	353; 272, 433 (n=36)	337; 298, 391 (n=20)	0.194

5.5.2 Schoolchildren (III)

The circulating concentrations of sL-selectin were higher in the progressors than in the non-progressors among the schoolchildren: 2.78 mg/l *vs* 1.79 mg/l ($p<0.05$), but this difference disappeared after adjustment for age (analysis of covariance after logarithmic transformation of the sL-selectin levels). The progressors among the subjects with three or more autoantibodies (n=7) had significantly higher sL-selectin concentrations than the non-progressors: 2.78 mg/l *vs* 1.77 mg/l ($p=0.04$), without any significant age difference.

5.5.3 Young children with HLA-DQ-conferred susceptibility to type 1 diabetes (IV)

5.5.3.1 Soluble adhesion molecules in relation to autoantibodies

The young genetically susceptible children had significantly lower concentrations of sICAM-1 and sL-selectin in their cord blood than in later samples, the medians [25^{th} and 75^{th} percentiles IQR] being 155 [134,182] μg/l *vs* 314 [269,368] μg/l ($p<0.001$) for sICAM-1 and 1.14 [0.93,1.31] mg/l *vs* 2.28 [2.00,2.65] mg/l ($p<0.001$) for sL-selectin, but the concentrations were of the same magnitude in the cases and controls. The total integrated concentrations of sICAM-1 correlated with those of sL-selectin ($r_s=0.37$, $p<0.001$).

concentrations similar to those observed in the children with less than two autoantibodies. The children (n=32) with three or more autoantibodies detected during the follow-up likewise had concentrations similar to those seen in the children with a maximum of two autoantibodies (data not shown). The children (n=38) with multiple autoantibodies in the first ICA-positive sample had integrated concentrations of soluble adhesion molecules similar to those seen in the other autoantibody-positive children (data not shown).

The children who seroconverted to positivity for ICA during the follow-up had higher integrated concentrations of sICAM-1 between 6 and 12 months of age than those who remained negative for ICA (*Table 8*), and those who seroconverted to positivity for IA-2A during the follow-up had higher integrated concentrations of sL-selectin during the second half of the first year of life than those who remained autoantibody-negative (*Table 9*). We did not find any differences between the ICA or IA-2A-positive converting children and those remaining seronegative with respective to the duration of exclusive or total breast-feeding or the age at introduction of cow's milk. No associations were seen between the concentrations of soluble adhesion molecules and IAA or GADA positivity.

Table 7. *Circulating concentrations of sICAM-1 and sL-selectin during the first 2 years of life in young children testing positive for at least one autoantibody before their fourth birthday vs control children. The values are medians; 25^{th}, 75^{th} percentiles.*

AUC (mg days/l)	Autoantibody-positive	Autoantibody-negative	p-value
Total AUC_{ICAM-1}	220; 196,259(n=65)	221; 192,247(n=65)	1.00
Total $AUC_{L-selectin}$	1600; 1410,1810(n=65)	1600; 1380,1880(n=65)	1.00
$AUC(6\text{-}12\ mo)_{ICAM-1}$	54; 48,65(n=56)	50; 45,60(n=56)	0.23
$AUC(6\text{-}12\ mo)_{L-selectin}$	439; 372,481(n=56)	401; 341,464(n=56)	0.25

Table 8. *Circulating concentrations of sICAM-1 and sL-selectin in young children testing positive for ICA during the follow-up vs control children remaining negative for ICA. The values are medians; 25^{th}, 75^{th} percentiles.*

AUC (mg days/l)	ICA-positive	ICA-negative	p-value
Total AUC_{ICAM-1}	228; 203,263(n=58)	218; 182,243(n=72)	0.24
Total $AUC_{L-selectin}$	1600; 1420,1800(n=58)	1600; 1380,1850(n=72)	1.00
$AUC(6\text{-}12\ mo)_{ICAM-1}$	55; 48,66(n=48)	50; 44,60(n=64)	0.07
$AUC(6\text{-}12\ mo)_{L-selectin}$	441; 374,481(n=48)	401; 341,464(n=64)	0.26

Table 9. *Circulating concentrations of sICAM-1 and sL-selectin in young children testing positive for IA-2A during the follow-up vs control children remaining negative for IA-2A. The values are medians; 25th, 75th percentiles.*

AUC (mg days/l)	IA-2A-positive	IA-2A-negative	p-value
Total AUC_{ICAM-1}	228; 195,268(n=20)	220; 195,247(n=110)	1.00
Total $AUC_{L-selectin}$	1740; 1530,1880(n=20)	1560; 1390,1820(n=110)	0.23
$AUC(6\text{-}12\ mo)_{ICAM-1}$	50; 44,65(n=16)	53; 46,63(n=96)	1.00
$AUC(6\text{-}12\ mo)_{L-selectin}$	466; 425,481(n=16)	406; 348,469(n=96)	0.07

5.5.3.2 Soluble adhesion molecules in relation to enterovirus infection

The median levels of sL-selectin were higher in the 3-month period after which an enterovirus infection was verified than in the previous and subsequent 3-month periods in the autoantibody-positive children (p=0.002), but not in the control children (p=0.071). The children who seroconverted to autoantibody positivity had higher sL-selectin concentrations in the time interval during which an enterovirus infection was diagnosed than did the autoantibody-negative children during the corresponding time interval [2.53 (2.22,2.68) mg/l *vs* 2.28 (1.94,2.47) mg/l; *p*=0.006]. The median concentrations of sL-selectin were also higher during this period in the children who seroconverted to positivity for IA-2A during the follow-up than in those who remained autoantibody-negative: 2.55 [2.46,2.71] mg/l (n=16) *vs* 2.31 [1.99,2.54] mg/l (n=39) (*p*=0.004). The median age at which an enterovirus infection was detected did not differ between the groups: median 12 months (range 9-24 months) for the IA-2A-positive children and median 12 months (range 6-24 months) for the IA-2A-negative control children.

The differences were no longer significant after exclusion of those children who seroconverted to autoantibody positivity during the interval with a concomitant enterovirus infection. A multiple regression analysis with the circulating concentrations of sL-selectin as the dependent variable and seroconversion to autoantibody positivity and enterovirus infections as independent variables showed that sL-selectin levels in the peripheral circulation were associated with seroconversion to autoantibody positivity (β=0.29; *p*=0.017) but not with enterovirus infections (β=0.09; *p*=0.47).

5.5.3.3 Soluble adhesion molecules in relation to diet in early infancy

We did not find any correlation between the integrated concentrations of ICAM-1 from 6 to 12 months and the duration of exclusive or total breast-feeding or age at introduction of cow's milk among the children who eroconverted to ICA positivity during the follow-up. Nor did we find any correlation between the integrated concentrations of sL-selectin from 6 to 12 months and the duration of exclusive or total breast-feeding or age at introduction of cow's milk among the children who seroconverted to IA-2A positivity during the follow-up (data not shown).

Total integrated sL-selectin concentrations among the progressors presenting with clinical diabetes by the age of 5 years tended to be inversely related to the age at the introduction of cow's milk (r_s=-0.40, p=0.071, n=21) and also to the duration of exclusive breast-feeding (r_s=-0.31, p=0.17, n=21), although the correlations remained non-significant. Among the progressors with clinical type 1 diabetes diagnosed after the age of 3 years, the total integrated sL-selectin concentrations were inversely related to the age at which cow's milk was introduced (r_s=-0.74, p=0.037, n=8), and there was also a tendency for an inverse relationship between total integrated concentrations of sICAM-1 and the duration of overall breast-feeding (r_s=-0.66, p=0.076, n=8). Inverse relationships were observed between integrated concentrations of sICAM-1 during the period 6-12 months and age at the introduction of cow's milk (r_s=-0.75, p=0.052, n=7), the duration of exclusive breast-feeding (r_s=-0.75, p=0.052, n=7) and that of overall breast-feeding (r_s=-0.86, p=0.012, n=7), and this was also true of the integrated concentrations of sL-selectin over the period from 6 to 12 months and age at the introduction of cow's milk (r_s=-0.75, p=0.052, n=7), and the duration of exclusive breast-feeding (r_s=-0.75, p=0.052, n=7), while the correlation with overall breast-feeding remained non-significant (r_s=-0.59, p=0.16, n=7).

6 Discussion

6.1 Soluble adhesion molecules in relation to HLA risk markers

The genetic determinants of soluble adhesion molecule levels are largely unknown. A trend was observed here for lower sICAM-1 and sL-selectin concentrations in antibody-negative siblings with the HLA-DR3/non-DR4 phenotype than in those who were heterozygous for DR3/4 or carried the DR4/non-DR3 or non-DR3/non-DR4 combinations, but the difference remained non-significant for both. DR3 patients have been observed to present with a milder disease at diagnosis than those with DR3/4 or DR4, having ketonuria or ketoacidotic symptoms less often and showing a subsequent partial remission more often (Ludvigsson et al. 1986).

In line with the present observations, Lampeter et al. (1992) did not observe any significant differences in the circulating concentrations of sICAM-1 or sL-selectin between healthy blood donors (n=100) with or without HLA-DR3 and/or DR4 risk alleles. On the other hand, we observed increased levels of sL-selectin in autoantibody-negative schoolchildren representing the general population who had the HLA-DQB1 genotypes associated with a low or decreased risk of type 1 diabetes, although we did not find any association between soluble adhesion molecules and specific HLA-DQB1 alleles conferring either protection against (DQB1*0602, *0301) or susceptibility to type 1 diabetes (DQB1*0302, *02), suggesting that the increased sL-selectin concentrations are not associated with any specific HLA-DQB1 allele. In contrast, Krętowski et al. (2000a) reported a positive relationship between high levels of sL-selectin and the presence of HLA alleles conferring a high risk of clinical type 1 diabetes, i.e. DR3/4 or DQB1*0201/*0302 (n=3), among healthy adult controls of Polish origin. Intermediate high levels were seen in subjects with either the DR4-DQB1*0302 or the DR3-DQB1*0201 haplotype without the DQB1*0602 allele (n=21), and intermediate low levels in subjects with either the DR4-DQB1*0302 or DR3-DQB1*0201 haplotype in combination with the DQB1*0602 allele (n=11). The lowest concentrations were observed in those with a genotype comprising none of the high-risk alleles (n=23).

The conflicting results are difficult to explain, but the number of subjects investigated so far has been low, especially the number of individuals carrying high-risk genotypes, which increases the likelihood of chance findings. Polymorphism in the L-selectin gene has been implicated as being able to modulate circulating sL-selectin levels. The presence of the T668C mutation in the L-selectin gene located on the long arm of chromosome 1 was associated with lower levels of circulating sL-selectin in a subgoup of unaffected relatives, but also in healthy controls, relative to the concentrations observed in subjects without this mutation. Since the T668C mutation was associated with HLA-DQ-conferred protection against diabetes in unaffected relatives but not in patients with diabetes or in unrelated control subjects, it was thought to play a protective role in the development of type 1 diabetes (Krętowski et al. 2000a). The group headed by Krętowski and Kinalska (2000b) later confirmed in a larger series that unaffected first-degree relatives have a higher frequency of the L-selectin gene T668C mutation than do the probands themselves or healthy controls, as 45 unaffected first-degree relatives out of the 72 analysed

(63%) had the T668C mutation, while the corresponding proportion among relatives with type 1 diabetes was 32% (29/91) and that among the controls 38% (31/81). Further analysis showed that the highest frequency of the T668C mutation was seen in first-degree relatives with the protective HLA-DQB1*0602 allele (17/21; 81%) and the non-DR3/non-DR4 phenotype (4/6), while the frequency of this mutation was similar among the first-degree relatives with the high-risk DR4-DQB1*0302 haplotype and among controls. Based on these results, it was suggested that the T668C mutation could have an influence on soluble L-selectin levels and potentially provide protection from progression to type 1 diabetes.

6.2 Soluble adhesion molecules in relation to autoantibodies

The question of a relationship between the levels of soluble adhesion molecules and various autoantibody specificities has remained controversial in the family studies conducted so far. Myśliwiec et al. (1999) reported positive correlations between sICAM-1 concentrations and ICA titres (r_s=0.47), GADA titres (r_s=0.45) and the number of autoantibodies (r_s=0.56) in 26 first-degree relatives (mean age 21.0 ± 8.1 years) with ICA $\geq$ 20 JDFU, whereas Lampeter et al. (1992) did not find any clear association between the circulating concentrations of soluble adhesion molecules and ICA positivity in 33 first-degree relatives (six positive for ICA) of patients with type 1 diabetes, nor did Krętowski et al. (2000a) observe any difference in the levels of sL-selectin between subjects without autoantibodies and subjects with one or more autoantibodies or with two or more autoantibodies in a series of 90 first-degree relatives, of whom 50 tested positive for at least one autoantibody (ICA, GADA, IAA and/or IA-2A).

We found no differences in the concentrations of soluble adhesion molecules between the autoantibody-positive and negative children, either among the siblings of children with type 1 diabetes or among those derived from the general population. These observations are in line with the findings of Krętowski et al. and Lampeter et al. suggesting that beta-cell autoimmunity *per se* is not associated with elevated levels of soluble adhesion molecules, i.e. markers of endothelial and leukocyte activation. The siblings with an ICA level of 20 JDFU or more in their initial sample had higher sICAM-1 concentrations than those with a lower titre, and 62% (23/37) of these had progressed to clinical type 1 diabetes by the end of 1997. An ICA level of 20 JDFU or more has been observed to be highly predictive of progression to clinical disease in the DiMe series of siblings of children with type 1 diabetes (Knip et al. 1998), and a positive association between the risk of progression and the ICA titre has been demonstrated extensively among first-degree relatives, and is also implied in the background population (Karjalainen et al. 1990). We could not, however, confirm the association between high titres of ICA and increased concentrations of sICAM-1 in the present series of schoolchildren, although a tendency towards higher levels of circulating sICAM-1 was seen in subjects with an ICA level of 40 JDFU or more. Two out of 15 schoolchildren with an ICA titre of 40 JDFU or more progressed to clinical type 1 diabetes during a follow-up of an average of 3.4 years, which is substantially less than would be expected among siblings. The positive PPV reported for type 1 diabetes development associated with an ICA titre of 40 JDFU or more was 58% in the DiMe sibling series over a time period of 7.7 years (Kulmala et al. 1998). Taken together, these observations suggest that ICA positivity is associated with increased circulating concentrations of sICAM-1 when it reflects a substantially increased risk of progression to clinical type 1 diabetes.

IA-2A appear relatively late in the prediabetic phase and reflect a high risk of progression to clinical disease (Christie et al. 1997, Knip et al. 1998). Their emergence is associated with rapid progression to

overt diabetes (Bingley et al. 1994, Christie et al. 1994, Gardner et al. 1999). We observed a positive association of high sICAM-1 and sL-selectin concentrations with IA-2A positivity in siblings. Moreover, IA-2A titres were related to increased sICAM-1 concentrations in the peripheral circulation in schoolchildren who were positive for IA-2A. The association between sICAM-1 concentrations and IA-2A levels after seroconversion is interesting, since a direct correlation has been demonstrated between humoral and cellular immune responses to the IA-2 autoantigen, confirming the role of the latter in the pathogenesis of type 1 diabetes, where cellular mechanisms are most likely the principal mediators of beta-cell destruction (Ellis et al. 1998).

Positivity for multiple autoantibodies indicates a high risk of progression to overt diabetes, and also appears to be associated with accelerated beta-cell destruction and increased requirements for exogenous insulin over the second year of the clinical disease (Sabbah et al. 1999). Multiple autoantibodies have been reported to have a substantially higher predictive value for type 1 diabetes in a series of family studies than positivity for a single antibody (Bingley et al. 1994, Verge et al. 1996, Kulmala et al. 1998). Furthermore, recent observations on subjects derived from the general population support the concept that multiple autoantibodies are also good predictive markers of type 1 diabetes in the general population (Maclaren et al. 1999, Kimpimäki et al. 2000, Kulmala et al. 2001, LaGasse et al. 2002). Multiple autoantibodies have been reported to be more prevalent in subjects with the high-risk HLA genotype than in those with other genotypes (Kulmala et al. 2000b), and the HLA-DQB1 genotypes indicating high or moderate risk were also more frequent among the children with multiple autoantibodies in our series of schoolchildren (8/16) than in the children positive for only one autoantibody (8/88). In the family study we observed higher concentrations of sICAM-1 and sL-selectin in siblings with three or more autoantibodies in their initial sample than in those with a maximum of two autoantibodies, and proposed that positivity for three or more antibodies could reflect an on-going destructive immune process in the pancreatic islets. Only six out of the cohort of 29 subjects progressing to clinical type 1 diabetes during observation for an average of 7 years tested positive for one or two autoantibodies in their initial sample, while 23 had three or four autoantibodies. Contrary to our expectations, we could not confirm any association between high levels of soluble adhesion molecules and positivity for multiple autoantibodies in the general population. It is plausible that the autoimmune process may be milder in such a population than in siblings of affected children, possibly reflecting slower progression to clinical disease, and therefore no concomitant increase in the circulating concentrations of soluble adhesion molecules would be seen. Based on the degree of insulitis and proportion of insulin deficient islets in the pancreas in patients with recent-onset type 1 diabetes, an age-related heterogeneous course of the disease has been suggested (Foulis et al. 1986). On-going insulitis has been found in an anti-GAD65-positive adult patient with residual beta-cell function (Shimada et al 1999), further suggesting slowly progressing beta-cell destruction in older subjects.

Although the discrepancies in the present results may be due to the heterogeneous course of preclinical type 1 diabetes and different stages of autoimmunity, they are more likely to indicate that the concentrations of soluble adhesion molecules only partly overlap with traditional markers of an active autoimmune process, i.e. autoantibodies against islet cell antigens. They may reflect different characteristics of the autoimmune process from the conventional humoral immune markers, although intra-individual and inter-individual variation is substantial, suggesting that other, unrelated factors have a major impact on the circulating concentrations.

6.3 Soluble adhesion molecules in relation to impaired beta-cell function

Beta-cell function can be assessed from the insulin response to intravenous glucose to serve as an indirect measure of the remaining beta-cell mass. A reduced FPIR is highly predictive of rapid progression to overt type 1 diabetes in relatives of affected patients with multiple autoantibodies (Bingley et al. 1996), and progressive loss of beta-cell function has been observed a matter of months to years prior to the diagnosis of clinical diabetes (Srikanta et al. 1983). Early beta-cell failure soon after seroconversion to autoantibody positivity has recently been reported in young genetically susceptible children in the general population (Keskinen et al. 2002). On the other hand, subclinical and non-progressive (or very slowly progressive) beta-cell dysfunction has also been reported (Greenbaum et al. 1999). At least some of the impairment in insulin secretion may be functional, due to the inhibition of insulin secretion by cytokines and other soluble factors, as implied by Mehta et al. (1994).

Increased circulating concentrations of sL-selectin in autoantibody-positive schoolchildren with signs of impairment in their insulin secretory capacity may reflect activation of the leukocytes involved in an active process of insulitis. We were unable to observe any association between a reduced FPIR value (<45 mU/l) and circulating levels of soluble adhesion molecules in the sibling series, but the controversial results are most likely to be explained by differences in the timing of sampling in relation to the IVGTT, as the concentrations of soluble adhesion molecules were measured from the fasting IVGTT sample in the cohort of schoolchildren, whereas those in the series of siblings of affected children were analysed in the first sample taken close to the diagnosis of the index case, the IVGTT being performed on average of 6 months later.

6.4 Soluble adhesion molecules in the course of prediabetes

L-selectin plays a major role in the first two steps in the lymphocyte homing process, tethering and rolling (Springer 1990, von Andrian and Mackay 2000), but is also involved in the priming process of naïve T cells, which differentiate into antigen-specific armed effector T cells after having encountered an antigen. Many T cells lose their expression of L-selectin during this process. We observed elevated concentrations of circulating sL-selectin in the 3-month period preceding the detection of the first autoantibody in children with increased HLA-conferred susceptibility to type 1 diabetes, suggesting enhanced leukocyte activation, which may reflect a lymphocyte homing process and/or T-cell activation at the stage of the initiation of beta-cell autoimmunity. On the other hand, the children who seroconverted to autoantibody positivity by the age of four, and even those who presented with multiple autoantibodies during the follow-up or with the clinical disease by the age of five, did not have increased integrated levels of soluble adhesion molecules during their first two years of life, implying that early-onset beta-cell autoimmunity – even with rapid progression to clinical diabetes - is not accompanied by signs of general endothelial/leukocyte activation in the peripheral circulation. The randomized intervention trial with nasal insulin for the children in the DIPP cohort with persistent autoantibody positivity is likely to have had a minimal impact on the present results, since only ten of the 65 positive children had been randomized for the placebo-controlled double-blind trial by the end of the follow-up. The intervention was initiated at a mean age of 18.3 months (range 15-21 months) in the ten participants, and its mean duration during the follow-up was 6.6 months (range 3-9 months).

We did find elevated levels of sICAM-1 at the age of 6-12 months in the children belonging to the DIPP cohort who seroconverted to positivity for ICA before their second birthday, which may indicate the importance of this period for the initiation of beta-cell autoimmunity in young children. Increased levels of sICAM-1 at the age of 3, 6, 9 and 12 months have been reported earlier in infants who received cow's milk-based formula as compared with those observed in infants fed with a highly hydrolyzed formula until the age of 9 months (Paronen et al. 1996). There is increasing evidence in both man and rodents that the gut-associated immune system plays a major role in the development of autoimmune diabetes, probably because of disturbed development of oral tolerance, which is dependent on immunological homeostasis and normal maturation of the gut (Harrison and Honeyman 1999, Vaarala 1999). These are in turn influenced by growth factors and cytokines from breast milk, normal bacterial colonization, infections and diet, all of which are implicated as potential risk factors for type 1 diabetes. As the induction of adaptive immune functions, including oral tolerance, seems to be an age-dependent phenomenon in young children (Vaarala et al. 1995), there may be a critical window for the exposure of children prone to type 1 diabetes to dietary antigens in relation to weaning. This is also indicated by a recent study on the association between gluten exposure and the risk of the appearance of autoantibodies in infants with a HLA-defined predisposition to type 1 diabetes (Norris et al. 2003). The development of oral tolerance may also be influenced by other environmental exposures such as the establishment of a normal bacterial flora and viral infections, the rate of which increases drastically during the second half of the first year of life, as transplacentally transferred maternal IgG levels decrease gradually during the first 6 months.

Breast-feeding has been reported to have positive effects on the postnatal maturation of the immune system, e.g. the CD4/CD8 lymphocyte subset ratio has been observed to be lower in breast-fed infants than in formula-fed ones (Hawkes et al. 1999). Those eight children who progressed to clinical diabetes after the age of 3 years showed a relatively close inverse correlation between circulating concentrations of sICAM-1 and sL-selectin, particularly at 6-12 months of age, and both age at the introduction of cow's milk-based formula and the duration of exclusive and overall breast-feeding. This may be due to enhanced immune activation in infants of that age, induced by increased exposure to foreign dietary proteins, as proposed in an earlier report (Paronen et al. 1996), and indicates that early introduction of cows' milk or other foreign antigens and/or short duration of breast-feeding is associated with a proinflammatory state, predisposing young children who have an increased genetic risk of type 1 diabetes to a process of beta-cell destruction.

Enterovirus infections have also been reported to be temporally associated with the emergence of diabetes-associated autoantibodies, suggesting that they may trigger beta-cell autoimmunity (Lönnrot et al. 2000). We observed higher circulating concentrations of sL-selectin in association with enterovirus infections in those children who seroconverted positive for autoantibodies than in the control subjects. This difference became non-significant, however, after the exclusion of those cases who seroconverted to autoantibody positivity concomitantly with an enterovirus infection. A multiple regression analysis implied that increased sL-selectin levels were associated with seroconversion to autoantibody positivity but not with signs of an enterovirus infection. Altogether, these observations suggest that the transiently increased circulating sL-selectin concentrations seen in this cohort are related to the appearance of beta-cell autoimmunity rather than to enterovirus infections.

Inverse seroconversions were rarely seen in the IA-2A-positive cases in this cohort of young children, and of the individual autoantibodies, IA-2A had the highest specificity (100%) and a PPV of 92% for persistent multiple autoantibody positivity by the age of 2 years, indicating that they are markers of aggressive, rapidly progressing beta-cell autoimmunity (Kimpimäki et al. 2002). The children seroconverting to IA-2A positivity during the follow-up showed especially high integrated

concentrations of sL-selectin at the age of 6-12 months, and 14 out of these 16 children seroconverted to autoantibody positivity (for any autoantibody) during this period. The increased concentrations of sL-selectin at the age of 6-12 months in children developing signs of progressive beta-cell autoimmunity by the age of two years may imply that seroconversion during this period could be critical.

There was no obvious trend for increasing concentrations of soluble adhesion molecules towards diagnosis of the clinical disease among the children representing familial type 1 diabetes and presenting with autoantibodies at the beginning of the follow-up. A German survey has reported that the peripheral concentrations of sICAM-1 and sL-selectin were increased to approximately the same extent both in patients with recently diagnosed type 1 diabetes and in healthy first-degree relatives irrespective of autoantibody status or HLA-DR risk alleles (Lampeter et al. 1992). A similar observation regarding sL-selectin was reported by Krętowski et al. (2000a), who found no significant difference between patients with newly diagnosed type 1 diabetes and first-degree relatives (autoantibody-positive or negative), although both of them had increased levels relative to healthy controls. They observed no correlation between sL-selectin levels and HbA1c or blood glucose concentrations, indicating that hyperglycaemia does not lead to increased sL-selectin levels. The sL-selectin concentrations did not differ between the controls and patients with either type 2 or type 1 diabetes for more than 10 years (Krętowski et al. 2000a). In another study the levels of sICAM-1 were found to be higher in non-diabetic first-degree relatives with an ICA level of 20 JDFU or more than in age and sex-matched unrelated healthy controls, and even higher than in patients with newly diagnosed type 1 diabetes (Myśliwiec et al. 1999).

Although the source and physiological function of sICAM-1 remains to be established, increased levels in progressors over a 3.5 year period in the preclinical phase may reflect endothelial and leukocyte activation due to destructive insulitis, being most prominent about 1.5 years before diagnosis. This observation is in line with evidence from both *in vitro* and *in vivo* studies that suggest a role for ICAM-1-driven Th1 polarization (Samoilova et al. 1998, Arai et al. 1999, Balasa et al. 2000, Smits et al. 2002). A Th1-dominated cytokine profile has been documented in subjects at risk for type 1 diabetes (Hussain et al. 1998, Karlsson et al. 2000). Alternatively we cannot rule out that sICAM-1 may be elevated due to a defensive attempt of the immune system to reduce aggressive insulitis. Recombinant ICAM-1 proteins (5 µg three times/a week for 4.5 months) beginning at age 35 days have been shown to inhibit insulitis and the onset of autoimmune diabetes in NOD mice (Martin et al. 1998). Recombinant soluble ICAM-1 has also been shown to suppress the reactivity of autoimmune T cells from recent-onset type 1 diabetic patients in response to an islet-specific autoantigen in concentrations found in subjects at risk for type 1 diabetes (Lampeter et al. 1992, Roep et al. 1994).

We did not observe any increases in the total integrated concentrations of soluble adhesion molecules in the DiMe siblings, more than 80% of whom tested positive for one or more autoantibodies at the time of initial sampling. Neither was there any relationship between integrated soluble adhesion molecule concentrations and specific autoantibodies or the number of autoantibodies detected during the follow-up. This negative finding may be due to the limited number of cases in each group, but it is more likely to suggest that there are factors other than the autoimmune process which affect the levels of soluble adhesion molecules in the peripheral circulation. When observing separately the possible variation in nadir sICAM-1 levels in relation to different autoantibody specificities, a tendency was noted for higher levels in those who progressed to overt type 1 diabetes, although significantly so only among the ICA-positive siblings and the siblings with two or more autoantibodies detected in the initial sample or during the follow-up.

We observed both among the autoantibody-positive siblings and among the young genetically susceptible children who seroconverted to autoantibody positivity during the follow-up that there were conspicuous intra-individual and inter-individual variation in the concentrations of soluble adhesion

molecules. This indicates that there are factors other than the diabetic disease process that affect the circulating concentrations of soluble adhesion molecules. Viral infections could modify these, and at least enteroviruses, respiratory syncytial virus (RSV) and human rhinoviruses have been reported to use ICAM-1 as their receptor (Racaniello 1995, Smyth et al. 1997, Van Kempen et al. 1999). In addition, RSV infections have been observed to increase the serum concentrations of soluble ICAM-1 (without any correlation with disease severity) in a series of children less than 1 year of age hospitalized for acute bronchiolitis (Smyth et al. 1997). Kulander et al. (2001) did not find any elevation in circulating sICAM-1 concentrations in 35 adult patients with evidence of viral infections, in contrast to 66 patients affected by bacterial infections. In the sibling cohort, we suggest that the impact of viral infections cannot be substantial, since the peak and total concentrations of sICAM-1 were similar in the siblings younger than 5 years of age and the older ones. One would also expect that the impact of viral infections would be the same in both progressors and non-progressors, although there are certain indications that immunological abnormalities related to the autoimmune process preceding the clinical manifestation of type 1 diabetes, such as depletion of memory CD4-positive cells and defective killer-cell activity, could transiently impair host defence against viral diseases in high-risk first-degree relatives (Moutschen et al. 1992). In the DIPP cohort the effect of viral infections may have been more pronounced, since RSV infections are especially common in this age group (Tsai et al. 2001, van Woensel et al. 2003).

In conclusion, although complex interactions between adhesion molecules are most likely to be involved in the initiation and propagation of autoimmune processes, such as type 1 diabetes, no specific or unique over-expression of adhesion molecules confined to any particular situation has been identified. This lack of specificity is reflected in the levels of soluble adhesion molecules reported in the present work, as any type of inflammatory or infectious process will utilize the adhesion cascade in a similar manner. Furthermore, the lack of standardization of ELISAs, i.e. the various specificities and standards used, may account for some discrepancies between the findings reported in different studies. Therefore the present assays available for measuring the concentrations of soluble adhesion molecules seem to be of limited diagnostic value in the context of preclinical type 1 diabetes. Similar conclusions have been drawn in relation to other autoimmune and inflammatory diseases as well.

7 Conclusions

I. Type 1 diabetes-associated HLA class II alleles do not have a major impact on the circulating concentrations of sICAM-1, while HLA-DQB1 genotypes may modify sL-selectin concentrations in the peripheral circulation.

II. Autoimmunity *per se* is not associated with elevated circulating concentrations of sICAM-1 or sL-selectin. Autoantibody markers with strong predictive value, i.e. multiple autoantibodies, high ICA levels and positivity for IA-2A, are related to increased levels of soluble adhesion molecules in familial type 1 diabetes, but not in non-familial cases. This indicates that these markers only partly overlap with conventional humoral immune markers, i.e. autoantibodies against islet cell antigens. They may reflect other characteristics of the autoimmune process, although intra-individual and inter-individual variation is substantial, indicating that there are several other unrelated factors affecting the circulating concentrations.

III. Increased concentrations of sL-selectin in subjects with impaired beta-cell function may reflect the leukocyte activation associated with on-going destructive insulitis.

IV. Early-onset progressive beta-cell autoimmunity, or even progression to clinical type 1 diabetes by the age of 5 years, is not reflected in overall increased concentrations of soluble adhesion molecules in the peripheral circulation during the first 2 years of life in children with increased HLA-conferred disease susceptibility, although elevated concentrations of sL-selectin are temporally associated with seroconversion to autoantibody positivity.

The older children representing familial type 1 diabetes presented with increased levels of sICAM-1 6-48 months before diagnosis, which may reflect immune activation and/or tissue destruction going on in progressors. Alternatively it may be a defensive attempt to reduce aggressive insulitis. The substantial overlapping in the circulating levels of soluble adhesion molecules between the progressors and non-progressors on all occasions implies, however, that peripheral sICAM-1 and sL-selectin concentrations cannot provide a clinically meaningful tool for identifying those children who will develop clinical type 1 diabetes.

V. The increased levels of soluble adhesion molecules during the second half of the first year may reflect the importance of this age for the induction of early beta-cell autoimmunity in genetically susceptible children. Stronger immune activation due to exposure to dietary antigens during weaning, reflected in elevated concentrations of soluble adhesion molecules, may be detrimental in some children who manifest overt diabetes by the age of 5 years.

8 Summary

The aim of this work was to characterize the active autoimmune process preceding the clinical manifestation of human type 1 diabetes, both familial and non-familial, based on the quantification of circulating concentrations of sICAM-1 and sL-selectin, which are established markers of endothelial and leukocyte activation. We also aimed at assessing whether these markers could be used to discriminate between disease progressors and non-progressors, in order to improve the prediction of type 1 diabetes on an individual basis in children and adolescents.

We did not observe any relationship between sICAM-1 and HLA class II-conferred susceptibility to type 1 diabetes, but sL-selectin concentrations were higher in autoantibody-negative schoolchildren carrying HLA-DQB1 genotypes conferring low or decreased risk for type 1 diabetes.

Autoimmunity *per se* is not associated with elevated concentrations of sICAM-1 or sL-selectin. Autoantibodies with strong predictive value, i.e. multiple autoantibodies, high ICA levels and positivity for IA-2A, are related to increased concentrations of soluble adhesion molecules in familial type 1 diabetes, but not in non-familial cases, indicating that these markers only partly overlap with conventional humoral immune markers, i.e. autoantibodies against islet cell antigens. They may reflect other characteristics of the autoimmune process, although intra-individual and inter-individual variation is substantial, indicating that there are several other unrelated factors affecting the concentrations of soluble adhesion molecules in the peripheral circulation.

Increased concentrations of sL-selectin in subjects with impaired beta-cell function may reflect leukocyte activation associated with destructive insulitis.

Early-onset progressive beta-cell autoimmunity, or even progression to clinical type 1 diabetes by the age of 5 years, is not reflected in overall increased concentrations of soluble adhesion molecules in the peripheral circulation during the first 2 years of life in children with increased HLA-conferred disease susceptibility, although elevated concentrations of sL-selectin were found to be temporally associated with seroconversion to autoantibody positivity. The older children representing familial type 1 diabetes presented with increased levels of sICAM-1 6-48 months before diagnosis, which may reflect immune activation and/or tissue destruction going on in the progressors. Alternatively it may be a defensive attempt to reduce aggressive insulitis. The substantial overlapping in the circulating levels of soluble adhesion molecules between the progressors and non-progressors on all occasions implies, however, that peripheral sICAM-1 and sL-selectin concentrations cannot provide a clinically meaningful tool for differentiating those children who will develop clinical type 1 diabetes from those who will remain non-diabetic.

The increased levels of soluble adhesion molecules during the second half of the first year may reflect the importance of this age for the induction of early beta-cell autoimmunity in genetically susceptible children. The stronger immune activation due to exposure to dietary antigens in relation to weaning, reflected in increased concentrations of soluble adhesion molecules during this period, may be detrimental in some children who manifest overt diabetes by the age of 5 years. Soluble adhesion molecules do not seem to be helpful in identifying the environmental factors involved in the

pathogenesis of type 1 diabetes, since other non-specific factors, such as common viral infections, may have an impact on their concentrations in the peripheral circulation.

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