Autoantibodies related to type 1 diabetes in children

Camilla Skoglund

Division of Pediatrics
Department of Clinical and Experimental Medicine
Faculty of Health Sciences, Linköping University
SE-581 85 Linköping, Sweden
To my family
Abstract

Type 1 diabetes is an autoimmune disease resulting from destruction of the insulin producing beta cells in the pancreas. The patients need life-long heavy treatment and still complications, both acute and later in life, are common. The incidence of type 1 diabetes has increased rapidly during the last decades, especially among young children. The disease can be predicted by genes predisposing type 1 diabetes, mainly human leukocyte antigen (HLA) genes, together with presence of autoantibodies to beta-cell antigens, where multiple autoantibodies confer the highest risk. A number of immune system intervention trials are now ongoing aiming to halt the progression of the inflammatory process in the beta cells.

This thesis aimed to investigate the prevalence and levels of autoantibodies in healthy children and in children with type 1 diabetes. Another aim was to study different properties of one of these autoantibodies, such as to which epitopes the antibodies bind and the distribution of immunoglobulin (Ig)-G subclasses, after immunomodulatory treatment in children with type 1 diabetes.

We found that positivity to autoantibodies against glutamic acid decarboxylase (GADA) and tyrosine phosphatase like protein islet antigen-2 (IA-2A) was associated with HLA risk genotypes in 5-year old children from the general population. HLA risk genotypes seemed important for persistence of autoantibodies and for development of type 1 diabetes, while emergence of autoantibodies, especially transient autoantibodies, seemed to be more influenced by environmental factors. Improved methods for detection of autoantibodies are needed, for prediction of diabetes and for identification of high-risk individuals suitable for prevention treatments. Therefore, an assay for measurement of insulin autoantibodies (IAA), based on surface plasmon resonance (SPR), was developed. The main advantages of this method are that there is no need for labelling and that it is time-saving compared to the traditionally used radioimmunoassay (RIA), but further development of the method is needed.

Treatment with GAD-alum (Diamyd) in children with type 1 diabetes has shown to preserve residual insulin secretion. This clinical effect was accompanied by an increase in GADA levels. We investigated the epitope reactivity of GADA in both GAD-alum and placebo treated children, and found that binding to one of the tested epitopes was temporarily increased after injection of GAD-alum. This result suggests that the quality of GADA was, to some extent, transiently affected by the treatment. On the other hand, no changes in binding to epitopes associated with stiff person syndrome (SPS) were observed, which together with the lack of change in GAD65 enzyme activity further strengthens the safety of the treatment. We also observed that the distribution of IgG subclasses was changed by GAD-alum treatment, with a lower proportion of IgG1 and higher IgG3 and IgG4. Lower IgG1 and higher IgG4 suggest a temporary switch towards a protective Th2 immune response, which has previously been observed in the same individuals for other immunological markers.

In conclusion, measurement of autoantibodies related to type 1 diabetes is an important tool for studying the autoimmune process in pre-diabetic and type 1 diabetic children. In addition to the use as markers of disease progression, the autoantibodies may be used for studying the effects of immunomodulatory treatments on the humoral immune response.

Målet med den här avhandlingen var att undersöka förekomst och nivåer av diabetesrelaterade autoantikroppar hos friska barn och hos barn med typ 1 diabetes. Därutöver studera olika egenskaper hos en av dessa autoantikroppar, t e x olika bindningsställen (epitoper) på ett kroppseget protein (antigen) dit antikroppar kan binda, och subklasser av immunoglobulin G (IgG)-antikroppar, hos barn med typ 1 diabetes som genomgått en immunmodulerande behandling.

Resultaten visade att förekomst av autoantikroppar mot glutaminsyredekarboxylas (GADA) och mot det tyrosinfosfatas-liknande proteinet IA-2 (IA-2A) var associerat med HLA-riskgener hos 5-åriga barn från den allmänna befolkningen. HLA-riskgener verkade viktiga för bestående autoantikroppar samt för utveckling av typ 1 diabetes. Uppkomsten av autoantikroppar verkade däremot påverkas mer av miljöfaktorer än av HLA-riskgener, vilket gällde särskilt för de så kallade övergående (transienta) autoantikropparna, dvs de som senare försvann.


Barn som nyligen fått typ 1 diabetes och som har behandlats med GAD bundet till aluminium hydroxid som vaccin-adjuvans (GAD-alum; Diamyd), har visats kunna behålla sin egen insulinproduktion bättre än de som inte fått denna behandling. Förutom denna kliniska effekt observerades också att GADA-nivåerna ökade. Vi undersökte vilka epitoper GADA band till både hos de barn som fått behandling och de utan behandling. GADA-bindning till en av de testade epitoperna visade sig öka efter GAD-alumbehandling, vilket skulle kunna tyda på att antikropparnas kvalitet till viss del har ändrats av behandlingen. Däremot var det ingen förändring i GADA-bindning till de epitoper som är kopplade till sjukdomen stiff person syndrome (SPS), vilket tillsammans med oförändrad enzymatisk aktivitet hos GAD65.
ytterligare bekräftar behandlingens säkerhet. Fördelningen av IgG-subklasser ändrades efter behandling med GAD-alum, med en lägre andel IgG1 och högre IgG3 och IgG4. IgG1 och troligtvis även IgG3 är kopplade till så kallade T-hjälpar 1 (Th1)-cellers immunsvar medan IgG4 är kopplat till Th2-svar. Den lägre andelen IgG1 och högre IgG4 som observerades i vår studie skulle kunna tyda på en tillfällig förändring av immunsvaret till ett skyddande Th2-svar, vilket också har setts hos dessa individer för andra immunologiska markörer.

Sammanfattningsvis, att mäta autoantikroppar som är relaterade till typ 1 diabetes är viktigt för att studera den autoimmuna processen hos barn som håller på att få diabetes och hos de som redan har diabetes. Utöver att använda autoantikropparna som markörer för utveckling av typ 1 diabetes kan de även användas för att studera immunmodulerande behandlingars effekter på det humorala immunsvaret.
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<th>Description</th>
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<tbody>
<tr>
<td>ABIS</td>
<td>all babies in southeast of Sweden</td>
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<tr>
<td>alum</td>
<td>aluminum hydroxide</td>
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<tr>
<td>CM</td>
<td>carboxymethylated</td>
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<tr>
<td>C-peptide</td>
<td>connecting peptide</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
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<td>CTLA-4</td>
<td>cytotoxic T lymphocyte antigen-4</td>
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<tr>
<td>DAISY</td>
<td>the diabetes autoimmunity study in the young</td>
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<td>DASP</td>
<td>diabetes autoantibody standardization program</td>
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<td>DIPP</td>
<td>the diabetes prediction and prevention study</td>
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<tr>
<td>DPT-1</td>
<td>the diabetes prevention trial</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ENDIT</td>
<td>the European nicotinamide diabetes intervention trial</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
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<td>GAD65</td>
<td>65 kDa isoform of GAD</td>
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<td>GADA</td>
<td>autoantibodies to GAD65</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>IA</td>
<td>insulin antibodies</td>
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<tr>
<td>IA-2</td>
<td>tyrosine phosphatase like protein islet antigen-2</td>
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<td>IA-2A</td>
<td>autoantibodies to IA-2</td>
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<tr>
<td>IAA</td>
<td>autoantibodies to insulin</td>
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<tr>
<td>ICA</td>
<td>islet cell antibodies</td>
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<td>IDS</td>
<td>immunology of diabetes society</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>INS</td>
<td>insulin gene</td>
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<td>LADA</td>
<td>latent autoimmune diabetes in adults</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MODY</td>
<td>maturity-onset diabetes of the young</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
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<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
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<tr>
<td>PLP</td>
<td>pyridoxal 5-phosphate</td>
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<tr>
<td>PPV</td>
<td>positive predictive value</td>
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<tr>
<td>PTP</td>
<td>protein tyrosine phosphatase</td>
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<td>PTPN22</td>
<td>protein tyrosine phosphatase N22</td>
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<tr>
<td>rFab</td>
<td>recombinant Fab</td>
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<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>RU</td>
<td>resonance units</td>
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<tr>
<td>SAM</td>
<td>self-assembled monolayer</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<td>SPS</td>
<td>stiff person syndrome</td>
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<tr>
<td>SPSS</td>
<td>statistical package for the social sciences</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TEDDY</td>
<td>the environmental determinants in diabetes of the young</td>
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<tr>
<td>Th</td>
<td>T-helper</td>
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<tr>
<td>Treg</td>
<td>naturally occurring regulatory T cells</td>
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<tr>
<td>TRIGR</td>
<td>the trial to reduce type 1 diabetes in the genetically at risk</td>
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<tr>
<td>U/ml</td>
<td>units/ml</td>
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<tr>
<td>VNTR</td>
<td>variable number of tandem repeats</td>
</tr>
<tr>
<td>WHO</td>
<td>the world health organization</td>
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<tr>
<td>ZnT8</td>
<td>zink transporter 8</td>
</tr>
<tr>
<td>ZnT8A</td>
<td>autoantibodies to ZnT8</td>
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</table>
Original publications

This thesis is based on the following four papers, which are referred to in the text by their roman numerals:

I. **Camilla Gullstrand**, Jeanette Wahlberg, Jorma Ilonen, Outi Vaarala and Johnny Ludvigsson
   Progression to type 1 diabetes and autoantibody positivity in relation to HLA-risk genotypes in children participating in the ABIS study.
   Pediatric Diabetes 2008; 9(Part I): 182-190

II. Jenny Carlsson, **Camilla Gullstrand**, Gunilla T. Westermark, Johnny Ludvigsson, Karin Enander and Bo Liedberg
   An indirect competitive immunoassay for insulin autoantibodies based on surface plasmon resonance.
   Biosensors and Bioelectronics 2008; 24: 882-887

III. **Camilla Skoglund**, Mikael Chéramy, Rosaura Casas, Johnny Ludvigsson and Christiane S. Hampe
   GAD-alum treatment-induced increase in GAD autoantibody (GADA) titers of type 1 diabetes children and adolescents is accompanied by a transient change in GADA epitope pattern.
   Submitted

IV. Mikael Chéramy, **Camilla Skoglund**, Ingela Johansson, Johnny Ludvigsson, Christiane S. Hampe and Rosaura Casas
   GAD-alum treatment in patients with type 1 diabetes and the subsequent effect on GADA IgG subclass distribution, GAD65 enzyme activity and humoral response.
   Clinical Immunology 2010; 137(1): 31-40
INTRODUCTION TO THE IMMUNE SYSTEM

The human body is continuously surrounded by microorganisms, where some of them are good and others harmful (Janeway et al. 2005; Mölne et al. 2007). The disease-causing microorganisms have been divided into four categories: viruses, bacteria, pathogenic fungi and parasites. The body has to defend itself against these pathogens. A well functioning immune system has been developed, consisting of a wide range of different white blood cells. These cells perform different tasks in the immune system. Communication between immune cells occurs via receptors on the surfaces of the cells and via different signaling molecules, such as cytokines and chemokines.

The immune system consists of three lines of defense (Janeway et al. 2005; Mölne et al. 2007). The first line of defense comprises mechanical and chemical barriers protecting the skin and mucosa, together with a microbiological barrier of non pathogenic bacteria. Epithelial cells are joined by tight junctions, mucus is transported from the lungs by cilia, fatty acids and antibacterial peptides are produced etc. These barriers are very effective and most of the microorganisms are therefore unable to enter the body, but those who manage to break through encounter the second line of defense, namely the innate immune system. This non-specific immune system consists of granulocytes (neutrophils, eosinophils, basophils and mast cells) and macrophages. Macrophages have a wide variety of cell-surface receptors that recognize microbial structures on the pathogen, such as lipopolysacharide (LPS), peptide glucane and virus-RNA. The pathogen is attached to a receptor and then engulfed by phagocytosis and eliminated by the macrophage. This is followed by activation of the macrophage with release of cytokines, chemokines and other mediators that initiate inflammation in the tissue and bring neutrophils and plasma proteins to the site of an infection, aiming at elimination of the microorganisms.

The third line of defense is the adaptive immune system, which can be activated by almost any microbial structure (Janeway et al. 2005; Mölne et al. 2007). The adaptive immune system consists of lymphocytes (T cells and B cells) and provides a specific response. Mature lymphocytes circulate between blood vessels, lymph vessels and lymph nodes. The antigen is
transported to the lymph nodes by antigen presenting cells, primarily macrophages. The environment of the lymph node is ideal for interaction between antigen and lymphocyte. The lymphocytes interact with antigen presenting cells through their antigen-specific receptors. Upon contact with the specific antigen, the lymphocytes are activated, mature and divide creating a clone of active cells, which leave the lymph node and migrate to places in the body where they can have their effect (MacLennan et al. 1997). The T cells mature into antigen-specific effector cells, often producing cytokines, and the B cells into antibody-secreting plasma cells.

**T cells**

Precursors of the T cells are developed in the bone marrow (Janeway et al. 2005; Mölne et al. 2007). They migrate to the thymus, where they mature into T cells and get their unique specificity. The T cells are selected based on their binding affinity to major histocompatibility complex (MHC)-peptide complexes. If T cell receptors (TCRs) on a T cell recognize and bind antigen presented by MHC molecules the cell will survive positive selection, while the other T cells will die. However, if the T cell reacts strongly with self antigens, it will instead die by negative selection, thereby maintaining tolerance to self antigens.

T cells can be divided into CD4-positive T cells, which react with the antigen bound to MHC-II molecules, and CD8-positive T cells, which recognize the antigen on MHC-I molecules (Janeway et al. 2005; Mölne et al. 2007). MHC-I molecules are present on all kinds of cells in the body, except red blood cells, while MHC-II are mainly expressed on antigen presenting cells. Human MHC molecules are referred to as human leukocyte antigen (HLA). When CD4-positive T cells are activated they become either helper T cells or regulatory T cells (Janeway et al. 2005; Mölne et al. 2007). Helper T cells can be divided into T-helper 1 (Th1), T-helper 2 (Th2) and T-helper 17 (Th17) cells, depending on their function in the immune system (Mosmann et al. 1996; Rautajoki et al. 2008; Korn et al. 2009).

Activation of helper T cells is central for all immune reactions, by producing cytokines and by cell-to-cell interactions. Th1 cells are important in cell-mediated immunity against intracellular pathogens, by activating and attracting macrophages and cytotoxic cells to the site of infection (Szabo et al. 2003; Rautajoki et al. 2008). In addition, Th1 cells stimulate the production of immunoglobulin (Ig)-G antibodies that are involved in opsonization and
phagocytosis. Further, cytokines produced by Th1 cells, such as interferon-gamma (IFN-γ), activate macrophages to increase their production of bactericidal substances, such as enzymes and free radicals, to eliminate those microorganisms that survived inside the macrophage (Mölne et al. 2007). Th2 cells are instead important in the humoral immune response (Mowen et al. 2004). Cytokines produced by Th2 cells, such as interleukin-4 (IL-4), IL-5, IL-9 and IL-13, assist B cells in their maturation and antibody production for elimination of extracellular pathogens. Further, a predominant Th2 response is associated with atopic diseases and allergies. The recently described Th17 cells produce cytokines including IL-17, IL-21 and IL-22, and seem to have a proinflammatory role (Korn et al. 2009). In addition, IL-17 has been shown to be important for host defense against some pathogens. Further, both Th17 and Th1 cells are associated with autoimmune diseases, including type 1 diabetes (Szabo et al. 2003).

Regulatory T cells control and down regulate various immune responses. Naturally occurring regulatory T cells (Treg) constitutively express CD25 (a subunit of the IL-2 receptor) on their surface and transcribe the FOXP3 gene (Sakaguchi 2004). Expression of the transcription factor FOXP3 is vital to the development and function of Treg (Fontenot et al. 2003; Hori et al. 2003). Defects in the function of Tregs have been hypothesized to be involved in the pathogenesis of numerous autoimmune diseases, including type 1 diabetes (Sakaguchi 2004).

Activated CD8-positive T cells mature into cytotoxic T cells, which can eliminate infected cells (Janeway et al. 2005; Mölne et al. 2007). Maturation of cytotoxic T cells is both dependent on cytokines, such as IL-2 and IFN-γ, and on cell-to-cell contact with the helper T cell.

**B cells**

B cells are developed in the bone marrow, where they also get their unique specificity (Janeway et al. 2005; Mölne et al. 2007). The B cell receptor for recognition of an antigen consists of a membrane bound antibody. When an antigen has bound to the B cell receptor, the antigen is internalized and processed into peptides (Figure 1). The B cell presents the peptides on MHC-II molecules and expresses cytokine receptors and the co-stimulatory molecule CD40 on its surface. This enables a helper T cell that recognizes the antigen, to attach to the B cell and then produce cytokines. The cell-to-cell contact between the B and the T cell, together with cytokines binding to the receptors, activates the B cell to mature into a
plasma cell that produces antibodies. These antibodies have the same specificity as the membrane bound antibody used as a receptor.

**Figure 1.** Schematic illustration of the process for the maturation of a B cell into an antibody producing plasma cell. The antigen is bound to the B cell receptor followed by internalization and processing into peptides, which are then presented on MHC-II molecules on the surface of the B cell. The interaction between an antigen-specific T cell and the B cell, together with cytokines produced by the T cell, leads to activation of the B cell and maturation into a plasma cell that produces antibodies. TCR = T cell receptor, CD40L = CD40 ligand, MHC-II = major histocompatibility complex-II.

**Antibodies**

Antibodies are gamma globulin proteins that are found in blood or other body fluids, and are used by the immune system to identify and neutralize foreign objects, such as bacteria and viruses (Cohen 1963; Janeway et al. 2005). The immune system creates billions of different antibodies with a limited number of genes by rearranging DNA segments during B cell development, prior to antigen exposure (Janeway et al. 2005). Mutation can also increase genetic variation in antibodies. Antibodies can react with almost any chemical structure in nature, including our own proteins. Antibodies that recognize self antigens are called autoantibodies (Milgrom et al. 1963).

Antibodies consist of two heavy chains and two light chains of amino acids, in a Y shaped form, with a molecular mass of approximately 150 kDa (Figure 2) (Janeway et al. 2005; Schroeder et al. 2010). The two ends of the antibody, linked via flexible hinge regions, have different functions. The Fc region determines the mechanisms used to destroy antigen, such as activation of complement and binding to Fc receptors, and the two Fab regions bind to a
specific epitope on the antigen. Differences in antigenic and structural properties of the heavy chains determine the class and subclass of the molecules. There are five antibody isotypes known as immunoglobulin A (IgA), IgD, IgE, IgG and IgM, which have different roles in the immune system.

**Figure 2.** A schematic presentation of an antibody. It is composed of two heavy chains and two light chains. The Fab regions, containing the antigen-binding sites, are linked by hinge regions to the Fc part of the antibody. Adapted from (Alberts et al. 2002).

**IgM** are relatively low-affinity antibodies that are associated with a primary immune response and are found in blood and sometimes in extracellular fluids (Schroeder et al. 2010). IgM consists of five antibody units in a pentamer structure, which makes it very efficient in opsonizing antigens for destruction and fixing complement.

**IgD** antibodies are found at very low levels in serum and the function of IgD is unclear (Schroeder et al. 2010).

**IgG** antibodies, which usually are of higher affinity, are produced later in the immune response and this isotype is predominant in blood and extracellular fluids (Schroeder et al. 2010). IgG can be divided into four subclasses; IgG1, IgG2, IgG3 and IgG4, which are numbered according to the rank order (IgG1>IgG2>IgG3>IgG4) of the serum levels of these antibodies in the blood of healthy individuals (Figure 3). The major structural differences between the IgG subclasses occur in the hinge region. IgG1 has a freely flexible hinge region consisting of 15 amino acids (Brekke et al. 1995). IgG3 has an elongated hinge region of 62 amino acids, giving this subclass the greatest flexibility. The hinge region of IgG4 is shorter...
than IgG1, and IgG2 has the shortest, making this molecule rigid (Roux et al. 1997). The flexibility of the molecule may be important for the function of the antibody (Brekke et al. 1995). IgG can activate the complement system, neutralize toxins, viruses and bacteria, and opsonize them for phagocytosis (Schroeder et al. 2010). They bind Fc-receptors on neutrophils, monocytes and macrophages, which facilitates opsonization of particles that are covered with IgG antibodies. The Fc regions of IgG, as well as IgM, can bind complement factor C1 and thereby activate complement via the classical way, leading to that also encapsulated bacteria can be opsonized by the complement system. Not all IgG subclasses can bind complement; IgG1 and IgG3 are effective complement activators, IgG2 is a weak complement activator and IgG4 is unable to activate complement.

![Figure 3](image-url)

**Figure 3.** Schematic structure and properties of IgG subclasses. IgG1 is predominant in serum, IgG3 is the subclass with the longest hinge region and the shortest half life and IgG4 is unable to fix complement. Adapted from (Hamilton 1987; Lin et al. 2010; Schroeder et al. 2010).

**IgA** antibodies are found as monomers in blood and extracellular fluids and as dimeric molecules at mucosal surfaces and in secretions, such as saliva and breast milk (Schroeder et al. 2010). IgA has two subclasses, which differ mainly in their hinge regions; IgA1 and IgA2, where IgA2 is more resistant to proteolysis. IgA provides a first line of defense against a wide variety of pathogens by protecting mucosal surfaces from toxins, viruses and bacteria, via direct neutralization or prevention of binding to the mucosal surface.
IgE is a very potent immunoglobulin, although it is present at the lowest serum concentration of all isotypes (Schroeder et al. 2010). It binds with high affinity to FcεRI receptors on mast cells, basophils, Langerhans cells and eosinophils. IgE antibodies are associated with hypersensitivity and allergic reactions and with responses to parasitic worm infections.

During an antibody response the isotype and subclass of antibodies can be shifted without changing the specificity (Janeway et al. 2005). The dominance of IgM in the beginning of an antibody response is after re-exposure of the antigen switched into other classes of antibodies, for example IgG in serum. Cytokines regulate the generation of different Ig isotypes and IgG subclasses (Purkerson et al. 1992; Snapper et al. 1993). The distribution of various isotype-specific antibodies may therefore reflect whether the immune response is Th1- or Th2-biased. The Th2 cytokine IL-4 induces the synthesis of IgG4 and IgE (Lundgren et al. 1989; Gascan et al. 1991), and the Th1 cytokine IFN-γ stimulates IgG1 and seems to stimulate IgG3 production (Widhe et al. 1998), but further studies are needed to clearly define the antibody subclass association with Th1 or Th2 response in humans.

Antibodies can be monoclonal or polyclonal (Janeway et al. 2005). Polyclonal antibodies are produced by different cells and are therefore immunochemically different from each other, while monoclonal antibodies are the product of an individual clone of plasma cells and thus immunochemically identical. Antibodies that bind to the same antigen but to different epitopes, or that bind to similar antigens, have different binding capacity, so called specificity. Affinity defines the strength of binding of the antibody to its antigen in terms of a single antigen-binding site binding to a monovalent antigen. The total binding strength of a molecule with more than one binding site is called its avidity. Anti-idiotypic antibodies recognise the antigen-binding site of a specific antibody and can thereby interfere with the binding to the corresponding antigen (Geha 1985). It has been suggested that there is a lack of anti-idiotypic antibodies in type 1 diabetes (Oak et al. 2008).

In conclusion, T cells and B cells are important in the adaptive immune system. Activated T cells become helper T cells or regulatory T cells, while activated B cells mature into plasma cells that produce antibodies. The five antibody isotypes IgM, IgD, IgG, IgA and IgE have different roles in the immune system.
INTRODUCTION TO DIABETES MELLITUS

Definition and diagnosis

Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (WHO 1999; American Diabetes Association 2008). Several pathogenic processes are involved in the development of diabetes. These include destruction of the beta cells of the pancreas with consequent insulin deficiency and abnormalities that result in resistance to insulin action.

Symptoms indicating diabetes mellitus are signs of hyperglycemia, such as thirst, polyuria, glucosuria, weight loss and others such as fatigue, blurred vision and recurrent infections (WHO 1999). In severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to drowsiness and coma, and in absence of effective treatment to death. Effects of diabetes mellitus are long-term damage, dysfunction and failure of various organs, especially the eyes, nerves, kidney, heart and blood vessels (Glastras et al. 2005; Melendez-Ramirez et al. 2010; Skyler 2010).

The criteria for the diagnosis of diabetes are symptoms of hyperglycemia and a plasma glucose value of ≥11.1 mmol/l or fasting plasma glucose of ≥7.0 mmol/l (in whole blood 6.1 mmol/l) or 2-h plasma glucose ≥11.1 mmol/l during an oral glucose tolerance test (OGTT) (American Diabetes Association 2008). In the absence of symptoms, the diagnosis must be confirmed another day by one of the three methods above. Diabetes may present differently in different individuals, ranging from severe symptoms and gross hyperglycemia to a lack of symptoms and with blood glucose values just above the diagnostic cut-off value. In children, diabetes often presents with severe symptoms, very high blood glucose levels, marked glycosuria and ketonuria (WHO 1999). Diagnosis is usually confirmed with blood glucose measurements and treatment is initiated immediately. Rarely, children and adolescents lack symptoms and are then diagnosed with a fasting blood glucose measurement and/or an OGTT. This happens, for example, when patients are found by screening of autoantibodies to identify individuals with increased risk of developing type 1 diabetes.
Classification

Diabetes mellitus can be classified into four types based on etiology; type 1 diabetes, type 2 diabetes, gestational diabetes and other types of diabetes. The majority of cases of diabetes fall into type 1 diabetes or type 2 diabetes (American Diabetes Association 2008).

Type 1 diabetes

Type 1 diabetes is characterized by an absolute deficiency of insulin secretion (Notkins et al. 2001; American Diabetes Association 2008). The most common form of type 1 diabetes is immune-mediated diabetes and accounts for 5-10% of all individuals with diabetes. This disease is considered autoimmune (Rose et al. 1993; Bach 1994) and results from a cellular-mediated autoimmune destruction of the beta cells of the pancreas (Knip 1997; Knip et al. 2008). Autoimmune destruction of beta cells has multiple genetic predispositions, mainly strong HLA associations, and is influenced by environmental factors (Knip 1997; Akerblom et al. 2002; Ilonen et al. 2002; Achenbach et al. 2005). Autoantibodies against beta-cell proteins, such as glutamic acid decarboxylase (GAD) (Baekkeskov et al. 1990), the tyrosine phosphatase like protein islet antigen-2 (IA-2) (Lan et al. 1996; Notkins et al. 1996), insulin (Palmer et al. 1983) and zink transporter 8 (ZnT8) (Wenzlau et al. 2007), are produced during the autoimmune destruction of the beta cells. One or more of these autoantibodies are present in 90-95% of individuals when hyperglycemia is initially detected (Notkins et al. 2001). Type 1 diabetes occurs predominantly in children and adolescents, usually as a rapidly progressive form, but may also occur in adults, often as a slowly progressive form, referred to as latent autoimmune diabetes in adults (LADA). A minority of patients with type 1 diabetes fall into the category named idiopathic diabetes (American Diabetes Association 2008). This form of diabetes is most common in non-Caucasians and is strongly inherited, lacks immunological evidence for beta-cell autoimmunity, and is not HLA associated.

Type 2 diabetes

In type 2 diabetes, the cause is a combination of resistance to insulin action and defects in insulin secretion (American Diabetes Association 2008). In adults, type 2 diabetes is much more prevalent than type 1 diabetes, but it is rare in children and adolescents in Sweden. Individuals with type 2 diabetes do initially not need insulin treatment to survive, and often not later in life either. Most patients are obese, which causes some degree of insulin resistance. Type 2 diabetes is often associated with a strong genetic predisposition and the risk
of developing this disease increases with age, obesity and lack of physical activity. Individuals with this form of diabetes are often undiagnosed for many years because their hyperglycemia is not severe enough to present symptoms, but they have increased risk to develop macrovascular and microvascular complications. Insulin sensitivity may be increased, but not restored to normal, by weight reduction, increased physical activity and/or pharmacological treatment of hyperglycemia.

**Gestational diabetes**

Any degree of glucose intolerance with onset or first recognition during pregnancy is referred to as gestational diabetes (American Diabetes Association 2008).

**Other specific types of diabetes**

Some forms of diabetes may be associated with genetic defects of the beta cell, which are often referred to as maturity-onset diabetes of the young (MODY) (Stride et al. 2002). These individuals have impaired insulin secretion with minimal or no defects in insulin action. Other types of diabetes are associated with genetic defects in insulin action or diseases of the exocrine pancreas, endocrinopathies, drug- or chemical-induced diabetes, infections, other genetic syndromes sometimes associated with diabetes, and uncommon forms of immune-mediated diabetes (American Diabetes Association 2008). The last category includes patients with stiff person syndrome (SPS) who also have developed diabetes. SPS is a rare neurologic disorder characterised by muscle rigidity and episodic spasms involving axial and limb musculature, and the patient has usually very high levels of GADA (Levy et al. 1999). About one third of patients with SPS will develop diabetes.

**Epidemiology of type 1 diabetes**

The International Diabetes Federation has estimated the number of children globally aged 0-14 years with type 1 diabetes to be 480 000 in 2010, with 76 000 newly diagnosed cases a year (IDF 2009). One quarter of the cases come from South East Asia and more than one fifth from Europe. The incidence of childhood onset type 1 diabetes is increasing worldwide, with an overall annual increase of about 3% (Onkamo et al. 1999; The DIAMOND Project Group 2006; IDF 2009; Patterson et al. 2009). The DIAMOND project group has examined incidence and trends of type 1 diabetes worldwide for the period of 1990-1999, giving an annual increase in incidence of 2.8% (The DIAMOND Project Group 2006), and the
EURODIAB study group has done the same for Europe during 1989-2003, resulting in an annual increase of 3.9% (Patterson et al. 2009).

The increase in incidence has been observed in countries with both high and low prevalence, with a more steeply increase in some of the low prevalence countries, such as those in central and eastern Europe (IDF 2009). The highest increase was found in children younger than 5 years of age, particularly in European populations (Karvonen et al. 1999; Green et al. 2001; 2006; Patterson et al. 2009). Finland has the highest incidence in the world; in 2005 the annual incidence was 64.2/100 000 per year in children before 15 years of age (Harjutsalo et al. 2008). Sweden has the second highest incidence, with an incidence in 2009 of 44.0/100 000 per year in children 0-14.9 years of age (SWEDIABKIDS 2009).

In general, the incidence of type 1 diabetes increases with age, peaking at puberty (Soltesz et al. 2007). The overall sex ratio for type 1 diabetes is roughly equal in children, with a minor male excess in incidence in Europe. A seasonality of onset has been reported, with a peak occurring in winter, and it is more pronounced in countries with marked differences between summer and winter temperatures (Dahlquist et al. 1994; Soltesz et al. 2007).

In conclusion, type 1 diabetes is a serious disease resulting from destruction of the beta cells in the pancreas. The incidence of type 1 diabetes is increasing worldwide and Sweden has the second highest incidence in the world.

PATHOGENESIS OF TYPE 1 DIABETES

The etiology of type 1 diabetes is largely unknown, but a combination of genetic predisposition, environmental factors and a dysregulated immune system is believed to play an important role for development of the autoimmune process leading to the disease (Figure 4). The genetic susceptibility of type 1 diabetes is mainly dependent of HLA class II genes, but other genes are also involved (Rich et al. 2009). Environmental factors that are suggested to influence the development of type 1 diabetes include viral infections, early infant diet, toxins and psychological stress (Peng et al. 2006). The autoimmune process, causing an inflammation in the beta cells (insulitis), is characterized by infiltration of CD8-positive cytotoxic T cells, CD4-positive T cells, B cells and macrophages (Imagawa et al. 1999; Moriwaki et al. 1999). This process, which may be initiated several years before clinical onset
of type 1 diabetes, leads to decreased beta-cell mass, reduced insulin production and finally to clinical type 1 diabetes. During the pre-clinical period, autoantibodies against beta-cell antigens often circulate in the peripheral blood, and measurement of these autoantibodies can be used for identification of individuals at risk for the disease.

**Figure 4.** Schematic illustration of the development of type 1 diabetes. Interaction between genetic predisposition and environmental triggers, together with a dysregulated immune system, may induce an autoimmune response with autoantibody production, leading to loss of beta cells and progression to type 1 diabetes. Modified from (Atkinson et al. 2001).

**Genetic risk**

Most of the children who develop type 1 diabetes have genetic predisposition for the disease. The HLA gene complex is responsible for about 50% of the genetic risk for type 1 diabetes and the remaining genetic susceptibility is conferred by a large number of loci, where most of them have minor effects (Ilonen et al. 2002; Rich et al. 2009). Other genes known to have effect on the risk of type 1 diabetes include insulin (INS), cytotoxic T lymphocyte antigen-4 (CTLA-4) and protein tyrosine phosphatase N22 (PTPN22) (Redondo et al. 2002; Onengut-Gumuscu et al. 2004).
**HLA**

The HLA gene complex is located on the short arm of chromosome 6 (6p21) and contains genes encoding HLA class I (HLA-A, -B and -C) and class II (DR, DQ and DP) molecules, which are peptide presenting molecules for T cells, consisting of an α- and a β-chain (She 1996; Undlien et al. 2001). These genes are the most polymorphic genes known in humans and there is a strong linkage disequilibrium between the genes, which means that the alleles at one HLA locus are non-randomly associated with alleles at other HLA loci. Polymorphisms in the HLA genes affect the conformation of the molecule and especially the peptide binding groove, and define which peptide that will be bound and presented to T cells, thus shaping the T cell repertoire (Ilonen et al. 2002).

Type 1 diabetes has strong HLA associations, with linkage to the DQA and DQB genes, and is influenced by the DRB genes (She 1996; Ilonen et al. 2002). The strongest determinant of genetic risk is the presence of risk associated HLA class II haplotypes DR3-DQ2 (DRB1*0301-DQA1*0501-DQB1*0201) and DR4-DQ8 (DRB1*04-DQA1*0301-DQB1*0302) or especially the combination of them both (Table 1) (Sanjeevi et al. 1995; She 1996; Ilonen et al. 2002; Redondo et al. 2002). About 90% of individuals with type 1 diabetes have at least one of these two high-risk haplotypes compared to approximately 20% of the general population (Redondo et al. 2002). The DRB1 allele modifies the risk conferred by the DQ8 molecule; the DRB1*0401, *0402 and *0405 are associated with high susceptibility, the DRB1*0404 with moderate susceptibility and DRB1*0403 with protection against type 1 diabetes.

Other class II haplotypes are protective, such as DR2-DQ6 (DRB1*15-DQA1*0102-DQB1*0602), which is the most significant in the Northern European population (Table 1) (Baisch et al. 1990; Redondo et al. 2002). This haplotype seems to confer dominant protection, since it is protective also in the presence of a high-risk haplotype in the same individual (Baisch et al. 1990; Pugliese et al. 1995; Redondo et al. 2002). About 20% of Europeans have DR2-DQ6 while less than 1% of children with type 1 diabetes have this allele (Redondo et al. 2002). Besides the susceptible and protective haplotypes, there are also neutral HLA class II haplotypes, which do not affect the risk of type 1 diabetes (Table 1).
**Table 1.** Susceptibility, protective and neutral associated haplotypes (according to Jorma Ilonen, personal communication 2005).

<table>
<thead>
<tr>
<th>Susceptibility</th>
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<tbody>
<tr>
<td>DRB1<em>0401/2/5-DQB1</em>0302 (DR4-DQ8)</td>
</tr>
<tr>
<td>DRB1<em>0404-DQB1</em>0302 (DR4-DQ8)</td>
</tr>
<tr>
<td>DRB1<em>0301-DQA1</em>0501-DQB1*0201 (DR3-DQ2)</td>
</tr>
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<table>
<thead>
<tr>
<th>Protective</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1<em>15-DQA1</em>0102-DQB1*0602 (DR2-DQ6)</td>
</tr>
<tr>
<td>(DR5)-DQA1<em>05-DQB1</em>0301</td>
</tr>
<tr>
<td>(DR7)-DQA1<em>0201-DQB1</em>0303</td>
</tr>
<tr>
<td>(DR14)-DQA1<em>0101-DQB1</em>0503</td>
</tr>
<tr>
<td>DRB1<em>0403-DQB1</em>0302</td>
</tr>
<tr>
<td>(DR1301)-DQB1*0603</td>
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<table>
<thead>
<tr>
<th>Neutral</th>
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<tbody>
<tr>
<td>(DR4)-DQA1<em>0301-DQB1</em>0301 (DR4-DQ7)</td>
</tr>
<tr>
<td>(DR1)-DQB1*0501</td>
</tr>
<tr>
<td>(DR7)-DQA1<em>0201-DQB1</em>02</td>
</tr>
<tr>
<td>(DR1302)-DQB1*0604</td>
</tr>
<tr>
<td>(DR9)-DQA1<em>03-DQB1</em>0303</td>
</tr>
<tr>
<td>(DR8)-DQB1*04</td>
</tr>
<tr>
<td>(DR7)-DQA1<em>02-DQB1</em>02</td>
</tr>
<tr>
<td>(DR4)-DQA1<em>03-DQB1</em>0301</td>
</tr>
<tr>
<td>(DR2)-DQB1*0601</td>
</tr>
<tr>
<td>(DR16)-DQB1*0502</td>
</tr>
<tr>
<td>DQB1*0609</td>
</tr>
</tbody>
</table>

**Other genetic factors**

**INS:** The insulin gene (INS) is located on chromosome 11p15 and includes a non-coding region with a variable number of tandem repeats (VNTR) (Bennett et al. 1995; Redondo et al. 2002; Ziegler et al. 2010). Polymorphisms in this region are associated with risk of diabetes and influence thymic insulin messenger RNA (mRNA). There are three main types of the insulin VNTR defined by the number of repeats, class I, class II and class III, where class III has the highest number. The class I VNTRs are most common in Caucasians and the class II alleles are rare (Stead et al. 2002). Homozygosity for class I alleles is associated with high risk for diabetes, while class III alleles confer dominant protection (Redondo et al. 2002). Class III alleles are associated with higher expression of insulin mRNA within the thymus and high concentration of thymic insulin might lead to negative selection of high-avidity autoreactive T cells and thus to the development of tolerance.
**CTLA-4:** The cytotoxic T lymphocyte antigen 4 (CTLA-4) gene is located on chromosome 2q33 and encodes a receptor expressed by activated T cells (Redondo et al. 2002). This receptor can limit the proliferative response of activated T cells, some of which could be autoreactive, upon binding to B7 molecules, and can also mediate T cell apoptosis. Polymorphisms or mutations that alter the activity of CTLA-4 are believed to play a role in the risk for developing autoimmunity. A polymorphism in the first exon of CTLA-4 (49 A/G) results in an amino acid change (threonine/alanine) in the leader peptide of the expressed protein. The presence of an alanine at codon 17 of CTLA-4 has been associated with genetic susceptibility to type 1 diabetes (Nistico et al. 1996; Donner et al. 1997).

**PTPN22:** The protein tyrosine phosphatase N22 (PTPN22) gene, located on chromosome 1p13, encodes a lymphoid-specific phosphatase known as Lyp (Onengut-Gumuscu et al. 2004; Ladner et al. 2005). This protein is a negative regulator of T cell activation by dephosphorylating T cell receptor activation-dependent kinases. The single nucleotide polymorphism C1858T of the PTPN22 gene have been associated with type 1 diabetes. Individuals lacking the C allele of PTPN22 may have reduced capacity to downregulate T cell responses and may therefore be more susceptible to autoimmunity.

**Environmental factors**

The rapid increase in incidence of type 1 diabetes cannot be explained by changes in genetic predisposition, but rather by environmental factors. In addition, the relatively low concordance (with both twins affected) in monozygotic twins, 21-70%, and 6% of siblings to type 1 diabetic patients that develops the disease (Redondo et al. 1999; Redondo et al. 2002; Aly et al. 2006), further emphasize a role of environmental factors in the etiology of type 1 diabetes. Several environmental factors, including viral infections, cow’s milk, gluten and psychological stress, have been suggested to trigger the autoimmune response and the development of type 1 diabetes, as reviewed in (Akerblom et al. 2002; Peng et al. 2006; Soltesz et al. 2007). A number of studies are ongoing aiming to further investigate the role of different environmental factors on the development of type 1 diabetes, see the section “Prediction and prevention studies”.
Review of the literature

**Viral infections**

Viral infections have been suggested to trigger autoimmunity and type 1 diabetes (van der Werf et al. 2007). Enterovirus infections, including Coxsackie B4, are the most studied, and have been associated with seroconversion to islet autoantibody positivity and with diabetes onset (Hyoty et al. 1995; Salminen et al. 2003; Moya-Suri et al. 2005). Congenital rubella infection has been found to associate with a high rate of subsequent type 1 diabetes, but effective immunization programs have eliminated this virus in most Western countries (Menser et al. 1978). Enteroviral infections during pregnancy have been associated with increased risk for type 1 diabetes in the offspring (Dahlquist et al. 1995; Hyoty et al. 1995). Interestingly, it has been observed that in populations with high incidence of type 1 diabetes, the frequency of enterovirus infections has tended to decrease over the last decades and is lower than in populations with low incidence of type 1 diabetes (Viskari et al. 2000; Viskari et al. 2004). This is in line with the polio hypothesis, which suggests that the complications of enterovirus infections become more common in an environment with a decreased rate of infections leading to a lack of immunity in the population.

The hygiene hypothesis suggests that the increased hygiene, leading to a lack of normal background infections, predisposes the immune system to autoimmunity, including type 1 diabetes (Ludvigsson 2006). Probably, changes in the gut bacterial flora influence the maturation of the immune system, leading to imbalance and thereby autoimmune reactions in genetically predisposed individuals (Vaarala 1999b). Further, the increased hygiene might lead to a lower immunity against certain viruses (Viskari et al. 2005).

**Dietary factors**

A number of dietary factors have been suggested to influence the development of beta-cell autoimmunity and type 1 diabetes, as reviewed in (Virtanen et al. 2003). Factors that seem to decrease development of autoimmunity include greater intake of breast milk, nicotinamide, zinc, and vitamins C, D and E. On the other hand, increased islet autoimmunity seems to be influenced by factors as early introduction of cow’s milk, gluten, nitrate, nitrite, and increased calories causing increased linear growth and weight. Nitrate and nitrite are mainly found in food, but may also originate from cigarettes, car interiors and cosmetics.

Breast-feeding has been suggested to have a protective effect; high frequency of breast-feeding has been associated with low incidence of type 1 diabetes and short duration of
breast-feeding has been reported to increase the risk of type 1 diabetes (Borch-Johnsen et al. 1984; Virtanen et al. 1993; Virtanen et al. 2003). In a Finnish study, the effects of duration of breast-feeding and the age at introduction of supplementary milk were studied (Virtanen et al. 1993). The results indicated that early introduction of cow’s milk increased the risk for type 1 diabetes, and that this factor may overcome the protective effects of breast-feeding. Further, Wahlberg et al found that early introduction of cow’s milk based formula and high consumption of cow’s milk were associated with higher levels of beta-cell autoantibodies (Wahlberg et al. 2006). It has been proposed that an early exposure to cow’s milk formula may result in an immune response to bovine insulin, and that this could trigger an immune response to human insulin, which has a very similar amino acid sequence as bovine insulin (Vaarala et al. 1999a). In addition, feeding with cow’s milk formula during infancy has been associated with increased weight gain (Johansson et al. 1994), which might induce beta-cell stress, see section below. Further, introduction of gluten too early (before 3 months) (Ziegler et al. 2003), or too late (after 6 months) (Wahlberg et al. 2006), seems to be a risk factor for induction of autoantibodies.

**Beta-cell stress**

Beta-cell stress has been suggested as a risk factor for the development of type 1 diabetes (Ludvigsson 2006). During psychological stress and periods of rapid growth, such as infancy and puberty, the beta cells have to work hard to produce insulin. This increased insulin production may result in beta-cell stress and stimulation of the autoimmune process, leading to overt diabetes. This beta-cell stress hypothesis is an extension of the accelerator hypothesis (Wilkin 2001), which states that increased insulin resistance, associated with childhood overweight and obesity, creates greater insulin secretory demand on the islets, leading to acceleration of beta-cell destruction and type 1 diabetes. The accelerator hypothesis suggests that the increased incidence of type 1 diabetes may be caused by an accelerated progression rather than by an increase in the absolute lifetime risk.

Psychological stress may, in certain individuals, cause insulin resistance and thereby beta-cell stress and diabetes-associated autoimmunity. Psychological factors that have been found to influence the development of autoantibodies, in one or two and a half year old children of the general population, include high parental stress and serious life events (Sepa et al. 2005; Sepa et al. 2005).
In conclusion, genetic risk factors, mainly HLA, together with environmental factors, such as viral infections and dietary factors, seem to play an important role for the development of type 1 diabetes.

**Beta-cell autoantigens and autoantibodies**

Pancreatic beta-cell autoantigens are the targets of immune-mediated destruction of beta cells (Yoon et al. 2005). One of the most common immunologic markers of individuals with autoimmune diabetes is the presence of autoantibodies against beta-cell autoantigens. These autoantibodies can also be used for prediction of type 1 diabetes, both in high-risk individuals and in the general population, where positivity to multiple autoantibodies confer the highest risk (Bingley et al. 1994; Bingley et al. 1997; Verge et al. 1998; Kulmala et al. 2001; LaGasse et al. 2002). The autoantibodies most commonly studied are directed against glutamic acid decarboxylase (GADA) (Baekkeskov et al. 1990), the tyrosine phosphatase like protein islet antigen-2 (IA-2A) (Lan et al. 1996; Notkins et al. 1996) and insulin (IAA) (Palmer et al. 1983). Islet cell antibodies (ICA) (Bottazzo et al. 1974) were previously widely used to study the clinical course and pathogenesis of type 1 diabetes, but were to a large extent replaced by GADA and IA-2A, when methods for detection of these autoantibodies were developed. Recently, autoantibodies against ZnT8 (ZnT8A) were discovered as an additional marker for type 1 diabetes (Wenzlau et al. 2007).

**Islet cell antibodies (ICA)**

Islet cell antibodies (ICA), recognizing islet cytoplasmic antigens, were detected many years ago in newly-diagnosed type 1 diabetic patients (Bottazzo et al. 1974) and comprise autoantibodies to a number of antigens, with a predominance of GADA and IA-2A (Notkins et al. 1996; Notkins et al. 2001). The presence of organ-specific pancreatic antibodies provides evidence for type 1 diabetes as an autoimmune disease (Bottazzo et al. 1974; MacCuish et al. 1974). ICA is measured using immunofluorescence, by incubation of sera from type 1 diabetic patients with frozen tissue sections of normal blood group 0 pancreas, which leads to staining of the pancreatic islets (Notkins et al. 2001). IAA is not recognized in the ICA test, because insulin and c-peptide leach out from the unfixed frozen tissue sections during sample preparation.
Glutamic acid decarboxylase (GAD)

Glutamic acid decarboxylase (GAD) is a biosynthesizing enzyme of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) and has been the most extensively studied beta-cell autoantigen (Baekkeskov et al. 1990; Yoon et al. 2005). This antigen was first discovered in serum from children with type 1 diabetes in Linköping, and was described as an antigen with the weight of 64kDa (Baekkeskov et al. 1982). Later, Baekkeskov et al. discovered that 64 kDa actually was GAD (Baekkeskov et al. 1990). This enzyme is expressed at high levels by pancreatic beta cells and a subpopulation of central nervous system neurons. There are two isoforms of GAD, GAD65 and GAD67, with molecular masses of 65kDa and 67kDa (Bu et al. 1992). In human pancreatic islet cells only the GAD65 isoform is expressed (Hagopian et al. 1993). GAD65 is an intracellular membrane anchored protein consisting of 585 amino acids (Figure 5) and the GAD65 gene is located on chromosome 10p11 (Bu et al. 1992; Christgau et al. 1992). The protein is synthesized within the cytoplasm as a soluble hydrophilic molecule, but becomes membrane anchored after a two-step modification at the NH2-terminal domain. GAD65 is located to the membrane of small synaptic-like microvesicles in the islet beta cells.

Autoantibodies in type 1 diabetes primarily recognize the GAD65 isoform (Hagopian et al. 1993). In addition to autoantibodies, GAD-specific CD4-positive T cells and HLA-A*0201-restricted CD8-positive cytotoxic T cells reactive against GAD have been observed in recently diagnosed type 1 diabetic patients and in high-risk individuals (Yoon et al. 2005). These results indicate that GAD may be an important target antigen and GAD-reactive T cells may play a pathogenic role in the destruction of pancreatic beta cells. Further, there is molecular mimicry between GAD (amino acids 247-279) and Coxsackie B4 virus (amino acids 32-47 of the P2-C protein of Coxsackie B virus), which might be a link between enterovirus infections and development of type 1 diabetes (Atkinson et al. 1994).
Figure 5. Schematic representation of the GAD65 protein. The regions of membrane anchoring, maximum divergence with GAD67, Coxsackie virus similarity and PLP binding are shown in the figure, as well as the cysteine rich regions (CYS) and the major conformational epitope regions recognized by GADA. Adapted from (Leslie et al. 1999).

**GAD autoantibodies (GADA)**

GADA has been found in approximately 50-80% of newly diagnosed type 1 diabetic patients (Bonifacio et al. 1995; Sabbah et al. 1999; Strebelow et al. 1999; Winter et al. 2002; Holmberg et al. 2006), and often persists in sera for many years after the diagnosis (Savola et al. 1998). Besides the presence of GADA in type 1 diabetes, this autoantibody has also been found in other autoimmune diseases, such as SPS (Levy et al. 1999), where approximately 80% of the patients have GADA (Rakocevic et al. 2004).

**Epitopes:** Diabetes-related GADA bind mainly to conformation dependent epitopes on GAD (Figure 5), where the middle region of the protein is the most important (Padoa et al. 2003; Ronkainen et al. 2004; Schlosser et al. 2005a). However, the GADA response in the preclinical stage of type 1 diabetes is dynamic, with epitope spreading accompanied by an increase in the number of epitopes recognized (Schlosser et al. 2005a). Initial reactivity of GADA appears to target mainly the middle region of GAD (Bonifacio et al. 2000) and spreads often rapidly to the C-terminal region (Ronkainen et al. 2006). Children with high risk of developing type 1 diabetes, defined by having more than one autoantibody, often show GADA reactivity both to epitopes in the middle and C-terminal part of the GAD molecule (Hoppu et al. 2004a; Schlosser et al. 2005a). In children with both autoantibodies and genetic risk for the disease this GADA reactivity generally spreads towards epitopes on the N-terminal part and other epitopes located in the middle (Schlosser et al. 2005a). However, the
most important epitope region on GAD at diagnosis and the following years after diagnosis is the middle region, often in combination with the C-terminal region (Falorni et al. 1996; Ronkainen et al. 2004). After disease onset the GADA epitope binding pattern seems to be quite stable (Hampe et al. 2002).

**Subclasses:** IgG1 is the dominant IgG subclass of GADA during the early antibody response in prediabetic individuals and in newly diagnosed type 1 diabetic individuals (Bonifacio et al. 1999; Hoppu et al. 2004a; Ronkainen et al. 2006). Antibodies of subclasses IgG2 and IgG3 often appear together with IgG1 or soon after the initial IgG1 response, while IgG4 is the last subclass to appear (Ronkainen et al. 2006). In addition, a broad initial response with three or four IgG subclasses and the lack of an emerging IgG4 response during follow-up have been associated with increased risk for progression to type 1 diabetes.

**The tyrosine phosphatase like protein islet antigen-2 (IA-2)**

The tyrosine phosphatase like protein islet antigen-2 (IA-2) is a transmembrane protein consisting of 979 amino acids with a molecular mass of 106 kDa and is a major autoantigen in type 1 diabetes (Lan et al. 1996). IA-2 belongs to the protein tyrosine phosphatase (PTP) family and is expressed in pancreatic islets and brain tissues (Notkins et al. 1996). The IA-2 gene is located on chromosome 2q35 and encodes a protein consisting of a signal peptide and an extracellular, a transmembrane and an intracellular domain (Figure 6) (Lan et al. 1994; Notkins et al. 2001). The PTP core sequence, a highly conserved region of 11 amino acids located within the intracellular domain of IA-2, differs from other PTPs primarily in the substitution of aspartic acid for alanine, which might be the cause of the lack of enzyme activity for IA-2 (Notkins et al. 1996). IA-2β, which is closely related to IA-2, is another member of the PTP family and does also act as an autoantigen (Lu et al. 1996). ICA 512 represents a fragment of IA-2, 453 amino acids shorter, and covers the region from amino acid 389 to amino acid 914 (Notkins et al. 1996). The extracellular domain of ICA 512 appears to reside within secretory granules and the intracellular domain is located in the cytoplasm (Solimena et al. 1996).
Review of the literature

Figure 6. Schematic representation of the IA-2 protein. The signal peptide and the extracellular, transmembrane and intracellular domains are shown in the figure, as well as the cysteine rich regions (CYS), the PTP core sequence and the region containing epitopes recognized by IA-2A. Adapted from (Leslie et al. 1999).

**IA-2 autoantibodies (IA-2A)**

IA-2A is found in about 55-80% of newly diagnosed type 1 diabetic patients (Bonifacio et al. 1995; Sabbah et al. 1999; Strebelow et al. 1999; Winter et al. 2002). The frequency of IA-2A varies with age and HLA genotype. In young children and in patients with HLA DR4-DQA1*0301-DQB1*0302 genotype, the frequency and/or level of IA-2A is highest (Bonifacio et al. 1995; Genovese et al. 1996; Gorus et al. 1997; Savola et al. 1998). The close association between IA-2A and HLA DR4 indicates that IA-2A may be a more specific marker of beta-cell destruction than GADA. IA-2A seems to be a strong predictor for development of type 1 diabetes (Achenbach et al. 2004b). Further, high levels of IA-2A seem to be correlated with rapid progression of disease (Bingley et al. 1994; Christie et al. 1994; Kulmala et al. 1998).

Autoantibodies to IA-2β are found in 35-50% of patients with type 1 diabetes. Since more than 95% of the patients who have autoantibodies to IA-2β also have IA-2A, screening for IA-2beta autoantibodies for diagnosis is often omitted (Leslie et al. 1999). However, it has been shown that presence of IA-2β autoantibodies in IA-2A positive individuals can increase prediction of type 1 diabetes (Achenbach et al. 2004b).

**Epitopes:** IA-2A bind exclusively to epitopes located in the cytoplasmic domain of the molecule (amino acids 601-979) (Figure 6) (Zhang et al. 1997; Dromey et al. 2004). Two linear epitopes within the juxtamembrane domain (amino acids 611-620 and 621-630,
respectively) and conformational epitopes in the PTP domain toward the C terminus of the molecule (aa 931-979) and within the central region (aa 795-889) of the PTP domain have been identified (Dromey et al. 2004). The disulphide bonds within the intracellular domain seem important for maintaining the antigenic structure of IA-2, since loss of these bonds result in almost total loss of reactivity with type 1 diabetic autoantibodies (Xie et al. 1997). IA-2A present early in the disease process are often directed to juxtamembrane domain epitopes (Dromey et al. 2004), and IA-2A reactivity to these epitopes have been associated with an increased risk of progression to type 1 diabetes (Hoppu et al. 2004b). By the time of diabetes onset, IA-2A reactivity has spread to epitopes predominantly in the PTP domain and reactivity to the juxtamembrane domain epitopes are less frequent (Dromey et al. 2004).

**Subclasses:** IA-2A of the IgG1 subclass dominates the autoantibody response in prediabetic individuals and in newly diagnosed type 1 diabetic individuals (Bonifacio et al. 1999; Achenbach et al. 2004b; Hoppu et al. 2004b). One study showed a correlation between IA-2A response of IgG4 subclass and protection from diabetes (Seissler et al. 2002), but others do not (Bonifacio et al. 1999; Achenbach et al. 2004b; Hoppu et al. 2004b). High risk of progression to type 1 diabetes was in one of these studies associated with IgG2, IgG3 and/or IgG4 subclasses of IA-2A (Achenbach et al. 2004b).

**Insulin**

Insulin, one of the major autoantigens in type 1 diabetes, is a hormone that regulates energy and glucose metabolism in the body. The blood glucose level is lowered by insulin, by accelerating the transport of glucose into cells. Insulin is secreted by the beta cells, which constitute about 70% of the pancreatic islet cells. The protein consists of 51 amino acids and is encoded on chromosome 11p15 (Notkins et al. 2001). The insulin molecule is a heterodimer consisting of an A and a B chain of 21 and 30 amino acids, respectively, linked by two disulfide bridges (Figure 7). Insulin is synthesized as pre-proinsulin by the beta cells of the pancreas, and after cleavage of an NH2-terminal sequence, proinsulin is formed (Eisenbarth 2008). After folding of proinsulin into its correct secondary and tertiary structure, the 5.8 kDa insulin protein is formed by proteolytic cleavage and removal of the connecting peptide (C-peptide), which is a large peptide in the middle of the proinsulin molecule.
Review of the literature

**Figure 7.** The structure of proinsulin and insulin. Proinsulin is formed from pre-proinsulin and after removal of the connecting peptide (C-peptide) the insulin protein is formed, consisting of an A and a B chain of 21 and 30 amino acids (aa), respectively.

**Insulin autoantibodies (IAA)**

In 1983, IAA was identified in newly diagnosed untreated diabetic patients (Palmer et al. 1983). This is often the first autoantibody to appear in young children as a sign of beta-cell autoimmunity and the levels correlate inversely with age (Vardi et al. 1988; Ziegler et al. 1999). IAA is found in about 40-70% of newly diagnosed type 1 diabetic children (Sabbah et al. 1999; Strebelow et al. 1999; Winter et al. 2002; Williams et al. 2003; Holmberg et al. 2006).

**Epitopes:** The epitopes on insulin recognized by IAA are not well characterized. However, it has been observed that IAA specific to human insulin seems to bind epitopes located on the B chain of insulin, while antibodies cross-reactive to insulin of other species recognize both the B chain and conformational epitopes involving both the A and B chains (Potter et al. 2000). Further, IAA that bind epitopes dependent on threonine B30 seem not to be associated with type 1 diabetes, while those who bind conformational epitopes incorporating the A and B chains frequently are. Epitope analysis of IAA using a recombinant Fab (rFab) revealed that IAA in type 1 diabetic patients can bind an epitope located predominantly on the A chain (Padoa et al. 2005).

**Subclasses:** IAA of the IgG1 subclass is often predominant in both pre-diabetic and type 1 diabetic individuals, but the IgG3 subclass is also frequently occurring in pre-diabetic individuals (Bonifacio et al. 1999; Potter et al. 2000; Hoppu et al. 2004c). In one study,
genetically susceptible young children who progressed rapidly to clinical type 1 diabetes were characterized by strong IgG1 and IgG3 responses to insulin, whereas a weak or absent IgG3 response was associated with relative protection from disease (Hoppu et al. 2004c). In another study, the risk of progression to type 1 diabetes was higher in individuals with IAA of subclasses IgG2, IgG3 or IgG4 than in those without these IgG subclasses (Achenbach et al. 2004b).

**Affinity:** In IAA positive children from the general population, antibody affinity can identify those at high and low risk (Schlosser et al. 2005b). High affinity has been associated with HLA DRB1*04, young age of IAA appearance and progression to multiple autoantibodies or type 1 diabetes (Achenbach et al. 2004a). In addition, the A8-A13 region on insulin is important for binding of high-affinity IAA.

**Zink transporter 8 (ZnT8) and ZnT8 autoantibodies (ZnT8A)**

ZnT8, a multispansing transmembrane protein belonging to a large cation efflux family, was recently discovered to be a major autoantigen in type 1 diabetes (Wenzlau et al. 2007). Approximately half of the ZnT8 molecule comprises six membrane-spanning regions, which might hinder the folding of the protein in an aqueous environment. Therefore, C-terminal, N-terminal and N- and C-terminal fusion proteins have been constructed for use in radioimmunoassays to detect ZnT8A (Wenzlau et al. 2007; Achenbach et al. 2009). Autoantibodies to the C-terminal part of ZnT8 have shown a strong relation to disease development. ZnT8A has been found in about 60% of children with new onset type 1 diabetes. In the first report, ZnT8A was found in 26% of type 1 diabetic patients previously classified as autoantibody negative (Wenzlau et al. 2007). Both the levels and the prevalence of ZnT8A increase with age, and the antibodies appear frequently in children by 3 years of age. ZnT8A usually precedes the disease by many years and emerges often after GADA and IAA.

*In conclusion, autoantibodies against beta-cell proteins can be used as markers for the autoimmune process, where GADA, IA-2A, IAA and ZnT8A are the most commonly used today.*
PREDICTION AND PREVENTION OF TYPE 1 DIABETES

The risk of developing type 1 diabetes is increased in first degree relatives of type 1 diabetic patients compared to the general population. The risk of type 1 diabetes in the general population is approximately 0.5%, or even higher in high incidence countries, while the risk for siblings is about 6%, the risk for offspring of a diabetic mother is 1-4% and of a diabetic father 6-9% (Hawa et al. 2002; Redondo et al. 2002). For prediction of type 1 diabetes, a combination of genetic and immunological markers is useful.

Presence of HLA susceptibility genes in an individual increases the risk of developing type 1 diabetes, but only to a small extent, as reviewed in (Ziegler et al. 2010). For example, risk of type 1 diabetes in children without a family history of this disease can vary from about 0.01% up to more than 5%, depending on their HLA class II genotypes. Further, some of the HLA genotypes confer extreme protection, such as DQB1*0602. The risk of type 1 diabetes in a child carrying this allele and with a family history of type 1 diabetes is reduced to about 1% of the risk in children with similar family history but without this allele.

The most important increase in type 1 diabetes risk of a child occurs when beta-cell autoantibodies develop. Autoantibodies to insulin, GAD, IA-2 and ZnT8 are good markers for prediction of type 1 diabetes in children and young adults (Bingley et al. 1994; Bingley et al. 1997; Kulmala et al. 1998; Verge et al. 1998; LaGasse et al. 2002; Wenzlau et al. 2007). Presence of only one of these four autoantibodies is associated with a marginally increased risk, both in individuals with and without a family history of type 1 diabetes. However, positivity to two or more autoantibodies is highly predictive of type 1 diabetes (Verge et al. 1996; Bingley et al. 1997; Eising et al. 2010; Knip et al. 2010). Further, the risk of type 1 diabetes varies dependent on which of the antibodies that is present, and IA-2A has been associated with the highest risk (Achenbach et al. 2004b).

In addition to autoantibody positivity, characteristics of the autoantibodies such as greater titer and affinity, broadness of epitope reactivity and certain distribution of IgG subclasses are also associated with high risk of type 1 diabetes (Achenbach et al. 2004b; Achenbach et al. 2006; Achenbach et al. 2007; Mayr et al. 2007). For example, in one study, the risk of progression to type 1 diabetes was higher in individuals with autoantibodies binding multiple epitopes than single epitope reactivity, and in individuals with IA-2β autoantibodies than in those
without these autoantibodies (Achenbach et al. 2004b). The risk of developing type 1 diabetes can be stratified from <1% up to >70% by using various combinations of risk markers (Figure 8) (Ziegler et al. 2010).

**Figure 8.** Stratification of type 1 diabetes risk by islet autoantibody properties together with other risk markers, such as HLA genotype and age. Characteristics of the antibody response can increase prediction for disease progression. Ab = antibody. Adapted from (Ziegler et al. 2010).

**Prediction and prevention studies**

Accurate assessment of risk of type 1 diabetes in non-diabetic individuals is necessary to identify those individuals suitable for recruitment into intervention trials aiming to prevent the clinical onset of diabetes. A number of intervention trials have recently been performed and others are ongoing (Ludvigsson 2010a), e.g. in pre-diabetic individuals; the trial to reduce type 1 diabetes in the genetically at risk (TRIGR) (Knip et al. 2010), the European nicotinamide diabetes intervention trial (ENDIT) (Gale et al. 2004), the Diabetes Prevention Trial (DPT-1) (DPT-1 2002) and The Diabetes Prediction and Prevention study (DIPP) (Kupila et al. 2001), and in newly diagnosed type 1 diabetic patients; DiaPep277 (Schloot et
al. 2007), anti-CD3 (Herold et al. 2009), anti-CD20 (Pescovitz et al. 2009), IFN-α (Rother et al. 2009) and GAD-alum (Ludvigsson et al. 2008). Some of these studies are described below.

In several prediction studies, children at increased genetic risk of type 1 diabetes are followed with frequent sampling and autoantibody analysis. In the All Babies in Southeast Sweden (ABIS) study (Ludvigsson et al. 2001), children from the general population are followed without selection for genetic risk, see the section “Study populations” for more information about this study. Ongoing studies, such as the Diabetes Autoimmunity Study in the Young (DAISY) (Barker et al. 2004), the Environmental Determinants in Diabetes of the Young (TEDDY) (The TEDDY Study Group 2008) and ABIS, investigate the role of different environmental factors on the development of autoimmunity and type 1 diabetes.

DAISY
The Diabetes Autoimmunity Study in the Young (DAISY) is a prospective study in Denver, Colorado, USA that follows young first-degree relatives (siblings or offspring) of persons with type 1 diabetes and newborn children with high or moderate risk HLA genotypes. It was shown that the predictive value of the autoantibody measurements could be increased by blinded duplicate and independent sample retesting together with the level of the autoantibody. Approximately one third of the autoantibody positive individuals were false positive, one third transiently positive and one third persistently positive (Barker et al. 2004). Further, early or late introduction of cereal seemed to increase the risk of islet cell autoimmunity in children younger than 3 years of age (Norris et al. 2007), while no association was found for early introduction of cow’s milk protein (Norris et al. 1996).

TEDDY
The Environmental Determinants in Diabetes of the Young (TEDDY) study is an observational clinical trial that started 2004 and involves six international centres; Seattle, Denver and Augusta in North America, and Germany, Finland and Sweden in Europe (Hagopian et al. 2006; The TEDDY Study Group 2007). The aim is to identify environmental factors, such as infectious agents, dietary factors and psychosocial factors, which contribute to or protect from islet autoimmunity and risk of type 1 diabetes. Newborns with HLA risk genotypes or who are first-degree relatives of type 1 diabetic patients were recruited and will be followed regularly from birth to the age of 15 years. By the age of 15, it is estimated that
800 children will develop islet autoimmunity and 400 will progress to type 1 diabetes (The TEDDY Study Group 2008).

**BABYDIAB**

The BABYDIAB study is a German prospective study in which newborn offspring of parents with type 1 diabetes were followed for development of autoantibodies and diabetes (Hummel et al. 2004). GADA, IA-2A and IAA were measured at 9 months and at 2, 5 and 8 years of age. By the age of 5 years, 24 (1.5%) children had developed type 1 diabetes, and all but one had multiple autoantibodies before the onset of the disease. In addition, the frequency of autoantibodies at this age was 5.9% and the frequency of multiple autoantibodies was 3.5%. Children with multiple autoantibodies before the age of 2 years were at the greatest risk of developing type 1 diabetes.

**DIPP**

The Diabetes Prediction and Prevention study (DIPP) is a Finnish trial in which genetically predisposed children from the general population were closely followed for signs of diabetes-associated autoimmunity (Kupila et al. 2001). Children who developed signs of beta-cell autoimmunity, with at least two autoantibodies in two consecutive samples, were invited to a separate prevention trial, with administration of intranasal insulin. The first autoantibody that emerged was predominantly IAA, and in the majority of the children autoantibodies appeared in the fall and winter, indicating that infectious agents may play a role in the induction of beta-cell autoimmunity (Kimpimaki et al. 2001). By the age of 5 years, 13 (1.3%) children had presented with type 1 diabetes, and all of them had developed at least two autoantibodies before onset (Kukko et al. 2005). Further, it was found that nasal insulin did not prevent or delay development of type 1 diabetes (Nanto-Salonen et al. 2008).

**TRIGR**

In the trial to reduce type 1 diabetes in the genetically at risk (TRIGR) study, which is a multinational, randomized, double-blind, controlled trial, newborns with HLA-conferred susceptibility to type 1 diabetes and with at least one family member with type 1 diabetes were recruited. If exclusive breast-feeding was not possible during the first 6-8 months of life, the child was randomly assigned either hydrolyzed cow’s milk formula or a standard cow’s milk based formula. The participants will be monitored up to the age of 10 years for the appearance of autoantibodies and clinical type 1 diabetes. Final results of the study will be
available in 2017 (TRIGR 2010). Autoantibodies against GAD, IA-2, IAA, ZnT8 and islet cells are analyzed to investigate if the elimination of cow’s milk proteins during infancy can decrease the development of beta-cell autoimmunity during the first years of life. Results from a median observation of 10 years have shown that the hazard ratio for positivity for one or more autoantibodies was reduced in the group that received hydrolyzed formula (Knip et al. 2010).

**ENDIT**

The European nicotinamide diabetes intervention trial (ENDIT) was a double blind placebo-controlled trial that aimed to assess whether high dose nicotinamide during 5 years could prevent or delay clinical onset of diabetes in ICA positive individuals with a first-degree family history of type 1 diabetes (Gale et al. 2004). The results showed that there were no differences in development of diabetes between the nicotinamide and placebo treated individuals.

**DPT-1**

The Diabetes Prevention Trial (DPT-1) was started in 1994 with the aim of determining whether antigen based treatment with insulin would prevent or delay diabetes. Relatives to type 1 diabetic patients were treated with oral or parenteral insulin. The results showed that these treatments did not overall slow the progression to type 1 diabetes (DPT-1 2002; Skyler et al. 2005). However, a beneficial effect of treatment with oral insulin was observed in a subgroup of the treated individuals with higher IAA levels.

**Anti-CD3 monoclonal antibody intervention studies**

The humanized Fc mutated anti-CD3 monoclonal antibody OKT3γ (Teplizumab) was tested in a clinical trial, in which newly diagnosed type 1 diabetic patients were randomly assigned the antibody or placebo for 14 days (Herold et al. 2002; Herold et al. 2005). Still after two years, a majority of the treated patients had a maintained or improved C-peptide response, as well as reduced usage of insulin and improved HbA1c levels. Adverse events were common, often mild, but some patients had serious adverse events. Further, a study with high-dose Teplizumab in newly diagnosed type 1 diabetic patients showed to preserve the endogenous insulin secretion still 5 years after intervention (Herold et al. 2009). However, the recruitment of patients in that study was stopped after treatment of only 6 patients, due to more adverse events, probably as a result of the high dose. Very recently, a phase II/III randomized double-
blind controlled multicenter study with intravenous administration of Teplizumab for 14 days in two rounds was performed, in children and adults with recent-onset type 1 diabetes (http://clinicaltrials.gov 2010a). This trial was a 4-armed dose-ranging study, with one group of patients receiving placebo and the other three groups receiving different doses of Teplizumab. The aims were to assess the efficacy, tolerability and safety of Teplizumab. Efficacy was defined primarily by the capacity of Teplizumab to markedly reduce insulin requirements while maintaining relatively normal blood sugar levels. In October 2010, MacroGenics and Lilly announced that the analysis of one-year safety and efficacy data of the phase III trial of Teplizumab was completed with the result that the primary efficacy endpoint of the study was not met, indicating a lack of efficacy of Teplizumab (Press release Macrogenics and Lilly 2010). As a consequence, the companies have decided to suspend further recruitment and dosing of patients in two ongoing trials of Teplizumab in type 1 diabetes.

GAD-alum intervention studies

In non-obese diabetic (NOD) mice, one of the animal models for type 1 diabetes, several studies have shown that diabetes can be delayed or prevented via administration of GAD (Tisch et al. 1993; Tian et al. 1996; Tisch et al. 1999; Jun et al. 2002). This preventive effect is probably due to restoration of self-tolerance, which seems not to be restricted to the tolerogen, but spreads to other beta-cell autoantigens (Bach et al. 2001). Tian et al showed that injection of GAD65 in an adjuvant that induced Th2 responses to GAD65 could greatly inhibit the progression of the autoimmune process in NOD mice that already had an established autoimmune process (Tian et al. 1996). This and other experiments led to the GAD65 vaccine (GAD-alum; Diamyd) that DIAMYD is using in clinical trials on humans. Aluminum hydroxide (alum) is a commonly used vaccine adjuvant that preferentially induces a humoral Th2 response and a Th2-driven antibody isotype production against co-injected antigens, rather than a cellular immune response (McKee et al. 2008).

The safety of GAD-alum was first tested in a phase I trial in healthy humans (Diamyd Medical, unpublished). Then, the efficacy of GAD-alum treatment as well as further evaluation of the safety and investigation of the immunological effect was tested in a phase IIa dose-finding study in a group of LADA patients (Agardh et al. 2005). This study was followed by a phase IIb clinical trial in newly diagnosed type 1 diabetic children (further described in the “Study populations” section) (Ludvigsson et al. 2008). Results from the latter
study showed a better preservation of fasting and stimulated C-peptide in the individuals with less than six months duration of disease at inclusion. The effect persisted still 4 years after treatment (Ludvigsson et al. 2010b). At present, phase III clinical trials with GAD-alum are ongoing in both Europe and the USA. Results from the European study will be available in the spring or summer of 2011 (www.diamyd.com). The study comprises 334 type 1 diabetic patients (10-20 years of age) diagnosed within the last three months. They have GADA positivity and fasting C-peptide levels above 0.1 pmol/ml (http://clinicaltrials.gov 2010b). This is a three-armed trial with one group receiving four subcutaneous injections with placebo, another group GAD-alum at two occasions and placebo at the other two visits, and the last group four injections of GAD-alum. It will be interesting to follow the results from the GAD-alum phase III clinical trial.

In conclusion, improved prediction strategies for type 1 diabetes facilitate identification of individuals suitable for intervention studies. Unfortunately, the ENDIT, DPT-1 and anti-CD3 intervention studies had no effect on the development of type 1 diabetes, but other treatments, such as GAD-alum, are still promising.

DETECTION OF AUTOANTIBODIES

Autoantibodies related to type 1 diabetes are traditionally detected using radioimmunoassays (RIAs). The first radioassays for detection of IAA in prediabetic and new onset diabetic patients used $^{125}$I-labelled insulin in fluid phase assays, with poly(ethylene glycol) (PEG) for precipitation and relatively large volumes of serum were needed. Antibodies induced by insulin injection were generally measured with enzyme-linked immunosorbent assays (ELISAs). However, the detection limit of the ELISAs was higher than of the radioassays, and the levels of the insulin antibodies induced by insulin injections are approximately 10-fold higher than the commonly detected levels of IAA in individuals at diagnosis of type 1 diabetes (Winter et al. 2002). In 1992, Greenbaum et al showed that IAA measured by RIA were more related to type 1 diabetes than those measured by ELISA, and thus recommending RIA as the method of choice for measuring IAA associated with type 1 diabetes (Greenbaum et al. 1992). This could be explained by the fact that in RIA, the ligand (insulin) is limiting and the antibodies are in excess, favoring the binding of high-affinity IAA, and it has been observed that IAA of high affinity is disease specific (Achenbach et al. 2004a; Schlosser et al. 2005b). On the other hand, in ELISA, an excess of the antigen is fixed to the plate, enabling
binding of low-affinity antibodies as well (Sodoyez-Goffaux et al. 1988; Wilkin 1990). Further, almost all of the epitopes on the insulin molecule would be available for binding in the fluid phase RIA, while in ELISA some epitopes might be blocked for antibody binding due to binding of insulin to the solid surface (Greenbaum et al. 1992). To improve sensitivity and specificity for detection of IAA with RIA, competition with unlabelled insulin was introduced and micro-assays using smaller volumes of serum and protein A sepharose for precipitation were developed (Williams et al. 1997) and this method is the standard assay for IAA today.

When RIA had been established as the most suitable method for detection of IAA related to type 1 diabetes, RIAs were also developed for the detection of GADA and IA-2A. These immunoassays, which are widely used today, are based on in vitro synthesized radioactively labelled ($^{35}$S-methionine) antigen and protein A sepharose for precipitation of antibody-antigen complexes (Grubin et al. 1994). These fluid phase RIA’s have generally demonstrated higher disease sensitivity compared to ELISAs, which are solid phase assays (Schmidli et al. 1995; Bingley et al. 2003). More recently the ELISAs have improved and some assays with the same or better sensitivity and specificity than the conventional RIAs have been developed (Ankelo et al. 2003; Brooking et al. 2003; Luo et al. 2004; Mueller 2007). Two ELISAs are based on a divalent action of GADA that forms a bridge, between immobilized GAD65 and liquid-phase GAD65-biotin in one assay (Brooking et al. 2003) and between immobilized GAD65-biotin and liquid-phase europium-labelled GAD65 in the other (Ankelo et al. 2003). Another assay uses a biotin-GAD65 fusion protein, which is free in the liquid-phase during the incubation with antibody-containing serum, and is then bound to the surface for detection of GADA (Luo et al. 2004). In The Diabetes Autoantibody Standardization Program (DASP) 2007, one laboratory using ELISA for detection of GADA and IA-2A had the highest sensitivities and specificities among all participating laboratories (Mueller 2007). The high sensitivity and specificity of these assays opens the question whether new ELISA assays might replace RIAs for determination of GADA and IA-2A in the future.

RIAs for GADA, IA-2A and IAA are today widely used in laboratories around the world. To improve and standardize the measurement of type 1 diabetes related autoantibodies, a series of international workshops have been held (Greenbaum et al. 1992; Schmidli et al. 1994; Schmidli et al. 1995; Verge et al. 1998) and were followed by the establishment of The Diabetes Autoantibody Standardization Program (DASP). DASP is a collaboration of the
Immunology of Diabetes Society (IDS) and the US Centers for Disease Control and Prevention. The major goals of DASP are to help laboratories to improve methods for detection of autoantibodies by providing technical support, training and information, to organize workshops that evaluate laboratory performance of the assays and to provide reference material for the development of new measurement technologies (Bingley et al. 2003; Torn et al. 2008). The aim of the first DASP evaluation was to improve comparability of the autoantibody measurements between laboratories and to evaluate the new WHO international reference reagent for GADA and IA-2A (Mire-Sluis et al. 2000; Bingley et al. 2003).

There are some disadvantages with the RIAs for detection of GADA, IA-2A and IAA, including the use of radioactively labelled material, low sensitivity for the IAA assay and long assay times for a sample. Of the three RIAs, the assay for IAA has been and is still the most difficult for new laboratories to perform and to standardize (Liu et al. 2007; Schlosser et al. 2010). This could be due to the very low levels of IAA that are present in serum of both prediabetic and newly diagnosed individuals with type 1 diabetes, and the fact that there is a very small difference in concentration between autoantibody positive individuals and normal control subjects. Thus, there is a need for better methods to measure autoantibodies against GAD, IA-2 and insulin. Surface plasmon resonance (SPR) is an interesting candidate technique for this purpose (Shankaran et al. 2007). Biacore’s SPR technology is an efficient method that investigates biomolecular interactions in real-time and uses label-free detection (Liedberg et al. 1995) (www.biacore.com). This technique can identify binding partners to target molecules, but also provide quantitative information on specificity of the binding between two molecules, the concentration of the molecule, rate of association and dissociation and strength of the binding. Comparison of SPR with two conventional immunoassays, i.e. RIA and ELISA, has shown that SPR is a more powerful technique with several advantages, such as the low quantities of reagents needed, no labelling of interactants and measurements in real time (Revoltella et al. 1998).

In conclusion, RIAs are commonly used for detection of GADA, IA-2A and IAA. There is a need for improved methods for detection of these autoantibodies, and SPR is an interesting candidate.
Aims of the thesis

The general aims of this thesis were to investigate prevalence and levels of autoantibodies related to type 1 diabetes, in healthy children and in children with type 1 diabetes. In addition, we wanted to study different properties of GADA, such as binding epitopes and IgG subclasses, after immunomodulatory treatment of children with type 1 diabetes.

The specific aims were:

I. To elucidate the influence of HLA genotypes on the development of diabetes-related autoantibodies and manifest type 1 diabetes in children participating in the ABIS study. We therefore decided to study the longitudinal changes of GADA and IA-2A and their relation to HLA genotypes, both in healthy children and in children with type 1 diabetes.

II. To develop a sensitive and specific method for detection of diabetes-related autoantibodies based on the surface plasmon resonance (SPR) technique. We thought that SPR might be a suitable technique due to its label-free detection, the short analysis time for each sample and the possibility to simultaneously measure multiple antibodies.

III. To study how treatment with GAD-alum in children with recent onset of type 1 diabetes influences GADA epitope binding. We hypothesized that the epitope binding pattern would not be influenced by the treatment, since unaltered epitope binding was previously observed after GAD-alum treatment in LADA patients.

IV. To study if treatment with GAD-alum in children with recent onset of type 1 diabetes influences IgG subclasses of GADA and GAD65 enzyme activity. We hypothesized that the IgG subclass distribution would be altered towards a Th2-like phenotype with more IgG4, since an early GAD65 specific Th2 cytokine response has previously been observed following this treatment.
STUDY POPULATIONS

The ABIS study
The All Babies In Southeast of Sweden (ABIS) study is a prospective population-based follow-up study, in which 17055 out of 21700 infants (78.6%) born between 1 October 1997 and 1 October 1999 in Southeast of Sweden were included (Ludvigsson et al. 2001) (www.abis-studien.se). The aim was to study the role of environmental factors for development of type 1 diabetes, but also for other diseases, such as celiac disease, inflammatory bowel disease and allergy. A second aim was to increase the knowledge of how to identify individuals with risk of developing type 1 diabetes in the general population, using markers such as autoantibodies and HLA. In ABIS I, 1997-2005, children were followed from birth up to 5 years of age. The parents were asked to fill in comprehensive questionnaires and biological samples, such as blood and hair samples, were taken from the children at birth and at 1, 2.5 and 5 years of age. In the follow-up study, ABIS II, which started 2006, all children participating in ABIS I were asked to participate. The children were asked to fill in questionnaires and to contribute with biological samples at 8, 11 and 14 years of age. The prevalence of diabetes in the ABIS cohort will be investigated, by registration of the diagnosis from the clinics, until the end of 2017. Results from the study so far include a number of factors that have been related to development of beta-cell autoantibodies, such as virus infections early in life (Sepa et al. 2005), short duration of breast-feeding (Wahlberg et al. 2006; Holmberg et al. 2007), early introduction of cow’s milk and late introduction of gluten during the first year of life (Wahlberg et al. 2005; Wahlberg et al. 2006) and psychological stress (Sepa et al. 2005; Sepa et al. 2005).

In paper I, samples from subgroups of children who participated in the ABIS study were used for analyses. Samples were grouped as follows:
1) GADA and IA-2A were investigated in all children who had given at least one serum or whole blood sample at any age (1, 2.5 or 5 years of age). The relationship between HLA alleles and GADA and IA-2A was investigated.
Subjects and methods

2) The occurrence of permanent vs transient autoantibodies was studied in 2329 children from whom samples at all three ages were available, and the association to HLA genotype was investigated. In this group, children who had developed type 1 diabetes were excluded.

3) Analyses of GADA, IA-2A and HLA genotype were performed in samples from 1, 2.5 and/or 5 years of age in children who developed type 1 diabetes in the ABIS study.

In paper II, serum samples from children in the ABIS study who had not developed type 1 diabetes were used for measurement of IAA in an indirect competitive immunoassay based on SPR.

The phase II clinical trial with GAD-alum

A phase II clinical trial with injections of recombinant aluminum formulated human GAD65 (GAD-alum) has been performed in type 1 diabetic patients (Ludvigsson et al. 2008). The aim of the study was to investigate if GAD-alum treatment could preserve beta cell function in children with type 1 diabetes. The participants (n=70) were 10-18 years old at diagnosis of type 1 diabetes and had disease durations of <18 months, fasting c-peptide levels of >0.1 nmol/l and tested positive for GADA at the initial screening. In this randomized, double-blinded placebo-controlled trial, recent onset type 1 diabetic children and adolescents were treated with either 20 μg GAD-alum (Diamyd®, Diamyd Medical, Stockholm, Sweden) (n=35) or placebo (alum-formula alone) (n=35) subcutaneously in a prime and booster injection four weeks apart. At day 1 and at 3, 9, 15, 21 and 30 months, the patients underwent a mixed-meal tolerance test to stimulate residual insulin secretion (measured as C-peptide level). Adverse events were regularly registered throughout the study, and neurological examination was performed by a pediatrician at regular intervals. The outcome of the study demonstrated that GAD-alum treatment had a clinical effect on the preservation of beta-cell function in children with shorter disease duration, and immunomodulated the humoral immune response, inducing GADA levels in GAD-alum treated individuals (Ludvigsson et al. 2008).

In addition, the effect of GAD-alum on the immune system was studied. Blood samples from 69 patients (one placebo patient dropped out after the first injection) were collected before the first injection (day 0) and after 1, 3, 9, 15, 21 and 30 months. All samples were transported to Linköping within 24 hours, and serum samples were stored at −70°C for simultaneous analysis to avoid inter-assay variation.
Subjects and methods

In paper III, serum samples taken before the first injection (baseline) and at 1, 3, 9, and 15 months after the initial injection were included. Nine patients (7 from the placebo group and 2 from the GAD-alum group) with baseline GADA levels <60 units/ml (U/ml), were excluded because measurement of competition with rFab in samples with low GADA levels is unreliable. One additional patient in the placebo group was excluded, since this individual dropped out after the first injection. Thus, GADA epitope specificity was analyzed in 27 patients from the placebo group and 33 from the GAD-alum group using rFab fragments in an epitope-specific radioligand binding assay.

In paper IV, the IA-2A and tetanus toxoid titers were measured at baseline, 3 and 9 months. GAD65 enzyme activity and IgG subclass measurements were performed at baseline, 3 and 15 months. Total IgE was analysed at baseline and 21 months.

Type 1 diabetic patients

In paper II, samples taken from children with manifest type 1 diabetes were studied. These children have been recruited at the Pediatric Clinic at Linköping University hospital and the diagnosis was based on the WHO criteria from 1999. All samples used were taken within 10 days after diagnosis, to be sure that the IAA measured were antibodies against endogenous insulin and not antibodies produced after encounter of exogenous insulin.

METHODS

RIA for detection of GADA and IA-2A (Paper I and IV)

GADA and IA-2A were determined using a RIA with $^{35}$S-labelled human recombinant GAD65 and IA-2ic, respectively. These antigens were produced by in vitro transcription and translation of complementary DNAs of GAD65 and IA-2ic, cloned into vectors (pcDNA2 and pSP64 poly (A), respectively; kindly supplied by Prof. Å Lernmark, Seattle), in the presence of $^{35}$S-labelled methionine. Serum or whole blood samples diluted 1:25 were incubated in duplicates with GAD or IA-2 antigen at +4°C over night. This was followed by precipitation with protein A sepharose (Amersham Biosciencies, Uppsala, Sweden) to separate free labelled antigen from antibody bound labelled antigen. A standard curve was included on each plate, as well as a blank (containing only buffer) and positive and negative controls. The
Subjects and methods

Immunoprecipitated radioactivity was measured in a Wallac 1450 Microbeta Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences), and the results were expressed as U/ml in relation to the standard curve.

In paper I, cut-off for positivity at the 98th percentile for GADA and IA-2A was used. However, at 5 years of age, the 99th percentile was used for IA-2A since less than 2% of the samples had values above the detection limit. In the DASP workshop 2005, our GADA assay had, at the 98th percentile, a specificity of 95% and a sensitivity of 76% (Table 2). For IA-2A, at the 99th percentile, the specificity was 100% and the sensitivity 72%. Interassay variations for negative and positive controls were 10% and 8% for GADA and 11% and 12% for IA-2A, respectively.

Table 2. Specificities and sensitivities for our autoantibody assays from the Diabetes Autoantibody Standardization Program (DASP) during the years 2003-2010. Measurements of GADA, IA-2A and IAA were included all time points, while the three variants of ZnT8A; ZnT8(Arg)A, ZnT8(Gln)A and ZnT8(Trp)A, were included for the first time in DASP 2010. The percentiles used as cut-off for positivity varied between antibodies and years and are shown in the table. * = In 2007, the cut-off value used for IA-2A was 9.9 U/ml, which exceeded the 99th percentile.

<table>
<thead>
<tr>
<th>Year</th>
<th>GADA</th>
<th>IA-2A</th>
<th>IAA</th>
<th>ZnT8(Arg)A</th>
<th>ZnT8(Gln)A</th>
<th>ZnT8(Trp)A</th>
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</thead>
<tbody>
<tr>
<td>2003</td>
<td>Sensitivity</td>
<td>74%</td>
<td>48%</td>
<td>24%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>100%</td>
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<tr>
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<td>Percentile</td>
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<td>98th</td>
<td>99th</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2005</td>
<td>Sensitivity</td>
<td>76%</td>
<td>72%</td>
<td>28%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>100%</td>
<td>100%</td>
<td>-</td>
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<td></td>
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<td>99th</td>
<td>98th</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2007</td>
<td>Sensitivity</td>
<td>82%</td>
<td>65%</td>
<td>25%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>&gt;99th*</td>
<td>98th</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2009</td>
<td>Sensitivity</td>
<td>66%</td>
<td>64%</td>
<td>40%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>98th</td>
<td>99th</td>
<td>98th</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2010</td>
<td>Sensitivity</td>
<td>80%</td>
<td>66%</td>
<td>34%</td>
<td>52%</td>
<td>28%</td>
</tr>
<tr>
<td></td>
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<td>98th</td>
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<td>99th</td>
<td>99th</td>
</tr>
</tbody>
</table>
In paper IV, the cut-off for positivity determined at the 95\textsuperscript{th} percentile was regarded as 67.3 U/ml (corresponding to 23.1 WHO units) based on measurements from 1700 children, aged 5-6 years, participating in the ABIS study. Validation of our assay in DASP 2007 showed 94\% specificity and 82\% sensitivity for GADA and 98\% specificity and 65\% sensitivity for IA-2A (Table 2).

**HLA genotyping (Paper I)**

HLA DQB1, DQA1 and DRB1 alleles were determined with a time-resolved fluorometry based sandwich hybridization assay, and performed by the research group of Professor Jorma Ilonen in Turku, Finland. Details of the method have been described earlier (Sjoroos et al. 1998; Nejentsev et al. 1999; Laaksonen et al. 2002; Hermann et al. 2003). Briefly, the polymerase chain reaction (PCR) was carried out using a 3 mm in diameter disc from whole blood spots on a filter paper punched directly into the PCR reagent mixture or using DNA extracted from whole blood. For amplification of the genes, specific HLA-DQA1, DQB1 or DRB1 primer pairs, of which one was biotinylated at the 5’-end, were used. The PCR product was transferred into a streptavidin coated microtiter plate and heat denatured, followed by addition of detection probes labelled with various lanthanide chelates (Europium, Samarium or Terbium). After an incubation step, allowing hybridization of the PCR product and the probes, the time-resolved fluorescence was detected in a Victor\textsuperscript{TM} 1420 Multi label Counter (Wallac Oy, Turku, Finland) (Figure 9). Differences in the emission wave lengths of the lanthanides and the usage of a time delay before measurement enable the detection of the three lanthanide labels.

For genotype analysis, HLA haplotypes were categorized into susceptibility associated (S), neutral (N) and protective (P) ones. This classification was done based on the results of haplotype-conferred risk in 622 Finnish families with one child affected by type 1 diabetes (Hermann et al. 2003), but essentially similar results have been described in other European populations (Koeleman et al. 2004; Lambert et al. 2004). Susceptibility associated haplotypes included DR4-DQ8 (DRB1*0401/2/4/5-DQB1*0302) and DR3-DQ2 (DQA1*05-DQB1*02), protective haplotypes included DR2-DQ6 (DQB1*0602), DR11/12/1303-DQ7 (DQA1*05-DQB1*0301), DR7-DQ3 (DQA1*0201-DQB1*0303), DR14-DQ5 (DQB1*0503), DR403-DQ8 (DRB1*0403-DQB1*0302) and DR1301-DQ6 (DQB1*0603) (Table 1). Other haplotypes were defined as neutral.
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Figure 9. Principle of time-resolved fluorometry based sandwich hybridization assay. PCR amplification of DNA with a biotinylated primer was followed by hybridization with specific detection probes on streptavidin coated microtiter plates and thereafter measurement of time-resolved fluorescence. SA = streptavidin, bio = biotin.

**RIA for detection of IAA (Paper II)**

A competitive RIA, based on the method developed by Williams et al. (Williams et al. 1997) with some modifications (Holmberg et al. 2006), was used for detection of IAA. Serum samples in quadruplicates (7.5 µl/well) were incubated in a 96 deep-well plate with either $^{125}$I-insulin alone (2 wells) (25 000-30 000 cpm/well of human recombinant insulin, mono-iodated at thyrosine position A14, Amersham Biosciences, Buckinghamshire, Great Britain) or with $^{125}$I-insulin together with an excess of unlabelled insulin (2 wells) (human recombinant insulin, Sigma-Aldrich, St. Louis, MO, USA) shaking for 72 h at +4°C. A standard curve as well as positive and negative controls were included on each plate. After incubation, the insulin-antibody complexes were precipitated with Protein A Sepharose (Zymed, San Francisco, CA, USA) and washed five times by centrifugation at +4°C, followed by measurement of the radioactivity in a gamma counter (1282 CompuGamma, Wallac, Turku, Finland). Specific antibody binding were calculated by subtraction of mean counts per minute (cpm) of duplicate samples with an excess of unlabelled insulin from mean cpm of duplicate samples with labelled insulin. The results were expressed as U/ml calculated in relation to a standard curve. The cut-off limit for positivity corresponding to the 98th percentile of 114 healthy Swedish children was 6.45 U/ml. Validation of our IAA assay in DASP 2003 showed 100% specificity and 24% sensitivity (Table 2).
Surface plasmon resonance (SPR) (Paper II)

Surface plasmon resonance (SPR) is an optical surface phenomenon that can be used to monitor biomolecular interactions in real-time without the need for labelling (Liedberg et al. 1995; Shankaran et al. 2007) (www.biacore.com). The instrument used in our study, Biacore X, is one of the commonly used instruments for SPR-based assays. One of the interacting molecules, the ligand, is immobilized on a sensor chip consisting of a glass surface coated with a thin layer of gold usually covered with a dextran hydrogel. The sample containing the other interacting molecule, the analyte, is then injected over the surface in a controlled flow.

The SPR phenomenon occurs when light, under conditions of total internal reflection, strikes a thin electrically conducting gold layer (Figure 10). Here, this gold layer is at the interface between a glass slide and the flow channel with buffer, which have different refractive indexes. When light strikes the gold film through a glass prism docked to the slide, an electric field is generated, which extends from the opposite film interface into the buffer. At a certain angle of incidence, the resonance angle, this so-called evanescent field excites the surface plasmon, which is a collective oscillation (wave) of electrons that propagates along the gold interface. This excitation is called surface plasmon resonance. When monitoring the intensity of the reflected light as a function of the angle of incidence, the resonance angle can be identified from a minimum. This is because most of the light energy has coupled into the surface plasmon upon excitation at this angle. The resonance angle is dependent upon the refractive index of the medium (the buffer or analyte solution) close to the gold layer. In this way, changes of the refractive index at or close to the surface, for example due to binding of biomolecules, results in displacement of the reflectance minimum (Figure 10a), which is monitored as a function of time in a so-called sensorgram (Figure 10b). The displacement is expressed in resonance units (RU, 1 RU ~1 pg/mm² of any biomolecule). The contribution to the refractive index change by any biomolecule binding to or dissociating from the surface is usually proportional to the molecular mass. After the analysis, analyte molecules can be removed using an appropriate regeneration solution that, ideally, does not affect the activity of the immobilized ligand.
**Figure 10.** The principle of surface plasmon resonance, with a protein immobilized on the sensor surface and a sample containing antibodies injected over the surface. Polarized light that strikes the surface at a certain angle, the resonance angle, generates an electric field that excites a surface plasmon, resulting in that the intensity of reflected light is at a minimum (I). When antibodies bind to the protein, the refractive index close to the surface is changed, resulting in displacement of the reflectance minimum (from I to II in graph a), which is displayed in a sensorgram (b), expressed as resonance units (RU).

The SPR technique can identify binding partners to molecules of interest and monitor binding specificity, but also provide quantitative information on analyte concentration as well as kinetic and thermodynamic parameters describing the interaction.

The design of the sensor chip differs depending on the application. The carboxymethylated (CM) dextran chip CM5 is a commonly used commercially available general-purpose chip for interaction analysis involving all types of biomolecules (Biacore, Uppsala, Sweden). The sensor chip is coated with gold and covered with a CM dextran layer (Figure 11). It is suitable for concentration measurements, and is generally the first choice for protein immobilization via amine coupling.
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Figure 11. The structure of a CM5 chip. At the bottom, the sensor chip coated with gold. On top of that the dextran layer, which has been carboxylated.

Prior to immobilization of a ligand, a process called pre-concentration is often performed, to get high absorption of the protein into the dextran matrix, and is optimized by adjusting the pH-value. When using a CM5 sensor chip, the pH of the solution should be acidic to pre-concentrate the protein in the negatively charged CM dextran matrix. This is usually obtained at a pH of the antigen solution of approximately 1 pH unit below the isoelectric point of the antigen.

Competitive epitope-specific RIA for determination of GADA epitopes
(Paper III)

The capacity of different rFab fragments to inhibit GADA binding to GAD65 was tested in a competitive epitope-specific RIA. Serum samples were initially screened for GADA using the traditional RIA, at a dilution of 1/25. Samples with a GADA titer exceeding 1000 U/ml were diluted to <1000 U/ml and reanalyzed. This dilution was then used in the epitope-specific RIA. Six GAD65-specific rFab were used to determine the GADA epitope specificity in serum samples. The rFab were cloned, expressed and purified as described previously (Padoa et al. 2003). Four of these have been related to type 1 diabetes (b96.11, DPA, DPD and MICA3) (Richter et al. 1993; Padoa et al. 2003) and the other two (b78 and N-GAD65 mAb) have been found in SPS patients (Raju et al. 2005). These rFab recognize epitopes on different regions of GAD65, ranging from the N-terminal to the C-terminal part of the molecule (Fenalti et al. 2008) (Figure 12). DPA and MICA3 are derived from two different patients with type 1 diabetes, and recognize epitopes within the same epitope region on GAD65. The insulin-specific rFab CG7C7 (Padoa et al. 2005) was included on each plate to determine non-specific competition. The rFab were added at a concentration sufficient to compete binding of the originating intact monoclonal antibody (mAb) to GAD65 by at least 80%, resulting in 4,
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0.6, 1.7, 14, 7 and 2 μg/ml for rFab DPA, b96.11, DPD, MICA-3, N-GAD65 mAb, b78 and CG7C7, respectively.

Figure 12. Illustration of the six rFab used in the competitive epitope-specific RIA, showing the epitope regions on GAD65 recognized by each of them. The rFab recognize epitopes located at the N-terminus (N-GAD65 mAb and DPD), middle region (b96.11) and C-terminus (DPA, MICA3 and b78). DPA and MICA3 are derived from two different patients with type 1 diabetes, and recognize epitopes within the same epitope region on GAD65.

Serum samples were incubated with rFab to inhibit GAD65 binding, or without rFab for non-competed measurement, and the levels of GADA were determined by precipitation with Protein A Sepharose. For each sample, non-competed GADA as well as GADA competed with a GAD65-specific rFab, were measured in triplicates, from which a mean value was calculated. This was done for each timepoint: baseline and month 1, 3, 9 and 15. The intra-assay average coefficient of variation was 4%, with the highest value of 13% and the lowest being 0.03%. Binding of GADA to GAD65 in the presence of rFab was expressed as follows: ratio = GADA cpm in the presence of rFab (competed) / GADA cpm in the absence of rFab (non-competed). A higher binding to GAD65 in the presence of an rFab indicates a lower proportion of GADA binding to the respective epitope. The cutoff for specific competition was determined as >15% by using the negative control rFab CG7C7.

A modified RIA for detection of IgG subclasses of GADA (Paper IV)

GADA of the subclasses IgG1, 2, 3 and 4 were measured using a modification of the conventional RIA for GADA, based on a protocol kindly provided by Shilpa Oak (University of Washington, Seattle, USA), with some further modifications in our lab. Briefly, serum samples (5 µl) were incubated with 60 µl human recombinant ¹³⁵S-labelled GAD65 in a 96-
well plate (Nunc 96 MicroWell plate, Nunc A/S, Roskilde, Denmark) under vigorous shaking at 4°C over night. In parallel, biotinylated monoclonal mouse anti-human IgG1, IgG2 and IgG4 (BD PharMingen, San Diego, CA, USA) and IgG3 (Southern Biotech, Birmingham, AL, USA) were incubated with streptavidin agarose beads (Thermo Scientific, Rockford, IL, USA), under vigorous shaking at 4°C over night. Thereafter, 50 µl of the mixture with \(^{35}\)S-labelled GAD65-GADA complexes and 50 µl of the biotinylated anti-human IgG antibody coupled to streptavidin agarose beads were transferred into a 96-well filtration plate (Millipore, Bedford, MA, USA), and incubated under vigorous shaking at 4°C for 2 h, followed by washing 8 times with 150 µl/well assay buffer using a vacuum device (Millipore). Then, scintillation liquid (OptiPhase Supermix, Perkin-Elmer Life Sciences Wallac) was added to the wells, and the activity was measured in a liquid scintillation counter (1450 Microbeta Trilux, Perkin-Elmer Life Sciences, Wallac). All samples were run in duplicates. The cut-off value for each subclass was determined using a GADA negative control, which was included in each assay. Results were expressed as cpm, and positivity of each sample was calculated by subtraction of the mean cpm value plus three times the standard deviation (SD) obtained for the negative control.

**GAD65 enzymatic activity assay (Paper IV)**

GAD65 enzyme activity was measured by a \(^{14}\)CO\(_2\)-trapping method based on the enzymatic reaction of glutamate to GABA. GAD65 works as an enzyme together with the coenzyme pyridoxal 5-phosphate (PLP). GADA-positive serum from SPS patients have shown to inhibit this reaction (Raju et al. 2005). First, 150 µl of a solution containing \(K_2HPO_4\) (stock solution 50 mM), PLP (stock solution 3 mM, Fisher Scientific, Göteborg, Sweden) and GAD65 (stock solution 0.2 mg/ml; Diamyd Diagnostics AB, Stockholm, Sweden) was mixed with 15 µl serum and incubated for 1 hour at room temperature. Thereafter, 28 µl of a mix containing \(K_2HPO_4\) (stock solution 50 mM), L-glutamic acid (stock solution 5 mM, Fisher Scientific, Göteborg, Sweden) and L-[\(^{14}\)C-(U)]-glutamic acid (0.4 µCi; Perkin Elmer, Boston, USA) was added to each tube. All tubes were sealed with a rubber stopper attached with a center well (Kimble Chase Kontes, Vineland NJ, USA), in which a NaOH-soaked (50 µl of 1 M NaOH) filter paper (Camlab Ltd, Cambridge, UK) was placed, and the reaction was carried out in a water bath at 37°C for 1 hour with gentle agitation. The reaction was stopped by placing the tubes on ice for 10 minutes. The radioactivity of the \(^{14}\)CO\(_2\) captured on the filter papers was measured in a Wallac Microbeta Liquid Scintillation Counter (Perkin Elmer Life and
Analytical Sciences, Inc, Boston, MA, USA). Serum from one SPS patient was included in all assays as a positive control for inhibition.

**Analysis of tetanus toxoid antibodies (Paper IV)**

Determination of tetanus toxoid antibodies in serum was performed using an Immunozym Tetanus Ab ELISA according to the manufacturer’s instructions (IBL, Hamburg, Germany). The optical density was measured at 450 nm using a VersaMax microplate reader (MDS, Inc., Sunnyvale, CA, USA). Cut-off levels were determined at >0.070 IU/ml.

**Analysis of IgE (Paper IV)**

Total serum IgE was quantified using the ImmunoCap100 system (Phadia AB, Uppsala, Sweden). In short, the principle of the method is binding of total IgE to anti-IgE covalently coupled to ImmunoCap, followed by addition of enzyme-labelled antibodies against IgE, addition of a developing agent and finally measurement of the fluorescence of the eluate. The fluorescence is directly proportional to the concentration of IgE in the serum sample. The measuring range for the assay was 2-50 000 kU/l, and calibrators were run in duplicates to obtain a full calibration curve. Levels of total IgE ≥ 85 kU/l were regarded as positive.

**C-peptide measurement (Paper IV)**

C-peptide levels were measured in serum samples using a time-resolved fluoroimmunoassay (AutoDELPHIA™ C-peptide kit, Wallac, Turku, Finland). Briefly, 25 µl of standards, controls and patient serum samples were incubated together with anti-C-peptide-Eu tracer solution in a microtiter plate coated with antibodies directed against C-peptide. After washing and addition of enhancement solution, automatic measurement and result calculation was performed using a 1224 MultiCalc® program (Wallac), with results expressed in pmol/ml. The results in each assay were validated by inclusion of a C-peptide control module containing a high, a medium and a low-level control (Immulite, DPC, UK).
STATISTICS

To elucidate if the immunological markers were normally distributed, the D’Agostino & Pearson omnibus normality test was performed. One can also evaluate if a material seems to be normally distributed by examining the distribution of the material in a histogram by looking if the material has a similar shape as a normally distribution curve, or by comparing mean and median values, which should be close to each other to support normally distribution. When the material is large, the outcome data does not necessarily have to be normally distributed for using parametric tests based on normally distributions (Lumley et al. 2002). A sufficiently large material in this context could be less than 100, but for consideration of parametric tests, other parameters than actual size of the material are also important, for example the shape of the distribution in the groups to be compared.

As the immunological markers in paper III and IV were not normally distributed, non-parametric tests corrected for ties were used. In paper III, to investigate differences in variability of the ratios over time, the sums of absolute differences between consecutive measurements were compared using Mann-Whitney U-test. Unpaired analysis was performed using Mann-Whitney U-test and correlations with Spearman’s rank order correlation test. Dot plots and longitudinal plots were used to visualize the data. In paper IV, unpaired analyses were performed using Kruskal-Wallis followed by the Mann-Whitney U-test. Differences within the groups were analyzed by Friedman’s test followed by Wilcoxon-signed rank test.

In paper I, Fisher’s exact test was used to analyze frequencies of GADA and IA-2A, and the relationship of HLA genotypes to autoantibodies and type 1 diabetes. Standard curves for RIA were performed using GraphPad Prism 4 (GraphPad, Software Inc., San Diego, California, USA). Positive predictive value (PPV) in % was calculated from the formula: (antibody positive who developed type 1 diabetes) / (antibody positive who developed type 1 diabetes + antibody positive healthy individuals) * 100

In paper II, a cut-off for positivity for the 5-min method was calculated from the mean value of a number of low IAA positive samples subtracted by 3 SD. In the standard curve plot with error bars, the mean values are presented and the error bars show the range.
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All statistical calculations were performed using the statistical package for the social sciences (SPSS) for Windows (SPSS Inc., Chicago, Illinois, USA). In paper I version 13.0 was used, in paper IV version 16.0 was used and in paper III the version named PASW Statistics 18 was used. A two-tailed p-value of <0.05 was considered statistically significant.

ETHICAL CONSIDERATIONS

Since type 1 diabetes often develops in childhood, it is important to study environmental and immunological factors influencing the development of type 1 diabetes in children, and not only in adult individuals. It is very important to get more knowledge about why this disease arises, to be able to prevent children from developing type 1 diabetes in the future. Autoantibodies are used to predict the development of type 1 diabetes, which is crucial for the identification of risk individuals to be included in prevention trials when candidate drugs are available.

The protocol of the ABIS study was approved by the Research Ethics Committees of the Medical Faculties of Linköping University, Linköping and Lund University, Lund. Parents gave their informed consent to enter the study, and they were informed that the participation was totally voluntary and could be discontinued at any time point without any specific reason. The parents were told that they, upon active request, could receive information about genetic risk genotypes or presence of autoantibodies, but that they were not going to be informed about this automatically. The data from questionnaires and laboratory analyses were stored without personal identifications, with only an ABIS code that identified the children in the study. Ethical questions in the ABIS study have been studied separately (Stolt et al. 2002; Stolt et al. 2005) as well as the maternal attitude to participation (Ludvigsson et al. 2001).

Collection of samples from children with manifest type 1 diabetes was approved by the Research Ethics Committee of the Medical Faculty of Linköping University, Linköping and parents gave their informed consent.

Approval for the phase II GAD-alum trial was obtained from the Swedish National Regulatory authorities and the Research Ethics Committee of the Medical Faculty of Linköping University, Linköping. Written informed consent was obtained from participants and their parents according to the Declaration of Helsinki. The study was randomized and
double blinded. Since GAD-alum treatment seems to be safe, is easy to administrate and has shown to be effective for preservation of endogenous insulin production compared to a control group, we believe that the ethical benefits of the GAD-alum treatment exceed the risks.
RESULTS AND DISCUSSION

AUTOANTIBODY POSITIVITY AND RELATION TO HLA GENOTYPES
(PAPER I)

The emergence of autoantibodies against beta-cell antigens is a dynamic process (Yu et al. 1996; Knip 1997). So far, there is no consensus on how often and at what age children in the general population should be tested to achieve the best possible prediction value for clinical type 1 diabetes. The emergence of autoantibodies has been investigated in different study populations, including families with first-degree relatives of individuals having type 1 diabetes, and individuals with increased risk for type 1 diabetes determined by disease-associated HLA genotypes. First-degree relatives of patients with type 1 diabetes have for example been studied in the BABYDIAB (Hummel et al. 2004), DPT-1 (Krischer et al. 2003), Childhood Diabetes in Finland (DiMe) (Savola et al. 2001) and the Bart´s Oxford and the Munich family studies (Achenbach et al. 2004b). The DIPP study (Kimpimaki et al. 2001; Kupila et al. 2001; Kukko et al. 2005) investigated instead populations with disease-associated HLA genotypes. In the TEDDY (The TEDDY Study Group 2007) and DAISY (Barker et al. 2004) studies, both individuals with HLA-risk genotypes and those with first-degree relatives were included.

The rapid increase in the incidence of type 1 diabetes during the last decades cannot be explained by genetic factors, but is probably instead related to changes in the environment. Several environmental factors that seem to be related to development of type 1 diabetes have been found, including viral infections, introduction of cow´s milk and gluten, beta-cell stress and breast-feeding (Akerblom et al. 2002; Peng et al. 2006). To elucidate the influence of environmental factors in the development of beta-cell autoimmunity and type 1 diabetes, it would be appropriate to follow a general population that has not been selected for genetic risk. Therefore, we studied the emergence of autoantibodies in children from the ABIS study, and related the presence of autoantibodies to HLA-risk and -protective genotypes.
Results and discussion

GADA and IA-2A in children at three different ages

We started to investigate GADA and IA-2A in children from the ABIS study, from whom samples were collected at 1, 2.5 and/or 5 years of age. Positivity to an autoantibody was then related to HLA genotypes. We found that children positive for GADA or IA-2A at 5 years of age had the HLA risk haplotype DR4-DQ8 or the high-risk genotype DR3-DQ2/DR4-DQ8 more often than autoantibody negative (GADA and IA-2A negative) children. In addition, children at 5 years of age positive for IA-2A had the protective haplotype DR2-DQ6 more seldom and the risk associated genotype DR4-DQ8/DR4-DQ8 more often than autoantibody negative children. At 2.5 years of age, children positive for IA-2A had the protective genotype DR2-DQ6/DR2-DQ6 more seldom than autoantibody negative children.

Similar relations between autoantibodies and HLA-risk and -protective alleles have been found in other studies, as presented in Table 3. For example, GADA has been associated with the HLA DR3-DQ2/DR4-DQ8 genotype (Kulmala et al. 2001; Bakhtadze et al. 2006) and the DR4-DQ8 haplotype (Kulmala et al. 2001), which was also found in our study, as well as associations for IA-2A with the DR4-DQ8 haplotype (Bakhtadze et al. 2006). An explanation for differences in associations between autoantibodies and HLA alleles could be that in some studies the autoantibodies were measured in individuals at onset of type 1 diabetes (Hagopian et al. 1995; Genovese et al. 1996; Savola et al. 1998; Bakhtadze et al. 2006) while in other studies in pre-diabetic individuals (Kulmala et al. 2000; Kulmala et al. 2001). Not all of the healthy individuals with autoantibodies will develop diabetes, and therefore the HLA genotypes in non-progressors might differ from the ones observed in individuals recently diagnosed with type 1 diabetes. In our study, associations between beta-cell autoantibodies and HLA-risk alleles were found only in children at 5 years of age. The lack of association at 1 and 2.5 years of age suggest that HLA-risk alleles related to type 1 diabetes are not necessarily needed for presentation of beta-cell antigens and for induction of beta-cell autoantibodies.
Table 3. Associations for GADA or IA-2A positive children with HLA alleles, found in our study and/or in previous studies. aab = autoantibody.

<table>
<thead>
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<th>Autoantibody</th>
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</tr>
<tr>
<td></td>
<td>DR4-DQ8</td>
<td>Kulmala et al. 2001+our study</td>
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<tr>
<td></td>
<td>DR3-DQ2</td>
<td>Savola et al. 1998; Hagopian et al. 1995</td>
</tr>
<tr>
<td></td>
<td>DR2-DQ6 (negatively associated)</td>
<td>Kulmala et al. 2001</td>
</tr>
<tr>
<td>IA-2A</td>
<td>DR4</td>
<td>Genovese et al. 1996; Savola et al. 1998</td>
</tr>
<tr>
<td></td>
<td>DR4-DQ8</td>
<td>Bakhtadze et al. 2006; Kulmala et al. 2001+our study</td>
</tr>
<tr>
<td></td>
<td>DR4-DQ8/DR4-DQ8</td>
<td>our study</td>
</tr>
<tr>
<td></td>
<td>DR2-DQ6 (negatively associated)</td>
<td>our study</td>
</tr>
<tr>
<td></td>
<td>DR2-DQ6/DR2-DQ6 (negatively associated)</td>
<td>our study</td>
</tr>
</tbody>
</table>

Permanent and transient autoantibodies

To further evaluate the associations of HLA genotypes with the presence of autoantibodies, we investigated samples collected at all three time points (1, 2.5 and 5 years of age) from a group of non-diabetic children in the ABIS study (Figure 13). Eight of 2329 (0.3%) children were antibody positive at least at 2.5 and 5 years of age, i.e. had permanent autoantibodies. In addition, 143 of 2329 (6%) children were autoantibody positive only at one time point followed by autoantibody negativity, i.e. had transient autoantibodies. Children with permanent autoantibodies had more often the risk associated DR4-DQ8 haplotype than autoantibody negative children, while no associations with HLA risk or protective genotypes were found for transient autoantibodies.

A previous study has shown that more than 50% of the children with permanent autoantibodies had the high-risk genotype DR3-DQ2/DR4-DQ8, while no children with transient autoantibodies had this genotype (Yu et al. 2000). That study demonstrated the occurrence of permanent antibodies in 0.9% of children from the general population, which was slightly higher than the percentage observed in our study (0.3%). However, it should be noted that the study by Yu et al. differed in some aspects from our study. That study included measurements of three autoantibodies; GADA, IA-2A and IAA, the age interval of the children was 0-13 years, and the consecutive measurements were performed more often; annually in autoantibody negative children and every 3-6 months after autoantibody positivity was found. Further, in our study the group of children with permanent autoantibodies was small, with only eight individuals, which makes it difficult to draw conclusions. In a recent
study, both HLA risk genotypes and the T allele of the PTPN22 gene were shown to influence progression to permanent islet autoimmunity (Steck et al. 2009).

Figure 13. Selection of children from the ABIS study for the follow-up of GADA and IA-2A at 1, 2.5 and 5 years of age. Thirty-two children had developed type 1 diabetes by the age of 6-7 years. Of the 2329 children from whom samples were available at all three ages, permanent and transient autoantibodies were studied and related to HLA genotypes. A subgroup of autoantibody negative children were randomly selected for HLA genotyping.

We found that children with transient autoantibodies and autoantibody-negative children had a very similar pattern of HLA genotypes (Figure 14). This suggests that HLA-risk alleles are not necessarily needed for the induction of beta-cell autoantibodies, but is likely rather influenced by environmental factors or other genetic factors than HLA.
Results and discussion

Figure 14. Percentages of individuals with risk, protective, a combination of risk and protective and neutral HLA genotypes in children with type 1 diabetes, in healthy children with permanent autoantibodies or transient autoantibodies and in healthy autoantibody-negative children.

Children with type 1 diabetes

By the age of 6-7 years we identified 32 children with type 1 diabetes among the 17055 participants in the ABIS study. HLA risk genotypes associated with type 1 diabetes were frequently found in these children, whereas protective alleles were rare (Figure 14). The percentages of HLA-risk and -protective haplotypes corresponded well with other studies, showing that nowadays the contribution of high-risk HLA haplotypes is lower and the proportion of protective haplotypes is higher among type 1 diabetes patients compared to 50 years ago (Hermann et al. 2003; Gillespie et al. 2004). This changed distribution of HLA haplotypes could indicate that environmental factors are important for development of type 1 diabetes, not only in children at later ages, but also in young children as in our study. Three percent (1/32) of the diabetic children in our study did not have any HLA-risk alleles, in accordance with a study by Gillespie et al. where 5-8% of children diagnosed before 5 years of age lacked risk-associated alleles (Gillespie et al. 2004).

By studying samples from the time point preceding the onset of type 1 diabetes we found that 64% of the children were positive for GADA and/or IA-2A. This is lower than what have been reported by other studies (Bonifacio et al. 1995; Sabbah et al. 1999; Strebelow et al. 1999; Winter et al. 2002). The lower frequency of autoantibody positivity in our study might be due to a longer interval between collections of samples, and in many individuals the last sample before onset was taken a long time before onset (range 0-2.5 years). Thus, it cannot be excluded that autoantibodies may have developed short after the last sampling. Another factor that could contribute to the lower percentage of autoantibody positivity is that IAA was not
Results and discussion

measured in our study, because of the small volumes of EDTA blood that was collected from the children at 1 and 2.5 years of age.

In conclusion, the strong relation between HLA risk alleles and type 1 diabetes observed in our study confirms that HLA risk genotypes play an important role for development of type 1 diabetes. In contrast, HLA genotypes seem not to explain induction of autoantibodies, especially transient autoantibodies, in the general population. Our results indicate that other factors than HLA are involved in the initiation of autoimmunity.

DEVELOPMENT OF AN IMMUNOASSAY BASED ON SPR (PAPER II)

Despite a lot of research in the field of type 1 diabetes, there is still no prevention for the disease. However, a number of ongoing and planned trials raise the hope for successful prevention strategies (Schloot et al. 2007; Pescovitz et al. 2009; Rother et al. 2009; Tian et al. 2009; Ludvigsson 2011). If any treatment demonstrates to be beneficial for prevention, the need to identify risk individuals is large. Although HLA-risk genotypes define some of the risk individuals (Ilonen et al. 2002), the best way to identify risk individuals is through autoantibodies (Verge et al. 1996; Bingley et al. 1997). Thus, large scale screening of autoantibodies in children from the general population will be necessary to identify high-risk individuals suitable for prevention treatment. At present, screening for autoantibodies is commonly performed by the well established RIA methods. However, there are some disadvantages with these methods, including the use of radioactively labelled material, variations among the “in-house” batches of the GAD and IA-2 proteins, low sensitivity for the IAA assay and long assay times for a sample. Thus, there is a need for better methods for detection of autoantibodies.

The surface plasmon resonance (SPR) technology is an interesting candidate for detection of autoantibodies, mainly due to the label-free detection, the short analysis time for each sample and the possibility to simultaneously measure multiple antibodies. We therefore decided to develop a method based on this technology, by using a Biacore instrument, for detection of three of the autoantibodies commonly related to type 1 diabetes; GADA, IA-2A and IAA. We aimed to decrease the analysis time per sample, and if possible, to increase the sensitivity of the autoantibody measurement compared to RIA. We decided to start with GADA, since this autoantibody is one of the most commonly observed in pre-diabetic children. In addition, a
Results and discussion

better method for GADA was of special interest for us since the GAD-alum trial was about to start. This treatment might be a good candidate to be used in prevention trials with GADA positive individuals.

**Attempts to develop an SPR-based immunoassay for GADA**

We immobilized GAD65 at pH 5.0 via amine coupling, resulting in moderate levels of immobilized protein at the dextran surface. However, the injections of GAD65 mAbs and a GADA positive serum, to the surface immobilized with GAD65, induced low and inconsistent binding responses. A possible explanation for this could be that the conformation of the GAD65 protein might have changed upon binding to the surface. In addition, after regeneration with NaOH, the binding responses were different from the ones observed before regeneration. We tried to overcome these problems by attaching the GAD65 protein to the surface using another coupling chemistry. Instead of immobilizing GAD65 directly to the sensor surface, we immobilized streptavidin to the surface and then added biotinylated GAD65, which binds to streptavidin. However, this setup did not perform better than GAD65 immobilized directly to the surface.

A number of different regeneration solutions and cocktails, such as NaOH (pH ranging from 10.7 to 12.7), glycine pH 3.0 and different ionic solutions, were tested without finding any solution that regenerated the surface sufficiently. David Myszka, in cooperation with Chris Hampe, has previously observed difficulties to regenerate the surface by using acidic or basic regeneration solutions after interaction with GAD65 (unpublished report, partially published in (Raju et al. 2005)). The problems with the low binding of antibodies to GAD65 and the altered binding after regeneration, suggest that GAD65 is a pH-sensitive and unstable protein. Consequently, since the GAD65 protein seemed to be problematic in this kind of assay, we did not continue the development of the assay.

We did not develop an SPR-based method for detection of IA-2A, mainly due to difficulties to obtain the IA-2 protein and antibodies towards IA-2 to test the assay.
Results and discussion

Development of the SPR-based immunoassay for IAA

Pre-concentration of human recombinant insulin to the dextran matrix was optimized. Then, insulin (100 µg/ml) at pH 5.0 was immobilized via amine coupling to a CM5 chip. This procedure was started by activation of the carboxyl groups at the sensor chip with 0.2 M N-ethyl-N-(3-diethylaminopropyl) carbodiimide (EDC) and 0.05 M N-hydroxysuccinimide (NHS) during 7 minutes (Figure 15). Thereafter, insulin was injected during 7 minutes for immobilization, followed by blocking of excess activated carboxyl groups with ethanolamine hydrochloride (1 M, pH 8.5) for 7 min. The surface was finally washed twice with glycine-HCl (10 mM, pH 1.8) for 1 min. The level of immobilized insulin varied to some extent between different sensor chips, but stayed at 1300 ± 200 RU. This was the highest level possible to immobilize, and could not be increased by injection of higher insulin concentrations or by increasing the time that insulin was allowed to pass the surface.

![Figure 15. Immobilization of insulin followed by regeneration and binding of a monoclonal anti-human insulin antibody. Injection of EDC/NHS (1), insulin (2), ethanolamine (3), glycine-HCl (4) and finally a monoclonal anti-human insulin antibody (5) are shown in the sensorgram.](image)

To assure that binding to the immobilized insulin worked properly, a standard curve, with a monoclonal anti-human insulin antibody (Art. No. I7660-14, Biosite, recognizing an epitope including amino acid B30) diluted in running buffer, was run once (Figure 16A). In following experiments, new surfaces immobilized with insulin were checked for activity with the same insulin antibody at a concentration of 20 µg/ml.
Figure 16. The anti-human insulin antibody concentrations injected were 10000, 5000, 2500, 1250, 625, 313, 156 and 78 ng/ml, resulting in responses of 1352, 630, 290, 137, 66, 31, 14 and 6 RU, respectively, as shown in the standard curve (A). In panel B, the anti-human insulin antibody at concentrations of 10000, 5000, 2500, 1250, 625 and 313 ng/ml were diluted in an IAA-negative serum instead of buffer. The responses were 948, 361, 163, 102, 41 and 4 RU, after subtraction of the response for the serum alone (169 RU), and were presented in panel B (black squares) together with the standard curve from panel A (black circles).

Efforts to reduce non-specific binding of serum proteins to the sensor surface

The next step in the assay development was to introduce serum to the immobilized surface. Non-specific binding of serum components to the surface of the sensor chip often gives a large SPR signal, which masks the analyte response. This is especially evident when measuring molecules of low concentration in serum, which is the case for IAA. We observed high non-specific binding of serum proteins during initial experiments. It has been reported that when the concentration of a molecule is high enough to be detected after diluting serum 1/20, the problem with non-specific binding of serum matrix proteins can be overcome (Karlsson et al. 1993). However, when detecting IAA in human serum, the generally very low concentrations do not allow the use of diluted samples in the assays.

Other ways to overcome the problem with non-specific binding of serum proteins are to use a proper design of the surface chemistry on the sensor chip and/or to introduce additives to the analyte solution. Different non-sticky surface coatings, based on dextran (Löfås et al. 1990) or PEG (Herrwerth et al. 2003; Gobi et al. 2007; Larsson et al. 2007; Shankaran et al. 2007), have been developed. For the development of our SPR-based immunoassay we used the CM5 chip (Figure 11). A CM4 chip, with low carboxylation, is sometimes used to reduce non-
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specific binding (Raju et al. 2005) (www.biacore.com). However, since assays for determination of the concentration of an analyte generally require a high surface concentration of the immobilized molecule, this chip with fewer immobilization sites was not a suitable choice for our assay. Addition of CM dextran to a serum sample has shown to reduce the non-specific binding to CM-coated surfaces with up to 75% via a competition effect (Karlsson et al. 1993). We therefore chose to use CM dextran and NaCl for reduction of non-specific binding. When the concentrations of these additives were optimized (1 mg/ml CM dextran and 0.35 M NaCl) an evident reduction of the non-specific binding signal was observed.

After reduction of the non-specific binding it was possible to detect the insulin antibodies in serum. A standard curve, with different concentrations of the monoclonal anti-human insulin antibody diluted in an IAA-negative serum sample, was run. The shape of the curve was similar, but with lower responses, than the standard curve obtained with different concentrations of insulin antibody diluted in buffer (Figure 16B).

**Detection of IAA in serum using the 3-day and 5-min methods**

Since the non-specific binding fluctuated among sera from different individuals, it was difficult to measure IAA directly in serum. To overcome this problem, an indirect competitive immunoassay was developed. In this method, each serum sample was divided in two equal aliquots; one was mixed with buffer and the other with an excess amount of insulin, similarly to the procedure for detection of IAA by RIA. In RIA, the following step is incubation of the serum solutions for 72 hours. Since one of our objectives was to develop a method which was not as time consuming as RIA, we aimed to reduce the time required for analysis of one sample. Thus, the serum mixtures were incubated under agitation either for 72 hours at 4ºC (according to the 3-day method) or for 5 minutes at room temperature (according to the 5-min method). Thereafter, each serum mixture was diluted with CM dextran and NaCl for reduction of non-specific binding, and then injected over the sensor surface immobilized with insulin. When the portion of serum mixed with buffer was introduced to the surface, IAA in the sample was free to bind the immobilised insulin on the surface (Figure 17, lower left). In contrast, when serum mixed with insulin was injected, competition for binding of IAA between insulin in solution and insulin immobilised on the surface took place. However, due to the large excess of insulin molecules in solution, very little binding of IAA to the surface-
bound insulin would occur. The resonance signal obtained from this sample is instead a measure of non-specific binding to the immobilized insulin and to the dextran matrix.

The levels of IAA interacting with insulin were too low to be directly quantified. To enhance the response signal, a secondary polyclonal anti-human IgG antibody (50 µg/ml in HBS-EP) was injected immediately after the serum injection was completed. After a short regeneration-pulse with glycine-HCl at pH 1.8 the surface was ready to be used for a new serum injection. Regeneration of the surface removed both specifically and non-specifically bound serum components from the surface. The regeneration was very reliable, providing a stable baseline, and a chip could be used for numerous (at least 50) repeated cycles without any visible decrease in response.

To calculate the level of IAA in a serum sample (R), the secondary antibody response from serum mixed with buffer (Ba-Bb) was subtracted from the response obtained when the serum was mixed with excess insulin (Ia-Ib) (Figure 17), according to the formula:

\[ R = (B_a - B_b) - (I_a - I_b) \]

where Ba = response for sample incubated with buffer after secondary antibody, Bb = before secondary antibody, Ia = response for sample incubated with excess insulin after secondary antibody and Ib = before secondary antibody.

To assess if we were able to detect different levels of IAA in serum, we run a standard curve, using both the 3-day and the 5-min method. A pooled high IAA-positive serum was diluted to different concentrations of IAA (0-40 U/ml) in pooled serum from IAA-negative non-diabetic individuals. This was done to yield a range of positive samples with almost identical serum matrix composition. The SPR-run showed that the responses increased with increasing IAA concentrations, both for the 3-day (Figure 18A) and the 5-min method (Figure 18B).
Figure 17. The principle of IAA detection with the indirect competitive immunoassay based on SPR (lower part of the figure) as well as a full response cycle for a serum sample mixed with buffer (upper left) and with insulin (upper right) is shown. In the figure, the different steps during the cycle are illustrated as follow: 1 = start of injection of serum mixed with buffer, 2 = end of serum injection, 3 = start of secondary antibody injection, 4 = end of secondary antibody injection, 5 = start of regeneration, 6 = end of regeneration, 7 = start of injection of serum mixed with insulin, 8 = end of serum injection, 9 = start of secondary antibody injection, 10 = end of secondary antibody injection, 11 = start of regeneration and 12 = end of regeneration. The difference in secondary antibody response from a serum sample incubated in buffer (Ba-Bb) and insulin (Ia-Ib), respectively, gives a measure of the level of IAA in the sample.
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Figure 18. Standard curves from (A) the 3-day and (B) the 5-min methods. A pooled high IAA positive serum was diluted to concentrations of 0, 4, 6, 10, 20 and 40 U/ml for the 3-day method, resulting in SPR responses of -4, 8, 15, 17, 20 and 29 RU. For the 5-min method, the concentrations of the pooled high IAA positive serum were 0, 4, 6, 7.5, 10, 20 and 40 U/ml, giving SPR responses of 45, 44, 47, 54, 61, 67 and 80 RU. For both methods, the IAA positive serum was diluted in pooled serum from IAA negative non-diabetic individuals. U/ml is an arbitrary unit from the reference RIA method.

Since the non-specific binding of serum proteins to the surface is very different among different individuals, we wanted to evaluate if a similar standard curve could be obtained when using sera from 7 non-diabetic individuals instead of one pooled serum as background for the standard curve. Therefore, another set of standard curves was run, with both the 3-day and the 5-min method. Each IAA-containing sample was diluted in a single IAA-negative serum, and different single IAA-negative serum samples were used for the different concentrations of the IAA-negative sample. Again, the SPR responses increased with increasing IAA concentrations (Figure 19 and 20), confirming that the SPR-based indirect competitive immunoassay was suitable for the measurement of IAA in serum from different individuals. To determine variations in the method, we run the standard curve with the 5-min method three times (Figure 20). Reproducibility was found to be excellent, with low variation between the runs (Table 4).
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**Figure 19.** Standard curve from the 3-day method. Different concentrations of a pooled high IAA-positive sample (0, 4, 6.6, 10, 20 and 40 U/ml) were diluted in different single IAA negative serum samples from non-diabetic individuals, resulting in SPR responses of -8, 4.9, 15.9, 23.2, 37.6 and 45.2 RU.

**Figure 20.** Standard curve from the 5-min method, based on three repeated runs. A high IAA-positive sample was diluted to different concentrations in different single serum samples from IAA-negative non-diabetic individuals. The values of the mean responses from the SPR run for these samples are shown below in Table 4. Sample A, B, C and D are non-pooled sera from type 1 diabetic patients (A = 32.5 U/ml, B = 24 U/ml, C = 11.3 U/ml and D = 5.2 U/ml).
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Table 4. Test of reproducibility of a standard curve from the 5-min method, with different concentrations of an IAA-positive sample diluted in different single serum samples from IAA-negative non-diabetic individuals. The corresponding SPR responses for the three runs are shown, as well as mean and CV (coefficient of variation).

<table>
<thead>
<tr>
<th>IAA (U/ml)</th>
<th>Response (RU)</th>
<th>Mean</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>46.7</td>
<td>46.0</td>
<td>2.4</td>
</tr>
<tr>
<td>2</td>
<td>48.9</td>
<td>40.6</td>
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</tr>
<tr>
<td>40</td>
<td>136.0</td>
<td>134.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

High-affinity IAA

It has been shown that IAA of high affinity is related to progression to type 1 diabetes (Achenbach et al. 2004a; Schlosser et al. 2005b). Thus, methods for detection of IAA should be designed to measure high-affinity antibodies. We were therefore interested to find out if the IAA measured with the SPR-based immunoassay were of high affinity. The sites for antigen immobilization in an immunoassay might affect the nature of the antibodies being captured. In our experiments, possible coupling sites on the insulin molecule to the dextran matrix are the N-termini of the A and B chains and the lysine residue at position B29, since these positions contain amino groups. Thus, the A8-A13 region, which is important for binding of high-affinity IAA (Achenbach et al. 2004a), should be available for binding after immobilization of the insulin molecule. This speaks in favor of that the observed response in the SPR-based assays can be originated from high-affinity IAA. Other important arguments supporting this idea are that the same conditions as in the incubation step in RIA were used in our 3-day method, and that all the IAA-positive serum samples measured in the SPR-based system were from newly diagnosed type 1 diabetic patients, and consequently should be disease-specific and thereby IAA of high affinity.

The 5-min method compared to RIA

We evaluated differences between the responses detected by the 3-day and the 5-min methods. The methods showed to perform similarly, suggesting that the 5-min method probably also detect high-affinity IAA. Thus, we believe the 5-min method is suitable for SPR-based detection of IAA in serum. In RIA, the mixture of IAA-containing serum and
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labelled insulin is incubated for 3 days, capturing the high-affinity antibodies (Wilkin 1990). In the Biacore system, the continuous flow enabling repeated associations and dissociations between antigens and antibodies, might explain the functionality of the 5-min method, although the incubation time is shortened to only 5 minutes.

By using a calculated cut-off value for the 5-min method, the serum samples were classified as IAA-positive or -negative in accordance with the results obtained from the measurement with RIA (Figure 20). To determine the cut-off level for the 5-min method, a number of IAA-negative samples from non-diabetic individuals as well as low IAA-positive samples from newly diagnosed type 1 diabetic patients were measured with the SPR method. The cut-off for positivity was set to 6 U/ml, by calculating the mean value of the low IAA positive samples subtracted by 3 SD. None of the IAA-negative samples exceeded this value, indicating that this cut-off value was suitable for our assay. Since the RU value for the same sample might differ to some extent between different runs, depending on the amount of insulin immobilized on the surface, the cut-off value cannot be expressed as RU. In RIA, the cut-off for positivity for IAA was 6.45 U/ml, corresponding to the 98th percentile of 114 healthy Swedish children (Holmberg et al. 2006).

The similar cut-off levels, i.e. 6 and 6.45 U/ml, for the SPR-based method and RIA respectively, indicate that the sensitivity for detection of IAA with both methods was similar. However, the IAA levels differed to some extent between the 5-min indirect competitive immunoassay and the RIA. A possible explanation might be that with the SPR-based assay we probably detected IAA of the IgG3 subclass, which cannot be detected in RIA using protein A sepharose for precipitation. In a study by Hoppu et al, IAA of IgG1 and IgG3 subclasses were predominant in genetically susceptible young children who rapidly progressed to clinical type 1 diabetes, while a weak or absent IgG3 response was associated with relative protection from disease (Hoppu et al. 2004c).

The SPR-based immunoassay for detection of IAA was time-saving compared to RIA. The RIA procedure takes 4 days to perform, and 15 samples can be run simultaneously in one plate. The same number of samples in the SPR-based immunoassay takes only approximately 13 hours to run, since the time to run one sample in this system is about 50 minutes and the samples are run after each other. One disadvantage with the SPR-based immunoassay compared to RIA is the larger volume of serum needed for the assay; 100 µl compared to only
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30 µl in RIA. However, the advantages of the 5-min method, with no need for labelling of interactants and that it is time-saving compared to RIA, speak in favor for the use of the SPR-based assay when sample volume is not a limiting issue.

**Type 1 diabetes-related analytes measured with SPR: Results from other studies**

SPR has been used in several studies to measure biomolecules in diluted serum (Vikinge et al. 1998; Gomara et al. 2000; Jongerius-Gortemaker et al. 2002) and some have detected biomolecules in undiluted serum (Masson et al. 2007; Phillips et al. 2007). A number of studies have measured type 1 diabetes-related analytes with SPR. For example, Ayela et al detected a polyclonal antibody against a synthetic peptide from the juxtamembrane region of IA-2, in diluted serum (1/100), using mixed self-assembled monolayers (SAM) on a gold surface as the sensor surface (Ayela et al. 2007). Lee et al measured monoclonal anti-GAD65 antibodies diluted in buffer, using an alkanethiol SAM sensor surface, by immobilizing streptavidin on the surface followed by injection of biotin-GAD (Lee et al. 2005). Another study focused on measurement of insulin, both in buffer and in diluted serum (1/4 and 1/10), using a PEG-containing SAM sensor surface (Gobi et al. 2007). They used an indirect competitive immunoreaction principle, in which a monoclonal anti-insulin antibody was mixed with different concentrations of insulin prior to injection into the SPR instrument. Thereafter, the insulin concentration was indirectly measured by detecting the binding of the anti-insulin antibody to the sensor surface immobilized with insulin. In another study, the level and affinity of insulin antibodies (IA) in diabetic patients after receiving exogenous insulin were measured with SPR, using a CM5 chip (Kure et al. 2005). Since the levels of IA in diabetic patients after receiving insulin are generally much higher than the levels of IAA commonly observed in newly diagnosed type 1 diabetic patients and non-diabetic individuals, their assay did not detect low concentrations of antibodies, as we aimed to do. Also, due to the relatively high concentrations of IA, they were able to overcome the problems with non-specific binding of serum proteins to the dextran matrix by diluting the serum. Our SPR-based indirect competitive immunoassay for IAA is, to our knowledge, the only published report on IAA quantification in serum samples using SPR.
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*Further and future experiments with the 5-min method*

After publication of the methodological paper with the SPR-based indirect competitive immunoassay for detection of IAA (Paper II), further experiments were performed. To evaluate the capability of the method for large scale screening of serum samples, 86 serum samples were analysed. The method detected the majority of the IAA-positive samples, previously classified as positive by RIA, and also most of the IAA-negative samples were correctly classified. However, we observed that some serum samples showed aberrant high responses, higher than what is commonly observed in positive samples with high IAA levels. To date, we have no explanation for this result, but it was interesting to observe that some of the sera were collected from diabetic patients while others came from non-diabetic individuals. Further experiments are needed to clarify the reason for the aberrant high responses.

**In conclusion**, we have developed an SPR-based indirect competitive immunoassay for detection of IAA in serum. The main advantages with this assay are that there is no need for labelling of interactants and it is time-saving compared to RIA, but further development of the method is needed.

**EFFECTS OF THE GAD-ALUM TREATMENT ON THE HUMORAL IMMUNE RESPONSE (PAPER III AND IV)**

**Increased levels of GADA**

In the phase II clinical trial performed in children and adolescents with type 1 diabetes, treatment with GAD-alum resulted in preservation of residual insulin secretion in children with short duration of the disease, shown as a declined decrease in C-peptide levels. A number of effects on the immune system have been observed after this treatment (Axelsson et al. 2010; Hjorth et al. 2010), including increased levels of GADA (Ludvigsson et al. 2008) (Figure 21). The GADA levels started to increase already after the first injection of GAD-alum, and peaked at 3 months, after the booster dose. Further, still 30 months after the first injection the levels were higher compared to the placebo group.
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Figure 21. Median levels of GADA for the GAD-alum group (black circles) and the placebo group (white circles) at baseline and at 1, 3, 9, 15, 21 and 30 months after initial injection. GADA titers increased after injections of GAD-alum, and at month 3, the titers were significantly higher in the GAD-alum compared to the placebo group. This difference persisted throughout the 30 months study period.

In a GAD-alum dose-finding study in LADA patients, GADA levels did not increase after injection of 20 μg of GAD-alum, the dose that resulted in clinical effect (Agardh et al. 2005) and was used in our study. However, in the same study, increased GADA levels were observed in patients treated with 500 μg of GAD-alum, and were accompanied by a temporary increase in GADA-binding to one epitope specificity often related to type 1 diabetes (Bekris et al. 2007). We were therefore interested to study whether the epitope binding pattern of GADA was affected by the GAD-alum treatment in type 1 diabetic patients.

Temporary increase in binding to one rFab-defined epitope

To investigate if the epitope specificity of GADA was affected by the GAD-alum treatment, we used six rFab (b96.11, DPA, DPD, MICA3, b78 and N-GAD65 mAb) recognizing different epitope regions on GAD65 (Paper III). The b96.11-defined epitope was generally
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predominant and observed in the majority of the tested sera of both GAD-alum and placebo treated children (Figure 22). This was expected, since this epitope specificity has previously been shown to be predominant in type 1 diabetic patients (Padoa et al. 2003). Further, binding to the DPD- and MICA3-defined epitopes was also observed in our study, previously found in other studies with type 1 diabetic individuals (Richter et al. 1993; Padoa et al. 2003).
Figure 22. Binding to GAD65 in the presence of rFab b96.11 (A), DPD (B), MICA-3 (C), DPA (D), b78 (E) and N-GAD65 mAb (F) at baseline, 3 months and 15 months for GAD-alum and placebo groups, presented as ratio of competed / non-competed. A higher binding to GAD65 in the presence of an rFab indicates a lower proportion of GADA binding to the respective epitope. Ratios above 1.4 are placed within brackets outside the range of the y axis.

Overall, there were no longitudinal changes in binding to any of the epitopes, including b96.11, over the 15 months study period (Table 5). Binding levels to the DPA-, N-GAD65 mAb- and b78-defined epitopes were very low throughout the study (Figure 22) and were therefore excluded from the statistical analyses.

Table 5. The variability over time (baseline to 15 months) was investigated by calculation of the sum of absolute differences between consecutive measurements, and compared for the GAD-alum and placebo groups.

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Sum of absolute differences (min, max)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GAD-alum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td></td>
</tr>
<tr>
<td>b96.11</td>
<td>40.0 (3.6, 108.2)</td>
<td>0.07</td>
</tr>
<tr>
<td>DPD</td>
<td>37.3 (17.3, 76.6)</td>
<td>0.48</td>
</tr>
<tr>
<td>MICA3</td>
<td>42.1 (16.3, 108.7)</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Next, as GADA titers peaked at 3 months, we were interested to study whether this increase in GADA titer was accompanied by any change in GADA epitope recognition between two visits. For this analysis, binding of GADA to rFab-defined epitopes was compared between samples collected at two time points (baseline to 1 month, baseline to 3 months, and 3 months...
to 15 months). Our results showed that binding to the b96.11-defined epitope changed temporarily, with increased binding between baseline and 3 months in the GAD-alum treated group compared to the placebo group (Table 6). This effect was especially evident in the group of GAD-alum treated patients who experienced an increase of more than 100% in their GADA titer from baseline to 3 months. The increase in binding to the b96.11-defined epitope was followed by decreased binding between 3 and 15 months, resulting in similar levels at 15 months as the ones observed at baseline. Similar results were observed in the GAD-alum dose-finding study on LADA patients, in which treatment with the highest dose of GAD-alum (500 µg) was accompanied by increased GADA levels (Agardh et al. 2005), and temporarily increased binding to the b96.11-defined epitope (Bekris et al. 2007). The changes in b96.11 epitope recognition after treatment with 20 µg GAD-alum found in our study, was not seen in the group of LADA patients receiving the same dose of GAD-alum resulting in clinical effect. This might be due to the small number of individuals in the LADA study, or alternatively, changes in b96.11 epitope recognition might be explained by the increased GADA levels in the type 1 diabetic patients, which was not observed in LADA patients after the same dose of GAD-alum.

Higher GADA titers have previously been associated with broader epitope reactivity (Gilliam et al. 2004; Ronkainen et al. 2004). In our study, we found no correlation between GADA titer and epitope binding at 3 and 15 months in the GAD-alum group, but in the placebo group binding to the b96.11- and DPD-defined epitopes correlated with GADA titer (Table 7). We cannot explain the lack of correlation in the GAD-alum group after treatment. The treatment induced very high GADA titers, often much higher than the ones detected in the majority of GADA positive diabetic individuals. We could speculate that the binding to the b96.11-defined epitope did not increase in the same proportion as the induced GADA. It could also be possible that some of the induced GADA bind to other epitopes on GAD65 than those tested in our study. The temporary increased binding to the b96.11-defined epitope could be due to the activation and further upregulation of the already existing memory B cell responses. Although the increase in binding to the b96.11-defined epitope was only temporary, the GADA levels remained higher in the GAD-alum group compared to placebo even 30 months after treatment. It cannot be excluded that as a part of the immunomodulatory effect of the treatment, GAD-alum might have induced memory B cells producing GADA with other epitope specificities than those tested in our study. This is an interesting hypothesis to be confirmed in future studies.
Table 6. Differences in epitope binding between GAD-alum and placebo patients, from baseline to 1 month, baseline to 3 months, 3 months to 15 months and baseline to 15 months. Changes in median binding to GAD65 in the presence of rFab b96.11, DPD and MICA3 between two time points were calculated and the differences in median response were compared between the GAD-alum and placebo groups. This was also done for individuals in the GAD-alum group who experienced an increase in GADA titer of more than 100% between baseline and 3 months (high GADA increasers, HGI) compared to individuals in the same group without this increase (non-HGI).

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Months compared</th>
<th>GAD-alum</th>
<th>Placebo</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Difference in median response % (min, max)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0-1</td>
<td>-2.8 (-54.4, 52.6)</td>
<td>0.5 (-28.4, 28.2)</td>
<td>0.45</td>
</tr>
<tr>
<td>b96.11</td>
<td>0-3</td>
<td>-8.1 (-72.4, 39.6)</td>
<td>1.5 (-28.3, 28.6)</td>
<td>0.02 *</td>
</tr>
<tr>
<td></td>
<td>3-15</td>
<td>8.3 (-17.1, 36.7)</td>
<td>-2.4 (-32.8, 30.1)</td>
<td>&lt;0.05 *</td>
</tr>
<tr>
<td></td>
<td>0-15</td>
<td>-5.6 (-62.5, 62.4)</td>
<td>1.6 (-25.6, 35.7)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>0-3</td>
<td>0.9 (-24.1, 23.3)</td>
<td>3.2 (-15.3, 32.5)</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>3-15</td>
<td>3.2 (-15.9, 24.3)</td>
<td>2.6 (-37.4, 26.7)</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>0-15</td>
<td>-2.5 (-42.3, 42.1)</td>
<td>-0.1 (-49.8, 37.3)</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>0-1</td>
<td>5.6 (-21.8, 51.7)</td>
<td>1.2 (-9.5, 49.4)</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>0-3</td>
<td>3.1 (-31.1, 56.8)</td>
<td>0.1 (-36.6, 25.4)</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>3-15</td>
<td>5.3 (-34.3, 20.7)</td>
<td>-0.9 (-42.1, 36.1)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0-15</td>
<td>0.1 (-33.9, 39.2)</td>
<td>-0.1 (-36.2, 25.3)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Epitope</th>
<th>GAD-alum HGI</th>
<th>GAD-alum non-HGI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Difference in median response % (min, max)</td>
<td>p-value</td>
</tr>
<tr>
<td>b96.11</td>
<td>-10.8 (-72.4, 30.5)</td>
<td>3.1 (-7.9, 39.6)</td>
</tr>
</tbody>
</table>
Results and discussion

Table 7. Correlations between GADA titer and binding of GADA to GAD65 in the presence of rFab b96.11 or DPD for all individuals, and for the GAD-alum and placebo groups, at baseline, 3 months and 15 months after the first injection.

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Time point</th>
<th>Individuals</th>
<th>Correlation with GADA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>p-value</td>
</tr>
<tr>
<td>b96.11</td>
<td>baseline</td>
<td>all individuals</td>
<td>0.001 **</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAD-alum</td>
<td>0.021 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>placebo</td>
<td>0.073</td>
</tr>
<tr>
<td></td>
<td>3 months</td>
<td>all individuals</td>
<td>0.018 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAD-alum</td>
<td>0.688</td>
</tr>
<tr>
<td></td>
<td></td>
<td>placebo</td>
<td>0.005 **</td>
</tr>
<tr>
<td></td>
<td>15 months</td>
<td>all individuals</td>
<td>0.017 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAD-alum</td>
<td>0.283</td>
</tr>
<tr>
<td></td>
<td></td>
<td>placebo</td>
<td>0.019 *</td>
</tr>
<tr>
<td>DPD</td>
<td>baseline</td>
<td>all individuals</td>
<td>0.003 **</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAD-alum</td>
<td>0.011 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>placebo</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td>3 months</td>
<td>all individuals</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAD-alum</td>
<td>0.840</td>
</tr>
<tr>
<td></td>
<td></td>
<td>placebo</td>
<td>0.004 **</td>
</tr>
<tr>
<td></td>
<td>15 months</td>
<td>all individuals</td>
<td>0.025 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAD-alum</td>
<td>0.254</td>
</tr>
<tr>
<td></td>
<td></td>
<td>placebo</td>
<td>0.048 *</td>
</tr>
</tbody>
</table>

Changes in IgG subclasses of GADA

Since the GAD-alum treatment affected GADA levels and temporarily increased binding to the b96.11 defined epitope, we were interested to investigate if there were any further effects of the GAD-alum treatment on GADA. In humans, Th1 cells and their related cytokines seem to stimulate the production of IgG1 while Th2 cells stimulate the production of IgG4 and IgE (Gascan et al. 1991; Couper et al. 1998; Bonifacio et al. 1999; Hawa et al. 2000). Based on results from animal studies, a Th2 profile has been suggested to be protective against the aggressive damage inflicted on the insulin-producing cells (Cameron et al. 1997; Petrovsky et al. 2003). The GAD-alum treatment in type 1 diabetic children induced a Th2 response, with increased levels of IL-5 and IL-13 already 1 month after the first injection (Axelsson et al. 2010). A broad range of cytokines were induced at 3 and 9 months, but only the two Th2 associated cytokines IL-5 and IL-13 together with FOXP3 increased continuously over time.
Therefore, we were interested to study the IgG subclass distribution of GADA. Samples from patients in the GAD-alum and placebo groups were investigated at baseline and at 3 and 15 months after initial treatment (Paper IV). Frequencies of the different IgG subclasses were calculated with respect to total IgG in each sample at every time point. IgG1 was the predominant IgG subclass of GADA, both in GAD-alum and placebo individuals (Table 8). This is in accordance with previous studies showing a predominance of IgG1 both in prediabetic individuals and in newly diagnosed type 1 diabetic individuals (Bonifacio et al. 1999; Hoppu et al. 2004a; Ronkainen et al. 2006).

At baseline, the percentages of IgG1, 2, 3 and 4 were similar in both groups. Three months after initial injection of GAD-alum a reduced percentage of IgG1 and increased IgG3 and IgG4 were detected in GAD-alum treated individuals (Figure 23). Emergence of the IgG4 subclass of GADA has been observed in genetically susceptible children who remained non-diabetic, while children who developed type 1 diabetes often had a broad initial response of IgG subclasses and rarely developed IgG4 before onset of the disease (Ronkainen et al. 2006). GADA of the IgG4 subclass has been found to appear more often in LADA patients than in type 1 diabetic patients (Hillman et al. 2009), indicating that IgG4 might be associated with slower progression to clinical diabetes, perhaps due to a more balanced immune response in the pancreatic tissue (Couper et al. 1998; Hillman et al. 2004). Thus, an increased percentage of IgG4 and decreased IgG1 after GAD-alum treatment might further support the suggestion that a predominant protective Th2 response was induced as a result of the treatment. While IgG1 has been associated with Th1 and IgG4 with Th2 responses (Gascan et al. 1991; Couper et al. 1998; Bonifacio et al. 1999; Hawa et al. 2000; Hillman et al. 2004), the relation to specific T cell subsets of cytokines for IgG2 and IgG3 in humans is not well-defined. There are some studies supporting that IgG3 might be associated with Th1 responses (Hussain et al. 1995; Widhe et al. 1998).
Table 8. GADA subclass distribution. The median levels and range of IgG are represented in cpm. The numbers of individuals with detectable levels are shown for each time point. To determine positivity for the samples, the mean cpm value for a GADA negative control and 3xSD of the same control was subtracted from each sample. All values above 0 after this calculation were regarded as positive.
At 15 months, the distribution of IgG subclasses in the samples from GAD-alum individuals had returned to that observed at baseline, and no differences in distribution between the GAD-alum and placebo groups were observed. It will be interesting to see if this transient effect of GAD-alum on IgG subclass distribution is prolonged by treatment with more than two GAD-alum injections in the ongoing phase III GAD-alum trial.

A.

B.

GAD-alum
Results and discussion

C.

Figure 23. The effect of GAD-alum in the GADA subclass distribution. Differences in IgG1, 2, 3 and 4 subclasses between GAD-alum (black circles) and placebo (white circles) (panel A), and within the groups (panels B and C). The results are presented as median percentage of total IgG GADA. Total IgG was calculated with respect to the combined sum of all four subclasses in each sample.

No changes in SPS-related epitope binding or inhibition of GAD65 enzymatic activity

There has been concern that induction of high GADA levels by GAD-alum treatment might cause adverse events, especially neurological symptoms. However, no treatment-related adverse events have been observed during the trial (Ludvigsson et al. 2008). High GADA levels leading to neurological symptoms have been associated with a decreased production of GABA in the nervous system of SPS patients (Murinson 2004; Murinson et al. 2004; Raju et al. 2008). GADA in SPS patients inhibits the enzymatic activity of GAD65, which is not the case for GADA in type 1 diabetic patients (Raju et al. 2005). We therefore investigated the capacity of serum from patients in the GAD-alum trial to inhibit the enzyme activity of GAD65 in vitro (Paper IV). The results showed that serum from GAD-alum treated individuals did not affect the enzymatic activity of GAD65, at baseline, 3 or 15 months after treatment (Figure 24).
Results and discussion

Figure 24. GAD65 enzymatic activity assay for serum from patients treated with GAD-alum (black circles) and placebo (white circles). GAD65 enzyme activity is reported as radioactivity (cpm) from $^{14}$CO$_2$ released from 14C-glutamic acid measured at baseline and after 3 and 15 months. A control sample from one SPS patient was included in each run (triangles).

Further, GADA in SPS patients recognizes mainly linear epitopes while GADA in type 1 diabetic patients generally recognizes conformational epitopes (Kim et al. 1994; Li et al. 1994; Daw et al. 1996; Raju et al. 2005). As a part of the epitope binding study (Paper III), we therefore investigated if the treatment affected the epitope specificity of GADA regarding two SPS associated epitopes (by using rFab b78 and N-GAD65 mAb). Sustained low binding to the b78- and N-GAD65 mAb-defined epitopes throughout the study indicates that these antibody specificities did not contribute to the increased GADA levels after GAD-alum treatment. Thus, the treatment did not affect either the GAD65 enzyme activity or the binding of GADA to SPS associated epitopes, further strengthening the safety of the treatment.
Antigen-specific effect

Another result that speaks in favor of the safety of the GAD-alum treatment was that the immunomodulatory effect of GAD-alum was antigen-specific (Paper IV). The levels of IA-2A, tetanus toxoid and IgE antibodies did not change after treatment, neither in the GAD-alum nor in the placebo group (Figure 25), indicating a GAD65 specific effect on the immune system. At all time points the levels of IA-2A were higher in the placebo group than in the GAD-alum group, but there were no changes over time. In addition, levels of IA-2A did not correlate to C-peptide levels, GADA levels or responsiveness of the treatment.
C.

Figure 25. Levels of IA-2A (A), tetanus toxoid antibodies (B) and IgE antibodies (C) before and after treatment with GAD-alum. IA-2A and tetanus toxoid antibodies were analyzed at baseline, 3 and 9 months. IgE was analyzed at baseline and at 21 months. Antibody levels for each patient in the GAD-alum (black circles) and placebo (white circles) groups are shown as dot plots.

The role of GADA in the effect of GAD-alum

The role of positivity to autoantibodies in the effect of autoantigen immunomodulation in type 1 diabetes is unclear. In our study, it was interesting to observe that baseline GADA levels were higher in patients with better C-peptide preservation 15 months after the first injection of GAD-alum (Paper IV). Our results are in line with those from the DPT-1 study, where a beneficial effect of treatment with oral insulin was observed in individuals with high levels of IAA pre-treatment, although this study failed to prevent type 1 diabetes (Skyler et al. 2005). A possible explanation for the association between the response and GADA titres might be that higher levels of GADA in circulation resulted in abundant antigen-antibody complex formation. Complexes circulate effectively, resulting in a more efficient antigen processing and presentation. In an experimental model of autoimmune disease, injection of specific immune complexes was able to powerfully redirect immune responses (Barabas et al. 2007). Thus, it is possible that induction of a more potent immune response by GAD65-antibody complexes might be responsible for an increased response to GAD65. It cannot be excluded that GADA levels simply reflect the current immunological status, and patients with higher autoantibody levels may have more beta-cells left. However, this seems less likely, as it has
been shown that after the diagnosis of type 1 diabetes the presence of GADA is independent of whether or not patients still have measurable C-peptide levels (Jensen et al. 2007). Also, another study showed that presence of GADA at onset of type 1 diabetes was not correlated with rate of beta-cell destruction after clinical onset (Batstra et al. 1997). We have to be cautious for drawing further conclusions from our results regarding better C-peptide preservation in individuals with higher GADA levels, based on a very small group of patients. However, this is an interesting observation that should be further addressed in ongoing trials including larger number of patients.

**In conclusion**, we found that the increase in GADA levels after GAD-alum treatment was accompanied by a temporarily increased GADA-binding to the b96.11-defined epitope. In addition, a decreased percentage of GADA of the IgG1 subclass and increased IgG4 were observed, which might suggest a temporary switch towards a protective Th2 response. The safety of the treatment was further confirmed by the antigen-specific effect together with the lack of changes in SPS-related epitope binding and GAD65 enzymatic activity.
Summary and conclusions

Autoantibodies related to type 1 diabetes are commonly measured in healthy children, often with increased genetic risk, to predict the disease, and in new onset diabetic individuals for classification of type 1 diabetes. In this thesis, we investigated the prevalence and persistence of autoantibodies against GAD and IA-2, and studied the relation between these autoantibodies and HLA genotypes, both in healthy children and in newly diagnosed type 1 diabetic children from the ABIS study. Our results confirmed the importance of HLA risk genotypes for the development of type 1 diabetes, and indicated that HLA risk genotypes play an important role for persistence of autoantibodies. On the other hand, the induction of autoantibodies in the general population, especially transient autoantibodies, seems to be more influenced by environmental factors. It is possible that other genetic factors than HLA might also play a role in the induction of autoantibodies.

Radioimmunoassays (RIAs) are commonly used for detection of autoantibodies and are regularly validated and compared between research laboratories around the world. However, there are some disadvantages with these methods, such as the use of radioactively labelled material, low sensitivity for the IAA assay and that they are time consuming. Due to the need for better methods we developed an SPR-based assay for detection of IAA. The main advantages of this method are that there is no need for labelling, and thus no work with radioactivity, and it is time-saving compared to RIA. We were able to measure IAA with a similar sensitivity as RIA, but further optimization of the SPR-based method is required. We also made attempts to develop a method for detection of GADA, but unfortunately without successful results. I believe that this is an area where further efforts should be done in the future. It is important to have fast and well-functioning methods for detection of autoantibodies, especially in the view of the increased possibilities for prevention, when there will be a need to screen the general child population for identification of high-risk individuals.

Besides measuring the prevalence and levels of autoantibodies in type 1 diabetes-related studies, it is also of interest to investigate their properties, such as subclass distribution, affinity and epitope reactivity. During the time from emergence of autoantibodies to the clinical manifestation of diabetes, the properties of the autoantibodies changes gradually, generally with increasing affinity, changed distribution of subclasses and epitope spreading. A
Summary and conclusions

A new field of interest is to study autoantibodies induced by immunomodulatory treatments with autoantigens. The fact that GADA levels were increased after two injections of 20 µg GAD-alum in type 1 diabetic children, raised the question whether the antibodies were different after treatment than before. Our results showed a temporary increased binding to the b96.11-defined epitope in the GAD-alum treated type 1 diabetic children, which was also the most prevalent epitope for GADA binding before treatment. No other changes in epitope specificities were observed, including the two SPS-related epitopes, which together with the antigen specific effect of the treatment and the lack of changes in GAD65 enzymatic activity, further speak in favor of the safety of GAD-alum.

Since the GAD-alum treatment induced an early Th2-directed cytokine response, we wanted to clarify if the distribution of IgG subclasses was changed upon treatment. We investigated the effect of GAD-alum on the distribution of the subclasses IgG1, 2, 3 and 4. A decreased proportion of the predominantly occurring IgG1 subclass and increased IgG4, associated in humans with Th1 and Th2 responses respectively, suggested a switch towards a protective Th2 response. It was interesting to observe that the change in subclass distribution was only temporary, and occurred when GADA titers were highest.

Concluding remarks and future perspectives

This thesis mainly comprises measurements of autoantibodies related to type 1 diabetes. We observed that HLA risk genotypes play an important role for development of type 1 diabetes, while induction of transient autoantibodies seems to be more influenced by environmental factors. New improved methods for detection of autoantibodies are needed, where our SPR-based immunoassay seems to be a good candidate, but further development is required. The GAD-alum treatment induced changes in the humoral immune response, with increased levels of GADA and temporary changes in epitope binding and IgG subclass distribution. GAD-alum seems to be a promising treatment for type 1 diabetes, and hopefully many of the research questions originated from our results will be answered during the ongoing phase III trials.
I would like to end this thesis by expressing my sincere gratitude and thanks to all who contributed and helped me to accomplish this. In particular, I would like to thank:

My supervisors: Johnny Ludvigsson for giving me the opportunity to do research within the field of diabetes and to visit and do laboratory work at the University of Washington in Seattle for 6 weeks during 2006. Thank you for your enthusiasm and helpfulness. Then I would like to thank Rosaura Casas, my assistant supervisor during the second half of my research studies. Your enthusiasm and support have helped me a lot and you were always willing to discuss small and big matters whenever I needed. Outi Vaarala, my supervisor during the first half of my research studies. Thank you for your ideas and discussions and for sharing your knowledge in the field of immunology.

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