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Cross-reactive immune responses between enteroviruses and islet cell autoantigens

by

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Academic Dissertation

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to Tara

in memory of beloved father

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ABSTRACT

Previous scientific data suggests that enterovirus infections, especially those caused by coxsackieviruses have a role in the pathogenesis of type 1 diabetes. One mechanism by which viral infection could initiate or accelerate diabetogenic process in humans is 'molecular mimicry', induction of antiviral immune responses which cross-react with self-epitopes in insulin producing β-cells. This study was prompted by sequence homology discovered between enterovirus capsid/procapsid proteins VP1 and VP0 and two immunogenic diabetes-associated epitopes in β-cell autoantigens IA-2/IA-2β and HSP60. Possible cross-reactive immune responses were investigated using proteins and peptides derived from the homologous regions and extended to sera obtained with immunization with viral proteins and those derived from viral infections.

Our results showed that rabbit antibodies raised against HSPs recognized capsid protein VP1 of coxsackievirus A9 (CAV9) and coxsackievirus B4 (CBV4) and recombinant VP1 protein of CAV9.
The results obtained with VP1-, VP0- and IA-2-peptide induced rabbit antisera demonstrated that immunization of rabbits with these peptides induce cross-reacting immune responses. Among the cross-reactive immune responses studied, results obtained with coxsackievirus-derived VP1-peptide and IA-2-peptide induced antisera were most convincing.

Enterovirus induced humoral responses were further studied in rabbits and NOD mice, which is an animal model for human type 1 diabetes. Both CBV4- and CAV9-induced rabbit antisera reacted with immunogenic epitope in IA-2. Immunization of female NOD mice with the diabetes-associated strain CBV4-E2 resulted in recognition of epitopes in viral capsid proteins. Epitope mapping with partially overlapping peptides in CAV9 capsid proteins demonstrated that recognition of both VP0 and VP1 peptide sequences was prominent. Furthermore, epitope mapping of islet cell autoantigens showed that regions in IA-2 and HSP60, with sequence similarity with VP1 and VP0, were both recognized by CBV4-E2 immunized NOD mice.

Studies were further extended to humans. Our results indicate that enterovirus infections in humans may induce immune responses that cross-react with β-cell autoantigens.

CONTENTS

ABBREVIATIONS

aa	amino acid
APC	antigen presenting cell
ATCC	American Type Culture Collection
BSA	bovine serum albumin
CAV	coxsackie A virus
CBV	coxsackie B virus
CTL	cytotoxic T-lymphocyte
cDNA	complementary deoxyribonucleic acid
DC	dendritic cell
EIA	enzyme immune assay
ELISA	enzyme-linked immunosorbent assay
EV	echovirus
FCA	Freund's complete adjuvant
FCS	fetal calf serum
FIA	Freund's incomplete adjuvant
GAD	glutamic acid decarboxylase
GADA	autoantibodies to glutamic acid decarboxylase
GMK	green monkey kidney cell line
HLA	human leucocyte antigen
HSP	heat shock protein
IA-2	insulinoma-associated protein 2
IA-2β	a β-cell protein belonging to the family of PTPs
IA-2A	antibodies to IA-2
IAA	insulin autoantibodies
IAR	a β-cell protein belonging to the family of PTPs
ICA	islet cell antibodies
ICA512	islet cell antigen 512
ICA69	69 kDa islet cell autoantigen
IDDM	insulin-dependent diabetes
T1D	type 1 diabetes
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP	intraperitoneal
IPV	inactivated polio vaccine
MHC	major histocompatibility complex
NOD	non-obese diabetic
OPV	oral polio vaccine
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PTP	protein tyrosine phosphatase
PV	poliovirus
RNA	ribonucleic acid
SI	stimulation index
SC	subcutaneous
TCR	T-cell receptor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Härkönen T., Hovi T, Roivainen M. Expression of coxsackievirus B4 proteins VP0 and 2C in Escherichia coli and generation of virus protein recognizing antisera. J. Virol. Meth. 69:147-158, 1997

II Härkönen T., Puolakkainen M., Sarvas M., Airaksinen U., Hovi T., Roivainen M. Picornavirus proteins share antigenic determinants with heat shock proteins 60/65. J. Med. Virol. 62:383-391, 2000

III Härkönen T., Lankinen H., Davydova B., Hovi T., Roivainen M. Enterovirus infection can induce immune responses that cross-react with cell autoantigen tyrosine phosphatase IA-2/IA-2β. J. Med. Virol. 66:340-50, 2002

IV Davydova B., Härkönen T., Kaialainen S., Hovi. T., Vaarala O., Roivainen M. Enterovirus immunization protects NOD-mice from spontaneous diabetes. Submitted.

V Härkönen T., Paananen A., Lankinen H., Hovi. T., Vaarala O., Roivainen M. Cross-reactive humoral responses between enteroviruses and islet cell autoantigens. Submitted.

1. INTRODUCTION

Type 1 diabetes (T1D) is a chronic disease, characterized by progressive destruction of insulin-producing β-cells leading to hyperglycemia and diabetes. According to the most widely accepted hypothesis, T1D is an autoimmune disease mediated by autoreactive T cells, and is associated with appearance of autoantibodies to multiple β-cell autoantigens (Gorus *et al*., 1997; Leslie *et al*. 1999).

Type 1 diabetes is thought to be genetically determined disease, associated with HLA molecules, but in addition, environmental factors are thought to be associated with the pathogenesis of T1D.

Enterovirus infections, especially those due to the group B of coxsackieviruses, have long been suspected of having a role in the pathogenic process (Helfand *et al*., 1995; Hyöty *et al*., 1995; Yoon *et al*., 1990). The pathway(s) by which enteroviruses may induce or accelerate diabetes is not well understood. During infection enteroviruses may reach the pancreatic β-cells and destroy islet cells by cytolysis (Yoon *et al*., 1979; Szopa *et al*., 1990). Alternatively, β-cells could be damaged by virus-induced inflammatory reactions. Furthermore, enterovirus infection could induce antiviral response that cross-reacts with β-cell autoantigens, leading to disappearance of normal tolerance to self-antigens and miss-directed immune responses (Kaufman *et al*., 1992; Lönnrot *et al*. 1996).

A great challenge for the future is the better understanding of the mechanism(s) by which viruses could be involved in the pathogenesis of T1D or in other autoimmune diaseases.

REVIEW OF LITERATURE

1. ENTEROVIRUSES

1.1. The genome of enteroviruses and its expression

Human enteroviruses belong to the family of Picornaviridae and are classified into five subgroups, polioviruses (PV), coxsackieviruses A, coxsackieviruses B, echoviruses and enteroviruses 68-71, on the basis of antigenicity and different pathogenity in mice. According to a new taxonomy, largely based on phylogenetic relationships, human enteroviruses are divided into five species: PV and human enteroviruses A-D (King *et al.*, 1999) (Table1). Antigenic variation exists to various degree in all serotypes and even single virus isolates are actually a collection of genetic variants, caused by a high frequency of point mutations and recombination, which may have pathogenic potential (Rotbart, 1990; Szopa *et al.*, 1990).
The enterovirus genome consists of a single-stranded RNA of positive polarity of about 7500 nucleotides. The genomic RNA acts as a template for viral proteins needed in translation and replication. The complete viral protein-coding region is translated as a single large polyprotein, which is proteolytically cleaved to precursors P1, P2 and P3. P1 is cleaved into three capsid proteins VP0, VP3 and VP1, of which VP0 is cleaved to VP2 and VP4 in capsid assembly. The virus capsid is formed of 60 copies of each of four capsid proteins VP1-VP4. Precursor proteins P2 and P3 are further cleaved to seven nonstructural proteins, which have functions in virus replication and protein processing. The genomic RNA is also used as a template for the synthesis of negative strand RNA, which are then used in synthesizing multiple copies of positive strand RNA. These strands are used in translation of viral proteins, and are also encapsidated into the assembling virions.

Table 1. New classification of enteroviruses according to King *et al.* (1999)

Species	**Serotypes within the species**
Poliovirus	Poliovirus 1-3
Human enterovirus A	Coxsackievirus A 2-8, 10 12, 14, 16
Human enterovirus B	Coxsackievirus A9
	Coxsackievirus B 1-16
	Echovirus 1-7, 9, 11-21, 24-27, 29-33
	Enterovirus 69
Human enterovirus C	Coxsackievirus A 1, 11, 13, 15, 17-22, 24
Human enterovirus D	Enterovirus 68, 70

1.2. Clinical manifestations

Outbreaks of enterovirus infections can be worldwide or they can be restricted to small groups such as schools and day care centers. In temperate climates enteroviruses are most prominent during the late summer and early autumn. The percentage of different clinical virus isolates varies depending on the year and country, and clinical isolates do not necessarily predict the actual prevalence of virus infection. In Finland, according to a study by Hovi *et al*., (1996), the CBV5, echovirus 11, CAV9, CBV3 and echovirus 30 predominate in clinical samples. Enterovirus infection usually starts in the respiratory or gastrointestinal mucosa, spreads through the lymphatics to the circulation causing viremia and may spread to secondary sites of replication. A great majority of enterovirus infections are asymptomatic, but they can cause a variety of diseases from mild upper respiratory infections to poliomyelitis, meningitis, meningoencephalitis, myocarditis and skin and mucosal eruptions. Enteroviruses are generally considered to cause acute infections, but there have been reports indicating the role of enterovirus in chronic human diseases, some, e.g. myocarditis and autoimmune type 1 diabetes, with autoimmune nature (Why, 1995; Åkerblom & Knip, 1998a, respectively).
A widely used serological test of enterovirus infection is the comparision of neutralizing antibody titers of acute phase and convalescent phase serum samples. Many seroepidemiological studies are based on the detection of IgM antibodies by enzyme-linked immunosorbent assay (ELISA) as evidence of recent enterovirus infection. This method is considered sensitive in the detection of enterovirus antibodies, but not completely serotype specific (Valtanen *et al.*, 1999). Several different enteroviral antigens, virus-infected cell lysates, purified virus capsids and synthetic peptides are used in detection of IgM and IgG antibodies (Cello & Svennerholm, 1994; Frisk *et al*., 1989; Roivainen *et al*., 1993a; Samuelson *et al.*, 1987). Detection of IgG requires, however, paired serum samples to give proof of recent enterovirus infection. Molecular biology techniques, especially polymerase chain reaction (PCR) (Hyypiä, 1989), have greatly improved the detection of viruses in different specimens and are used in diagnosis and genome comparisons of enteroviruses (Rotbart, 1990).

1.3. Antigenic sites of enteroviruses

Virus infection can induce both B- and T-cell responses. Protective immunity against enteroviruses is created by the presence of serotype specific neutralizing antibodies. Enterovirus infection induces a rapid production of IgM, followed by IgG and IgA class antibodies. IgM antibodies usually disappear in 6 months, while IgG and IgA can persist for years. Apart from neutralizing antigenic sites there are other antigenic regions that induce immunity, but are not neutralizing and they often induce cross-reacting immune responses (Hovi & Roivainen, 1993).
Many B-and T- cell epitopes in various enteroviruses have been characterized (Huber *et al*., 1993b; Mahon *et al*., 1992; Roivainen *et al*., 1991; Simons *et al*., 1993). Most B-cell epitopes

are thought to be conformational, while T cells recognize shorter, usually linear peptides (Horsfall *et al.*, 1991). Antigenic determinants of enteroviruses have been investigated in humans, in mice by infection or immunization with enterovirus, and in rabbits by immunization with viruses (Huber *et al.*, 1993a; Pulli *et al.*, 1998; Roivainen *et al.*, 1991). Targets of humoral antigenic responses have been studied with escape mutants generated by neutralizing monoclonal antibodies (Minor *et al.*, 1986), with synthetic overlapping peptides and peptide scanning method (Pulli *et al.*, 1998; Roivainen *et al.*, 1991).

The major neutralizing antigenic sites in polioviruses exist in VP1, and to a lesser extent in VP2 and VP3 (Hogle *et al.*, 1985; Mateu, 1995; Rossmann *et al.*, 1985). By the peptide scanning techinique, the most reactive humoral epitopes in PV3 were in VP1 and VP3 capsid proteins, while none were found in the VP4 protein (Roivainen *et al.*, 1991).

Antigenic sites in other enteroviruses are less well known. Some information has been published on antigenic sites in CAV9, CBV4 and CBV3. Antisera raised against native CAV9 in rabbits recognized epitopes in VP2 and VP3 and three regions in VP1 (Pulli *et al.*, 1998). Polyclonal antisera raised against CBV3-derived peptides also recognized VP1, VP2 and VP3 capsid proteins (Auvinen *et al.*, 1993).

Enterovirus-specific T-cell epitopes have been analyzed in mice inoculated or infected with PV or CBV3, and investigated by proliferation of lymphocytes to peptides (Huber *et al.*, 1993; Mahon *et al.*, 1992). Most T-cell epitopes were identified to localize in VP1 and VP3 proteins. Some PV epitopes were serotype specific, located usually in VP1 and VP3, while cross-reactive T-cell epitopes were located in VP4 (Mahon *et al.*, 1992). Enterovirus infection in humans has been shown to induce both serotype specific and cross-reactive immune responses (Cello *et al.*, 1996; Graham *et al.*, 1993; Juhela *et al.*, 1998). T-cell epitopes in VP1 protein of PV1 and PV3 were shown to exist near neutralizing antigenic sites (Graham *et al.*, 1993; Simons *et al.*, 1993). Studies by Simons *et al.* (1993) showed that isolated peripheral blood lymphocytes from OPV-vaccinated humans respond to all isolated capsid proteins VP1-VP4. VP2 and VP3 and to a lesser extent VP1 were shown to contain T-cell epitopes by Cello *et al.*(1996). In a recent study, a panel of CBV4-specific T-cell lines were generated and their target epitopes were shown to be concentrated in regions, which are conserved among enteroviruses (Marttila *et al.*, 2002), confirming the earlier reports (Cello *et al.*, 1996; Juhela *et al.*, 1998) showing that T-cell epitopes are largely cross-reactive between various enterovirus serotypes.

2. TYPE 1 DIABETES (T1D)

Type 1 diabetes mellitus (T1D), or insulin-dependent diabetes (IDDM), is thought to be an autoimmune disease characterized by progressive destruction of insulin-producing β-cells of the Langerhans islets, most probably by autoreactive T lymphocytes directed against islet cells or their antigenic determinants (Atkinson & Maclaren, 1994). Only very rarely, disease develops abruptly and without characteristic immune markers. The β-cell destruction progress is

gradual, and it can take years before clinical presentation of diabetes, when about 80% of cells are already destroyed (Figure 1). Islet cell autoantigens are largely studied by investigation of specificities of circulating antibodies present in sera and PBMC derived from diabetic and prediabetic individuals (Figure 1). Considerable progress has been made in the identification of T1D associated antigens, such as ICA (Bottazzo *et al.*, 1974), GAD65 (glutamic acid decarboxylase) (Baekkeskov *et al.*, 1990), insulin (Palmer *et al.*, 1983; Palmer, 1987), tyrosine phosphatase IA-2 (or ICA512) and IA-2β (or IARββ, phogrin) (Christie *et al.*, 1992; Cui *et al.*, 1996; Hawkes *et al.*, 1996; Lu *et al.*, 1996; Payton *et al.*, 1995), heat shock proteins (Birk *et al.*, 1996; Elias *et al.*, 1990; Jones *et al.*, 1990), ICA69 (Pietropaolo *et al.*, 1993), and GLIMA (Aanstoot *et al.*, 1996).

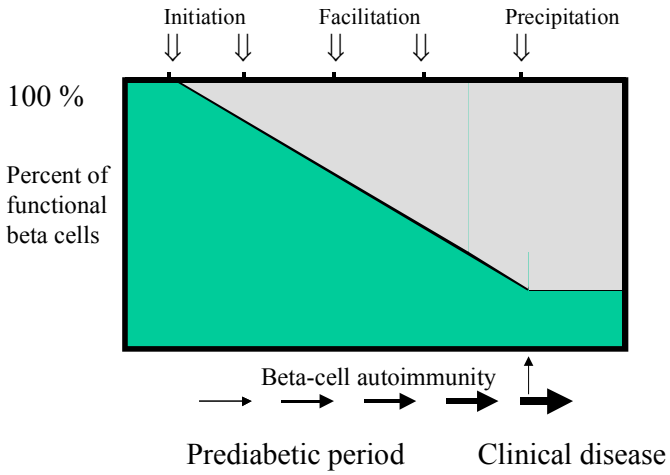

Figure 1. Phases of T1D pathogenesis

2.1. Insulitis

A common histopathological feature associated with the development of T1D is insulitis, characterized by the presence within and around the islets of mononuclear cells consisting mainly of T lymphocytes and macrophages (Bottazzo *et al.*, 1985; Foulis *et al.*, 1986), and a high proportion of the lymphocytes has been shown to secrete IFNγ (Foulis *et al.*, 1991). Human studies are largely confined to autopsies on patients who have died of recent onset diabetes, and most of the work in humans has been done using peripheral blood cells, although they do not necessarily represent the infiltrate very good.

Much of our knowledge on the pathogenesis of T1D in humans is interpolated from studies on non-obese diabetic (NOD) mice, an animal model for human T1D. Many genetic and immunological features, like pancreatic infiltrates and β-cell specific autoantibodies, are similar to those in human disease. In NOD-mice the development of diabetes is, however, spontaneous and female mice develop insulitis early in life (2-4 weeks) and develop overt diabetes from 12 weeks on. The incidence of diabetes varies between colonies (Pozzilli *et al.*, 1993), but typically 70-80% of female NOD-mice are diabetic by 30 weeks of age. As in human T1D, there is extensive infiltration of islets by T cells, macrophages, B cells and dendritic cells (Chatterjee *et al.*, 1992; See & Tilles, 1995).

2.2. Contribution of genetics to T1D

The susceptibility to T1D is thought to be determined by a combination of genetic and environmental factors. Twin studies, family studies, and animal models have helped to elucidate the genetics of autoimmune diabetes. The prevalence of type 1 diabetes is highest in identical twins (about 30-50%) decreasing to 16-20% in HLA identical siblings (Cavender *et al.*, 1984; Tarn *et al.*, 1988). The risk in the general population is 0.3-0.7% (Bingley *et al.*, 1993; Tillil & Kobberling, 1987).
Most of the genetic susceptibility is accounted for by human leukocyte antigen HLA alleles on chromosome 6. HLA class I molecules are composed of α-chain and β2-microglobulin that are expressed on all nucleated cells and which present antigenic peptides to cytotoxic $CD8^+$ T cells. The HLA class II molecule is also a heterodimer, composed of one α and one β chain, and they are expressed on antigen presenting cells (APC). HLA II molecules present antigenic peptides to $CD4^+$ T cells. Allelic variations in the binding pocket among HLA molecules accounts for differences in peptides preferentially bound by each class II molecule (Kwok *et al.*, 1996; Reijonen *et al.*, 1991; Todd *et al.*, 1987). Susceptible alleles may select potential autoreactive T cells during thymic selection, while protective/neutral alleles may either delete or fail to select these potential autoimmune T cells, thus reducing the possibility of developing disease. In the periphery, an HLA molecule derived from a susceptible allele may then recognize and present self peptide, or foreign peptide that cross-reacts with an autoantigen, leading to activation of autoreactive T cells. DQB1 and DRB1 are considered the most important genes in either protection or susceptibility to T1D (Awata *et al.*, 1992; Ilonen *et al.*, 1997; Karvonen *et al.*, 1993).

2.3. Role of immunopathology in T1D

There is a lot of evidence that many different sectors of the immune system take part in initiation and progression of β-cell destruction: presentation of antigens by HLA I and II mol-

ecules, proliferation of $CD4^+$ T cells specific for different cell autoantigens (Durinovic-Bello *et al.*, 1996; Harrison *et al.*, 1992; Roep, 1996), stimulation of β-cell specific autoantibodies, activation of cytotoxic $CD8^+$ T cells, recruitment of inflammatory cells and the production of cytokines. The availability of NOD-mice has greatly enhanced the understanding of the possible pathogenic mechanisms involved in human T1D. With the possibility of separating different cell types or blocking their activity it has been possible to investigate the roles of $CD8^+$, $CD4^+$, B cells, cytokines and β-cell autoantigens in the development of diabetes.
The exact mechanism involved in the initiation and progression of β-cell destruction and the main component of immune system, having the major role in the destruction process, is unresolved (Yoon *et al.*, 1998). Most probably T1D results from a dysregulation in humoral and cell-mediated immunity (Atkinson & Maclaren, 1994).

2.3.1. Cellular immune reactions in T1D

Strong evidence of the role of T cells in the pathogenesis of T1D comes from studies with NOD-mice, where diabetes can be transferred with T lymphocytes from diabetic mice into irradiated recipients (Elias *et al.*, 1990; Kaufman *et al.*, 1993; Wicker *et al.*, 1986). Moreover, there are plenty of examples of prevention of diabetes in NOD-mice including suppression of T-cell autoimmunity (Elias *et al.*, 1991; Kaufman *et al.*, 1993; Lampeter *et al.*, 1989; Tisch *et al.*, 1994; Zechel *et al.*, 1997). Studies with immune suppressive agents, and those involving the development of diabetes in recipients after bone marrow transplantation from T1D patient (Lampeter *et al.*, 1993), together with numerous studies of T-cell responses in diabetic patients to islet cell autoantigens, indicate that cellular immune responses are important in the pathogenesis of human T1D. (Eisenbarth, 1986; Honeyman *et al.*, 1998; Lampeter *et al.*, 1993; Roep, 1996).
In NOD-mice it has been shown that both $CD4^+$ and $CD8^+$ subsets of T cells play a role in development of disease. The absence of $CD4^+$ T cells prevented diabetes in NOD-mice (Phillips *et al.*, 2000; Shizuru *et al.*, 1988). Diabetes did not occur in $CD8^+$ deficient mice either (Katz *et al.*, 1993; Serreze *et al.*, 1994; Wang *et al.*, 1996). Controversies, however exist, and according to one group, $CD4^+$ T cells, but not $CD8^+$ have been shown to alone transfer disease (Christianson *et al.*, 1993), while Wong 96 *et al.* (1996) reported that transfer of $CD8^+$ T-cell clones from young NOD islets can cause rapid onset of diabetes in NOD-mice in the absence of CD4 cells.

CD4 cells are further divided into Th1 and Th2 types based on the cytokines they secrete. Th1 cytokines are classified as inflammatory cytokines. Secretion of these cytokines results in upregulation of MHC molecules on APC, enhancement of CTL killing, increased activation-induced cell death and proliferation, and recruitment of other cells that secrete Th1 cytokines (Mauricio & Mandrup-Poulsen, 1998; von Herrath & Oldstone, 1997). Th2 cells secrete IL4,

IL5, IL6, IL10 and IL13 and provide help for B-cell activation and for antibody production. Artificial introduction of cytokines or antibodies to cytokines that favor Th1 or disfavor Th2 cell development generally promotes diabetes, whereas the converse inhibits diabetes (Katz *et al.*, 1995; Sarvetnick *et al.*, 1990; Trembleau *et al.*, 1995).

2.3.2. Humoral immune responses in T1D

Antigen presenting cells, B lymphocytes, dendritic cells (DC), and macrophages have all been shown to take part in insulitis and T1D in rodents, and the removal of B cells reduces or prevents diabetes (Akashi *et al.*, 1997; Serreze *et al.*, 1996; Wong *et al.*, 1998). Serreze *et al.* (1998) later, showed that the diabetogenic role for the B lymphocytes in NOD-mice is based on the abilty of B cells to process and present certain β-cell autoantigens to autoreactive T lymphocytes. Trembleau *et al.* (2000) showed that T-cell response to IA-2 represents an early event in T1D in NOD-mice, as in humans, and conclude that autoantibodies to IA-2 in humans do not only reflect destruction of β-cells by exposed intracytoplasmic IA-2, but that B cells producing these autoantibodies have obtained help from pathogenic T lymphocytes (Trembleau *et al.*, 2000).

Antibodies to several β-cell antigens found in circulation is the most evident sign of β-cell autoimmunity, and they are used for assessing the risk for type 1 diabetes and in the search for the cause of the disease. Antibodies can have direct effector functions, and they may play a significant role in the processing and presentation of T-cell epitopes to T cells. Antibodies have been shown to increase the efficiency of antigen capture by APCs, and antigen-antibody complexes can substantially lower the amount of antigen needed to stimulate T-lymphocyte response (Amigorena & Bonnerot, 1998). This is supported by Reijonen *et al.*, (2000), who showed that GAD65-specific autoantibodies enhance the presentation of GAD65 to T cells and the enhancement was most prominent with sera from patients with high GAD65 antibody levels.

2.4. Target antigens

Much research has focused on identifying key antigen(s) initiating the immunological reactions leading to β-cell specific autoimmune responses. Although yet unidentified, β-cell autoantigens were shown to be important in development of diabetes and autoreactive T lymphocytes able to transfer diabetes in NOD-mice require the presence of target β-cells (Larger *et al.*, 1995). Islet cell autoantigens are largely studied by investigation of specificities of circulating antibodies present in sera and PBMC derived from diabetic and prediabetic subjects. Of several islet cell autoantigens described (Baekkeskov *et al.*, 1990 ; Birk *et al.*, 1996; Cui *et al.*, 1996; Elias *et al.*, 1990; Jones *et al.*, 1990; Palmer, 1987; Pietropaolo *et al.*, 1993) insulin,

GAD65, IA-2 and HSP60 (especially in NOD mouse) seem to be most relevant.
Inter- and intramolecular spreading of both B- and T-cell autoimmune responses has been shown to occur both in humans (Bonifacio *et al.*, 2000; Brooks-Worrell *et al.*, 2001; Durinovic-Bello *et al.*, 1996; Sohnlein *et al.*, 2000) and NOD-mice (Tian *et al.*, 1996; Tisch *et al.*, 1993; Zechel *et al.*, 1998b) and is associated with a greater risk for developing type 1 diabetes (Brooks-Worrell *et al.*, 2001; Durinovic-Bello *et al.*, 1996; Kawasaki *et al.*, 1998; Knip, 1998b).

2.4.1. ICA

Identification of autoantibody-positive individuals relied previously on histochemical detection of islet cell-specific autoantibodies (ICA) on frozen sections of human pancreas (Bottazzo *et al.*, 1974)**.** High and persistent levels of ICA have been shown to predict the development of type 1 diabetes (Bonifacio *et al.*, 1990; Knip *et al.*, 1994), especially in sibling pairs of affected individual with type 1 diabetes (Knip *et al.*, 1998b). ICA comprise autoantibodies to a number of antigens, of which GAD65 and IA-2 autoantibodies represent the major fraction. Screening for these two protein antibodies is suggested to replace ICA testing in future (Gorus *et al.*, 1997; Kulmala *et al.*, 1998).

2.4.2. GAD65

Immunoprecipitation with radiolabelled human islet cell extracts identified 65 kDa proteins as major targets for autoantibodies in T1D (Baekkeskov *et al.*, 1990), later identified as glutamic acid decarboxylase (GAD65), which catalyzes the synthesis of the inhibitory transmitter γ-aminobutyric acid (GABA). There are two isoforms of GAD, GAD65 and GAD67, which are mainly expressed in pancreatic β-cells and the central nervous system. Human islets predominantly express GAD65, while in mouse islets GAD67 appears to dominate (Kim *et al.*, 1993). Antibodies to GAD65 are detected in vast majority of T1D patients at the diagnosis of disease and in the preclinical phase (Bonifacio *et al.*, 2000; Seissler *et al.*, 1992; Verge *et al.*, 1994). Anti-GAD autoantibodies have been studied in relatives of T1D patients (Kulmala *et al.*, 1998) and GAD antibodies, combined with detection of both insulin and IA-2 antibodies, identify a large proportion of individuals at risk and nearly all patients with T1D (Verge *et al.*, 1998). The major humoral antigenic regions of GAD antibodies (GADA) seem to be directed to the middle and C-terminal part of GAD65 (Hawa *et al.*, 2000; Leslie *et al.*, 1999; Richter *et al.*, 1993). T-lymphocytes derived from diabetic patients have been shown to react with GAD65 proteins and various peptides within the protein (Atkinson *et al.*, 1994; Endl *et al.*, 1997; Harrison *et al.*, 1993; Lohmann *et al.*, 1994; Lohmann *et al.*, 1996). T cells from T1D patients have been reported to respond immunodominately to peptide 247-279 in GAD65, which show sequence homology expanding six amino acids (PEVKEK) with coxsackievirus 2C protein (Atkinson *et*

al., 1994). These finding were not, however, confirmed by Lohmann *et al.* (1994) and Schloot *et al.* (1997) whose results showed that reaction to this homologous peptide is not primarily associated with T1D.
The importance of GAD-induced immune responses in the development of diabetes and the epitopes eliciting these responses have been intensively studied in NOD-mice (Kaufman *et al.*, 1993; Zechel *et al.*, 1998a). The Th1 response to GAD was shown to develop in NOD-mice at the time of onset of insulitis (Kaufman *et al.*, 1993) and GAD expression in the islet cells was shown to be essential for induction of diabetogenic T-cells (Yoon *et al.*, 1999). Furthermore, diabetes could be prevented by intrathymic or intravenous immunization of young NOD-mice with purified GAD65, GAD67 or peptides derived from these proteins (Kaufman *et al.*, 1993; Tian *et al.*, 1996; Tisch *et al.*, 1993, 1994, 1999). Intraperitoneal injection of GAD65 at 8 weeks of age, even after the onset of insulitis, was able to inhibit disease progression (Tian *et al.*, 1996) and moreover, GAD65 also prevented the development of other immune reactions that usually occur in NOD-mice, such as those against HSP65. The mechanism of protection seems to involve activation of Th2 cells, which home to the islets and secretion inhibitory cytokines (Tian *et al.*, 1996, 1998; Tisch *et al.*, 2001). Anti-GAD antibodies have been detected also in NOD-mice but not all studies confirm this result (Bonifacio *et al.*, 2001).

2.4.3. IA-2/IA-2β

Sera of T1D patients were shown to immunoprecipitate 40 –and 37-kDa tryptic fragments from islet-cell lysates (Christie et al., 1990, 1993). These fragments were later identified to encompass most of the cytoplasmic domain of two protein tyrosine phosphatase (PTP) like proteins IA-2 (IA-512) and IA-2β (phogrin, IAR), which share a high degree of sequence homology in their intracellular part (Bonifacio et al ., 1995; Cui *et al.*, 1996; Hawkes *et al.*, 1996; Lu *et al.*, 1996; Payton *et al.*, 1995). These are transmembrane proteins primarly expressed in secretory granule membranes of Langerhans islet cells and in many parts of the central nervous system (Solimena *et al.*, 1996). Although IA-2 and IA-2β are members of PTP family, their have not been shown to have enzymatic activity and the function of native proteins is not known.
Many patients with type 1 diabetes have autoantibodies to IA-2 (Ellis *et al.*, 1998; Hawa *et al.*, 2000; Kawasaki *et al.*, 2001; Seissler *et al.*, 1996; Verge *et al.*, 1996) and antibodies are directed to intracellular part of the protein. Antibody prevalences as high as 86% have been detected in T1D patients at and prior to disease onset (Savola *et al.*, 1998) and they seem to be more prevalent in younger patients and in patients with HLA DR4 haplotype (Genovese *et al.*, 1996), and they have a high positive prediction value for type 1 diabetes (Gorus *et al.*, 1997).
The major humoral antigenic determinants of both IA-2 and IA-2β reside in the C terminus, but antibodies are also directed to the N-terminal and middle regions of cytoplasmic IA-2 (Bonifacio *et al.*, 1998; Kolm-Litty *et al.*, 2000; Leslie *et al.*, 1999; Seissler *et al.*, 2000). The

use of IA-2 deletion mutants and chimeric proteins showed determinant spreading and changes in epitope specificity during disease progression (Kawasaki *et al.*, 1998; Park *et al.*, 2000) Peripheral blood T-lymphocytes from patients with type 1 diabetes have been shown to react with IA-2 (Durinovic-Bello *et al.*, 1996). Diabetes-associated T-cell epitopes within intracellular part of IA-2 have been characterized by some groups either with the use of synthetic peptides or by elution of peptides from the T1D-associated HLA-DR4 molecule (Hawkes *et al.*, 2000; Honeyman *et al.*, 1998; Peakman *et al.*, 1999).

Recently, identification of T-cell responses to the intracellular part of IA-2 was reported in unprimed NOD-mice, detected already in 3-week-old mice by increased production of INF-γ (Trembleau *et al.*, 2000). They also showed that intraperitoneal (i.p.) injection of IA-2 into 8-day old mice significantly enhanced T1D development. T-cell clones with diabetogenic activity have also been generated from young female NOD-mice immunized with cytoplasmic region of recombinant phogrin , and some of these T-cell clones destroyed islet tissue *in vivo* (Kelemen *et al.*, 1999). Two main T-cell epitopes in phogrin were identified by the same group using recombinant phogrin deletion mutants and overlapping peptides (Kelemen *et al.*, 2001).
Low levels of IA-2 specific antibodies in the sera of NOD-mice were directed to cytoplasmic domain of IA-2 and were usually transient and noticed before the onset of hyperglycemia (Myers *et al.*, 1998). Reports on humoral responses to IA-2 antigens in NOD-mice are, however, controversial (Bonifacio *et al.*, 2001).

2.4.4. Insulin

Insulin and its precursor proinsulin are the only known β-cell specific autoantigens so far identified. Insulin is a 5.8 kDa protein hormone, which is synthesized from a prehormone, proinsulin, consisting of A-and B-chains and a C-peptide, which is proteolytically cleaved off to produce the mature protein. Antibodies to insulin are detected in 16-70% of newly diagnosed T1D patients (Palmer *et al.*, 1983; Sabbah *et al.*, 1999) and they are often the first antibodies to appear (Ziegler & Eisenbarth, 1990). T-cell responses to insulin have been more difficult to demonstrate in diabetic patients (Durinovic-Bello *et al.*, 1996), but there are reports indicating cellular responses to insulin (Keller, 1990; Schloot *et al.*, 1998).
T cells from β-cell infiltrates of NOD have been shown to be specific for insulin and most of them recognized B-chain peptide B9-23 (Daniel & Wegmann, 1996; Wegmann & Eisenbarth, 2000). Moreover, insulin specific Th1 $CD4^+$ T-cells can transfer the disease to NOD-*scid* mice (Daniel *et al.*, 1995). In another study, $CD8^+$ T- cells that transferred diabetes were shown to recognize the insulin B-chain residues 15-23 (Wong *et al.*, 1999)**.** The role of insulin in the development of diabetes is further suported by studies in which the administration of insulin, insulin B-chain, or an insulin peptide epitope was able to significantly delay or protect against diabetes in NOD-mice (Daniel & Wegmann, 1996, Hutchings & Cooke, 1998). Hutchings *et*

al (1998) also showed that the protection is dependent on the route of insulin administration (Hutchings & Cooke, 1998). Accumulating evidence suggests that proinsulin may be an important autoantigen in type 1 diabetes (Bohmer *et al.*, 1991; Chen *et al.*, 2001; Congia *et al.*, 1998; Dubois-LaForgue *et al.*, 1999). When transgenic mice expressing HLA-DR4 and human CD4 molecule were immunized with human preproinsulin, (PPI), proinsulin (PI), and insulin, it was shown that the main T-cell epitopes reside in preproinsulin-and proinsulin regions spanning part of the leader sequence and B-chain and another spanning C-peptide and A-chain (Congia *et al.*, 1998). Both the leader and the C-peptide are cleaved off from the precursor to give mature insulin. Chen *et al.* (2001) showed that proinsulin autoreactive T-cells are detected in young unprimed NOD-mice, and T-cell responses are mainly directed against proinsulin p24-33 epitope that spans the B-chain/C-peptide junction. These data are consistent with the notion that T-cell reactivity to mature insulin is rare in T1D (Durinovic-Bello *et al.*, 1996). The data presented by the International Workshop on autoantibodies in animal models of T1D (Bonifacio *et al.*, 2001) demonstrated that insulin autoantibodies are markers of autoimmunity in NOD-mice, and they appear in10-30% of mice at the age of 4 weeks and in 15-75% at the onset of diabetes.

2.4.5. HSP

Heat shock proteins (HSP) belong to a family of related proteins, which are inducible in response to stressful stimuli and/or constitutively expressed by prokaryotic and eukaryotic cells (Lindquist & Craig, 1988). HSP60 is expressed with insulin in the secretory granules of β-cells and its distribution within the β-cell has been shown to vary in prediabetic NOD-mice and is temporarily associated with the induction of humoral response to HSP60 (Brudzynski *et al.*, 1992; Brudzynski, 1993).

The critical role of immunity against HSP60 in NOD mouse diabetes was shown by Cohen and coworkers, who discovered that NOD-mice spontaneously develop autoantibodies and autoreactive T-cells to the autologous HSP60 (Birk *et al.*, 1996; Elias *et al.*, 1991), a molecule that cross-reacts with the 65 kDa heat shock protein (HSP65) of *Mycobacterium tuberculosis* (Elias *et al.*, 1991; van Halteren *et al.*, 2002). The 437-460 peptide sequence of HSP60, designated p277, was found to serve as an important target in mouse type 1 diabetes. T-cells reactive to p277 were capable of transfering diabetes, and modulation of the anti-HSP60 T cell response could arrest the autoimmune destruction of β-cells (Elias & Cohen, 1994; Elias *et al.*, 1990, 1991, 1997). Even a single subcutaneous administration of peptide 277, given late in the autoimmune process, could induce a shift in the cytokine profile of activated T-cells from Th1 type to anti-inflammatory Th2 type (Ablamunits *et al.*, 1998, Elias & Cohen, 1995; Elias *et al.*, 1997).

Studies investigating the role of HSP60 in human type 1 diabetes are fewer. Increased T-cell responses to intact human HSP60 and to peptide p277 were significantly increased in patients

with type 1 diabetes patients compared with the type 2 group and healthy blood donors (Abulafia-Lapid *et al.*, 1999). Recently, two regions in HSP60 and HSP65/*M.Bovis* were recognized by diabetic patients' sera using epitope scanning (Horvath *et al.*, 2002). The first region, aa 394-413 and aa 366-385 in HSP60 and HSP65 in *M. Bovis*, respectively, is similar to the sequence aa 520-534 found in GAD65 (Jones *et al.*, 1993). The second reactive region overlaps with p277 to a large extent HSP60 aa 435-454 and HSP65/*M.Bovis* aa 408-427. Interesting results were recently published from a randomized, double-blind, phase II study where patients with newly diagnosed T1D were treated with of HSP p277 (Raz *et al.*, 2001). The group showed that patients who had received p277 maintained their ability to produce C-peptide and needed significantly less exogenous insulin compared to patients in the placebo group. The mechanism was thought to be an epitope-specific shift from Th1 to Th2 cytokine production by autoimmune cells.

2.5. Environmental factors

Several lines of data support the role of environmental factors in the pathogenesis of type 1 diabetes. First, the concordance rate for T1D in monozygotic twins is less than 50% (Cavender *et al.*, 1984). Second, epidemiological data and seasonality of the disease indicate the contribution of external factors in T1D. Incidence varies even 10-fold within Europe, more than can be expected due to genetic differences in populations and the incidence is increasing rapidly both in low- and high-incidence populations, being highest in Finland (Onkamo *et al.*, 1999). The strongest putative contributors among the environmental factors in T1D are nutritional factors and viral infections.

2.5.1. Dietary factors

Nutrition, particularly animal proteins and nitrosamines in the diet have been considered as putative factors in T1D (Åkerblom & Knip, 1998). Enhanced immune responsiveness against several cow milk proteins, such as bovine serum albumin (BSA) and β-lactoglobulin, has been reported in type I diabetic patients (Karjalainen *et al.*, 1992; Vaarala *et al.*, 1996). One possibility is that milk proteins induce cross-reactive responses to β-cell autoantigens based on sequence similarities between milk proteins and autoantigens (Karjalainen *et al.*, 1992). On the other hand, cow's milk formulas might induce humoral immunity to bovine insulin, with subsequent cross-reaction with human insulin (Vaarala *et al.*, 1999).

2.5.2. Enteroviruses

2.5.2.1. Epidemiological data

Enteroviruses, especially those belonging to coxsackieviruses, have for long been suspected of having a role in the pathogenesis of T1D (Banatvala *et al.*, 1985; Yoon, 1990; Yoon *et al.*, 1979). By seroepidemiological studies several enteroviruses have been associated with type 1 diabetes (Hyöty *et al.*, 1995; Roivainen *et al.*, 1998; Sadeharju *et al.*, 2001). Increased prevalence and elevated levels of enterovirus IgM antibodies and enterovirus RNA have been reported in newly diagnosed type 1 diabetic patients (Clements *et al.*, 1995; Helfand *et al.*, 1995), implying that enterovirus infection could be a final hit in disease progression. The possible initiating role for enteroviruses in β-cell destruction was investigated in a prospective study carried out in Finland (Hyöty *et al.*, 1995). This study showed that enterovirus infections were more common both close to the time of diagnosis and even years before clinical presentation of the disease in children who later developed type 1 diabetes when compared to children who remained non-diabetic over the follow-up period. Enterovirus infections appeared to coincide with seroconversion or enhancement of immune responses to islet cell or insulin antibodies (Hiltunen *et al.*, 1997; Hyöty *et al.*, 1995; Lönnrot *et al.*, 2000).
A few studies of the T cell responses to enterovirus antigens have been published (Bruserud *et al.*, 1985; Jones & Crosby, 1996b; Juhela *et al.*, 2000). Results by Jones *et al.* (1996) and Juhela *et al.* (2000) indicate that patients with type 1 diabetes have stronger T-cell responses to CBV-infected cell lysates, whereas Bruserud *et al.* did not find a difference in T-cell responses to CBV4 antigen. HLA background may influence the host's susceptibility or resistance to EV infections (Bruserud *et al.*, 1985; Luppi *et al.*, 1999). This is supported by the study where seroconversion to ICA after enterovirus infection was seen more often in children with the high risk HLA-DQB1 genotype (Hiltunen *et al.*, 1997).

2.5.2.2. Studies in mice

Yoon *et al.*, (1979) reported of 10-year-old boy who had diabetic ketoacidosis days after having flu-like symptoms, and who died a few days later. Coxsackie B4 virus could be isolated from the pancreas and the isolate was shown to induce hyperglycaemia, inflammation in the islets of Lagerhans and β-cell necrosis in mice (See & Tilles, 1995; Szopa *et al.*, 1993; Yoon *et al.*, 1979). Although the nucleotide sequence of this CBV4-E2 has been determined (Kang *et al.*, 1994), the pathogenic determinants have not been reported. According to one study, mice that developed severe disease were more immunoresponsive than mice that survived infection with CBV4, suggesting that immunomediated mechanism may play a role in the severity of CBV4-induced disease (Halim & Ramsingh, 2000).

2.6. Possible mechanisms of β-cell destruction by viruses

Viruses may play a role at various stages of T1D pathogenesis. They may initiate the process in individuals with no previous β-cell damage, accelerate an already ongoing process, or be the final hit precipitating the symptoms of T1D. The mechanism(s) by which enteroviruses may trigger autoimmunity and onset of type 1 diabetes are not clear. First, enterovirus infection in the pancreas may cause direct β-cell lysis by the cytotoxic life cycle of the virus (Szopa *et al.*, 1990). Second, viral infection may induce a systemic immune response that cross-reacts with β-cell autoantigens (molecular mimicry) (Atkinson *et al.*, 1994; Vreugdenhil *et al.*, 1998). Furthermore, viral infections result in local infection of pancreas, leading to inflammation, tissue damage, and the release of normally sequestered antigens, which activate potentially self-reactive T cells (Horwitz *et al.*, 1998).

Alternatively, viral infections may locally induce production of cytokines and other inflammatory mediators in exocrine cells in pancreas that may affect β cells directly by their toxic effects, a mechanism also called 'innocent bystander damage' (Chehadeh *et al.*, 2000; Rabinovitch, 1992; See & Tilles, 1995).

2.6.1. Cell lysis

Virus variants that have a potential to induce a diabetes-like syndrome in mice have been primarily isolated from patients with rapid onset diabetes (Yoon *et al.*, 1979). Mice infected with coxsackievirus B4 usually develop acinar pancreati, but not diabetes (Caggana *et al.*, 1993). CBV4 strains can, however, be adapted to replicate in murine islets and β-cells *in vitro* (Yoon *et al.*, 1979). By this method, both Coxsackie B3 virus (Vuorinen *et al.*, 1992; Yoon *et al.*, 1998) and coxsackie B4 virus (Yoon *et al.*, 1979) were shown to be able to infect human β-cells. Coxsackie B viruses are not, however, the only enteroviruses affecting β-cells. Several strains of enteroviruses have been shown to infect cultured human β-cells and either kill them or impair their function (Roivainen *et al.*, 2000). Based on studies by Roivainen enteroviruses can be divided to those directly destroying β-cells by virus-induced cytolysis and to less aggressive viruses, which cause damage of β-cells through inflammatory reactions (Roivainen *et al.*, 2000). The most recent data indicate that even different isolates of the same serotype can differ in β-cell destruction (Roivainen 02, in press).

2.6.2. Bystander damage

Horwitz *et al.* (1998) infected different strains of mice with CBV4-E2 to investigate if molecular mimicry between GAD65 and CBV4 2C protein or bystander T-cell activation account for the diabetogenic nature of CBV4-E2. No viral acceleration of diabetes was shown in NOD-mice, indicating that molecular mimicry between GAD65 and CBV4-2C does not medi-

ate diabetes. Another mice used in this study was BDC2.5 transgenic mice with diabetogenic T-cell reseptor specific to an islet cell autoantigen that is different from GAD65 and does not cross-react with CBV4. These mice do not develop spontaneous diabetes but maintain a large number of resting islet-specific T-cells. CBV4-E2 infection of BDC2.5 transgenic mice rapidly induced diabetes in BDC2.5 transgenic mice through bystander damage of β-cells and reactivation of the resting autoreactive anti-islet lymphocytes. Another group observed that infection with CBV4-E2 can accelerate diabetes in NOD through the induction of bystander activation, but only after a critical threshold level of β-cell-autoreactive T cells have accumulated (Serreze *et al.*, 2000). Based on their studies the authors presume that coxsackievirus infection might not initiate β-cell autoreactive immunity, but could accelerate an already ongoing process, and timing of coxsackievirus infection in humans could be important factor in development of autoimmunity to β-cells. Horwitz's studies further confirmed that β-cell specific autoreactive T cells within the pancreas are activated due to the release of sequestered antigen from the damaged tissue and not due to Th1 cytokine induction following viral infection (Horwitz *et al.*, 2001). Recently, other groups have reported that preproinsulin and proinsulin are early targets in T1D and the authors propose that viral infection could release immunogenic prehormones or insulin from β cells, possibly inducing autoreactive T cells (Chen *et al.*, 2001; Congia *et al.*, 1998).

2.6.3. Molecular mimicry

2.6.3.1. Concept of molecular mimicry

Antigenic molecular mimicry is defined by cross-reactive immunity due to structural or functional homologies between molecules encoded by distinct genes. Several viruses and microbes have been reported to be associated with autoimmune diseases (Table 2), even though the interpretation is often complicated, since the viral or microbial infection that initiated the autoimmune response usually is not present any longe at the time of disease diagnosis. Viral infections may cause loss of self-tolerance by altering the cytokine profile, exposition of sequestered proteins, or by stimulating the production of autoreactive T cells.
One group of proteins associated with autoimmune diseases are HSPs. Immune responses to microbial HSPs are speculated to initiate chronic inflammatory disease, in which an autoimmune response to HSP60 may be central to the pathogenesis (Jones *et al.*, 1990). Both cellular and humoral antibody responses to autologous HSP60 peptides have been identified in patients with several chronic inflammatory diseases (Jones *et al.*, 1993). Human HSP60 shares sequence homology with many autoantigens, including those being target molecules in T1D, rheumatoid arthritis (RA) and multiple sclerosis, and this homology is thought to be involved in these autoimmune diseases through molecular mimicry. In each case the target antigen is specific for the cell type involved and has a region of similarity to human HSP60 (Table 2).

In diabetes, viral infection may induce expression of HSP60 in the islets. Bacterial HSPs may initially elict activation of HSP-specific T/B cells, which later react with autologous HSP via molecular mimicry principle. In healthy individuals, a balanced network of potentially self-reactive B cells and T cells exists. These cells have evaded clonal deletion in the thymus, but are usually eliminated by the host immune system or they remain anergic. However, tolerance to self-antigens may be distorted by encounters of the immune system with foreign microbial antigens.

Table 2. Examples of autoimmune diseases with indication of a microbial trigger (modified from report by Oldstone 1998) and examples of similarity between human HSP60 and autoantigens (modified from report by Jones 1993).

Disease	**Cross-reactive immune responses**	**References**
Myocardial	Cardiac myosin/ Streptococci, CBV3	Cunningham*et al.,* 1992 Huber *et al.,* 1993
Herpes stomal keratistis	Corneal antigen(s) Herpes simplex	Zhao *et al.,*1998
T1D	Islet cell antigens/ Coxsackieviruses Rotaviruses	Atkinson *et al.,* 1994, Honeyman *et al.* 1998

Disease	**HSP60/autoantigen region**	**References**
Coxsackie myocarditis	HSP60 aa 86-100/ Cardiac myosin heavy chain aa516-530	Beisel *et al.,*1990
T1D	HSP aa 391-405/ GAD65 aa 520-534	Baekkeskov *et al.*, 1990

2.6.3.2. Molecular mimicry in T1D

GAD65 has been reported to have cross-reactive determinants with many viruses and also with β-cell autoantigens (Jones & Crosby, 1996a; Kaufman *et al.*, 1992; Rudy *et al.*, 1995; Vreugdenhil *et al.*, 1998). Most attention has been focused on sequence homology revealed between GAD65 and the non-structural protein 2C of coxsackieviruses (Atkinson *et al.*, 1994; Tian *et al.*, 1994). This homologous sequence in GAD65 (PEVKEK) has been shown to be a part of a diabetes-related T-cell epitope both in NOD-mice and in man (Atkinson *et al.*, 1994; Schloot *et al.*, 1997; Tian *et al.*, 1994; Vreugdenhil *et al.*, 1998) and induced cellular and humoral cross-reactive immune responses with the 2C region (Atkinson *et al.*, 1994; Lönnrot *et al.*, 1996).

In contrast, Richter *et al.* did not find cross-reactivity to GAD65 and 2C specific antibodies or between antibodies to GAD65 and the homologous HSP60 peptide (Richter *et al.*, 1994). T-cell lines specific to CBV4 2C protein failed to respond to whole GAD65 protein and also to GAD65 peptides containg the shared PEVKEK sequence (Marttila *et al.*, 2001). Also, according to other groups, the importance of this PEVKEK homology in type 1 diabetes was not proved (Horwitz *et al*., 1998; Lohman *et al.*, 1994; Schloot *et al.*, 1997; Vreugdenhil *et al.*, 2000).

It has also been shown that epitope in proinsulin residues 24-36 and GAD65 residues 506-518 have sequence homology (Rudy *et al.*, 1995), which could induce cross-reactive immune responses in an early autoimmune event of T1D (Chen *et al.*, 2001).

Diabetes-associated T-cell epitopes in IA-2 have been characterized (Hawkes *et al.*, 2000; Honeyman *et al.*, 1998; Peakman *et al.*, 1999) and sequence comparison revealed similarities of these epitopes with various viruses and dietary proteins (Honeyman *et al.*, 1998). The predominant IA-2 epitope (amino acids 805-820) was found to have 56 % identity and 100 % similarity over nine amino acids with a sequence in VP7, a major immunogenic protein of human rotavirus. This epitope also has sequence homology to Dengue virus, CMV, measles virus, hepatitis C virus and with autoantigen GAD65. Further, other epitopes in IA-2 had sequence similarity with some viruses and dietary proteins of milk and wheat (Honeyman *et al.*, 1998). Later it was shown that there was a specific and highly significant association between rotavirus seroconversion and increase in antibodies to IA-2, insulin and GAD65 suggesting that RV infection may trigger or exagerate islet-cell autoimmunity in genetically susceptible children (Honeyman *et al.*, 2000).

In addition to GAD65 and IA-2, other homologous molecules have been detected between β-cell autoantigens and environmental agents associated with T1D (Table 3).

Table 3. Examples of identified homologies between β-cell autoantigens and environmental agents associated with T1D

β-cell autoantigen	**Sequence homology**	**References**
GAD65	Coxsackievirus 2C-protein,	Kaufman *et al.,* 1992
rotavirus, HSP60, proinsulin	Jones *et al.,* 1996	
ICA69	Bovine serum albumin, ββ casein	Karges *et al.,* 1995
		Karjalainen *et al.,* 1992
IA-2	Rotavirus, Denguevirus	Honeyman *et al.,* 1998
	Dietary proteins, GAD65	

3. AIMS OF THE PRESENT STUDY

By sequence comparison we found homologies between enterovirus capsid/procapsid protein VP1/VP0 and diabetes-associated epitopes in β-cell autoantigens IA-2/IA-2β and HSP60. The purpose of the study was to clarify if this sequence homology can induce cross-reactive immune responses (molecular mimicry) to enteroviruses and islet cell autoantigens, one possible mechanism by which enteroviruses could induce β-cell destruction. The specific aims were:

1. To study immunological cross-reactions between antisera raised against enterovirus proteins, islet cell proteins and peptides derived from homologous regions of these proteins.

2. To investigate if immunization with enteroviruses in rabbits and in NOD-mice induces cross-reacting immune responses.

3. To study if enterovirus infection in humans induces immune responses that cross-react with β-cell autoantigens.

4. METHODS AND SUBJECTS

4.1. Antigens

Viruses, proteins, peptides and corresponding antisera used in this study are described in Table 4.

4.1.1. Viruses

Virus strains used in this work were prototype strains obtained originally from the American Type Culture Collection (ATCC) or the World Health Organization (WHO), Reference Laboratory of Enteroviruses. African green monkey kidney cells (GMK) were used for propagation of coxsackievirus B4/J.V.B (CBV4), coxsackievirus B5/Faulkner (CBV5) and coxsackievirus A9/Griggs. A-549, a human lung carcinoma cell-line, was used for propagation of human parechovirus 1/Harris (HPE-1). Poliovirus type 3/Sabin (PV3) was grown in WHO-Hep2c cells. A diabetogenic strain of coxsackievirus B4 (CBV4-E2) was kindly provided by Dr. Yoon (University of Alberta, Canada). Crude virus preparations were cleared from cell debris by low-speed centrifugation and used as such or further purified in 5-20 % sucrose gradient (Abraham & Colonno, 1984).

4.1.2. Peptides

Peptides used in this work were synthetized and purified by high performance liquid chromatography at Department of Biochemistry, University of Helsinki or Department of Biochemistry, University of Turku, Finland. Peptides were derived from the conservative region of coxsackievirus B and poliovirus type 3 capsid protein VP1 (pVP1a, and pVP1b, pVP1c, respectively), procapsid protein pVP0, two overlapping peptides from C-terminal part or IA-2/IA-2β (pIA-2a, pIA-2b) and immunogenic region of HSP60 (pHSP60) (Table 4).

4.1.3. Proteins

Fusion proteins GST-VP0, GST-2C, His-VP0, His-VP1 were expressed and purified by us (see below). Recombinant HSP60, *Mycobacterium bovis,* was a gift from Dr. M. Singh, GBF, Braunschweig, Germany. Recombinant human HSP60 was from Stress Gen, Victoria, Canada. *Chlamydia pneumoniae* HSP60 was produced at the Vaccine Development Laboratory, National Public Health Institute, Helsinki, Finland. The intracellular region of human tyrosine phosphatase (ICD-IAR) was kindly provided by Dr. C. Pallen (National University of Singapore).

4.2. Expression and purification of virus proteins

4.2.1. GST-VP0 and GST-2C (I)

Sequences coding for CBV4 VP0 and 2C were amplified by polymerase chain reaction using CBV4 cDNA (a gift from Dr. J.Almond, University of Warwick, UK) using primers with *Bam*HI restriction sites at ends. PCR products were digested with restriction enzyme and cloned into pGEX-2T expression vector (Pharmacia P-L Biochemicals Inc.) for protein expression. *Bgl*II adapter shifting the frame by two nucleotides was used to get the 2C protein in frame with GST fusion protein.
pGEX-2T-VP0, pGEX-2T^{2+}-2C and pGEX-2T plasmids were transformed into *E.coli* BL-21 cells and expression of proteins was induced by IPTG. The cells were harvested by centrifugation and lysed with a French press and centrifuged to separate cell debris and supernatant. Fusion proteins were purified both in denatured form from insoluble fraction and in native form from supernatant. Insoluble fusion proteins in pellets were extracted with detergents and exised from SDS-PAGE gel. Purification of soluble fusion proteins and GST from supernatant was carried out by GST-sepharose columns basically according to manufacturis instructions (GST Gene Fusion System, Pharmacia Biotech Inc).

4.2.2. His-VP0 and His-VP1 (II)

Coxsackievirus A9 (CAV9) and coxsackievirus B4 (CBV4) cDNAs (kindly provided by Dr. G. Stanway, University of Essex, UK and Dr. J. Almond, University of Warwick, UK) were used as templates to amplify VP0 of CBV4 and VP1 of CAV9 by PCR. The amplified product of VP0 was cloned to *Bam*HI site, VP1 to *Bam*HI-*Eco*RI site of histidine tagged expression vector pPROEX-HTb (Gibco BRL, Gaithersburg, MD, USA). Transformed *E.coli* BL-21 cells were induced with IPTG, harvested after a few hours and the cells disrupted with a French press. Fusion proteins were finally extracted from pellet in soluble form with 8 M Urea, 100 mM NaCl, 20 mM Tris-HCL (pH 8.0) and purified by Ni-NTA agarose (Qiagen, Chatsworth, CA, USA) under denaturing conditions.

4.3. Antisera

For production of antisera three sequential doses of purified virus, peptide or recombinant protein were injected into rabbits in Freund's complete (FCA) or incomplete (FIA) adjuvant (Table 4). The insoluble pellets of GST-VP0 and GST-2C were run on a SDS-PAGE, exiced, homogenized and used in immunization. For production of His-VP0 and His-VP1 antisera, Ni-NTA purified proteins were run on SDS-PAGE and protein bands exiced from gel.

Two polyclonal antisera to *Mycobacterium bovis* HSP65 (rabbit number 13 and 19) were a gift from Dr. Q. Xu and Prof. G. Wick (Austria Academy of Science, Innsbruck, Austria).

4.4. Immunological methods

4.4.1. Enzyme immune assay (EIA) (II, III, IV, V)

High binding microtiter wells were coated with peptides, expression proteins or purified virus particles in 50 mM NaHCO3, pH 9.4. The plates were washed three times with PBS-Tween 20 (0.1%) and the wells blocked with PBS-BSA (0.1%) or PBS-casein (0.1%) for 30 min at RT. The wells were washed as above and incubated with test sera, diluted in the EIA buffer (PBS, 1% BSA, 0.1% Tween 20, 5 mM EDTA, 1% FCS), for 2 hours at 37 °C. The wells were washed and the reaction visualized by species specific conjugates. For blocking experiments antisera were preincubated with antigens before addition to wells.

4.4.2. Immunoblotting (I, II)

Proteins and viruses were separated by SDS-PAGE and transferred electrically to nitrocellulose membranes. Nonspecific binding was prevented with PBS-5% milk and membranes incubated with specific antisera diluted either to PBS-5% milk powder or 20 mM TBS, pH 7.4 containing 2% BSA. Detection of binding was achieved with HRP-conjugated anti-rabbit serum. Blocking experiments were done by preincubation of sera with antigens before addition to the membranes. Peptides were immobilized as spots on membranes and assays performed as describes above.

4.4.3. Immunoprecipitation (I)

GMK cells were infected with CBV4 and grown in methione-free medium for 4 h before addition of ^{35}S-methione and the labelled virus was immunoprecipitated as described in earlier (Roivainen *et al*., 1993b)

4.4.4. Epitope mapping (III, IV)

Partially overlapping peptides of capsid proteins VP1-VP4 of CAV9, nonstructural 2C protein of CBV4 and β-cell autoantigens IA-2, preproinsulin, GAD65 and HSP60 were synthetized on cellulose membranes in 384-spot format (SPOTs, Genosys, Inc) with a 12 amino acid window and three residue shift. The membranes were blocked with casein based blocking buffer (Genosys) or 5% dried milk, 5% sucrose and TBS-Tween20 (0.05%) pH 8.0) overnight at +4

°C and incubated with serum dilutions, either virus induced rabbit, NOD mouse or human sera. After washings with TBS-Tween20 (0.05%), the membranes were incubated with the HRP-conjugated antiserum, washed and finally covered with thin layer of ECL reagent (Amersham and NEN Life Science Products). The chemiluminiscence of spots was determined with a scintillation counter (Perkin Elmer Life Science, Wallac, Turku) and verified by autoradiography.

4.5. Plaque neutralization

Sera obtained from diabetic children were assayed for the presence of neutralizing antibodies to CAV9 and CBV4 as described earlier (Roivainen *et al.*, 1998).

4.6. Immunization of non obese diabetic (NOD) mice

F1-generation of NOD/-Bom mice, obtained from M&B A/S, Copenhagen, Denmark, were raised and maintained under pathogen-free conditions. Female offsprings were immunized intraperitoneally with inactivated CBV4-E2 or with PBS in Freund's incomplete adjuvant. The amount of virus used and immunization protocol differed in different groups and are shown in original publications III and IV. Onset of diabetes was monitored by measuring blood glucose consentration (>13 mmol/l hyperglycemic)

4.7. T-cell proliferation assay (IV)

Spleen cells from NOD-mice were incubated in triplicate with different antigens in RPMI-1640 supplemented with 25 mM Hepes pH 7.4, 50 μM β-mercaptoethanol, 5% FCS and 25 μg/ml gentamycin. After 48 hours stimulation tritiated thymidine (1 μ Ci/ml) was added and cells harvested 18 hours later for counting tritium incorporation with Victor (Perkin Elmer Life Science, Wallac, Turku)

4.8. Cytokine assay (IV)

Single cell suspensions of spleens were incubated with test antigens in culture medium and tested for presence of cytokines using commercial EIA assays (IL-2, 19211T; IFN-γ, 19301T; IL-4, 19231V; IL-10, 19281V; Pharmigen, San Diego, USA).

4.9. Adoptive transfer of diabetogenic splenocytes (IV)

Spleen cells of clinically diabetic mice were collected and mixed with an equal number of splenocytes isolated from 14 weeks old healthy NOD-mice immunized either with CBV4-E2

or PBS. T-lymphocyte mixtures (20 x 10^6), were transferred intravenously to female NOD-*scid* mice at 5 weeks of age. The development of diabetes was tested weekly by measuring glucose concentrations from urine (1-4 weeks) or blood (5-10 weeks). Recipients remaining normoglycemic were scored negative for adoptive transfer.

4.10. Human subjects (III and V)

Detailed data of human subjects and study groups are given in original papers (III and IV) and are only summarized here.
110 paired sera, collected during documented enterovirus infections, were obtained from the Netherlands, Sweden, and Finland (III, IV, V).
In report IV, blood samples were obtained from 73 Finnish children vaccinated according to the standard vaccination protocol (Piirainen *et al.*, 1999) with the Salk type of IPV given at the age of 4 and 7 months and OPV at 18 months old.

In report IV, sera were obtained from 20 children with diagnosed type 1 diabetes, previously tested for presence of antibodies to insulin (IAA), IA-2 (IA-2A) and GAD65 (GADA). A blood sample was also obtained from eight healthy children.

Table 4. Peptides, proteins, viruses and the corresponding antisera.

antigen	code	sequence	adjuvant	antisera codes
Coxsackie-derived VP1	p41/pVP1a	EAIPALTAVETGHTSQVC	FCA	482, 522,552, 392, 1331, 1231
poliovirus - P3/VP1	pB99-5/pVP1b	KEVPALTAVETGATC	FCA/OVA FIA	900, 910, 920, 642, 6337
poliovirus/P3 Sabin/VP1	p37/pVP1c	KEVPALTAVETGATNPLVC	FCA	1051, 112, 582, 602,
CBV4/VP0	p125/pVP0	VMIKSPALNSPTVEEC	FCA	154, 164, 983
IA-2/IA-2β	p331/ pIA-2	KEQFEFALTAVAEEVC	FCA FIA	4033, 4013 4600
IA-2/IA-2β	p332/ pIA-2	EFALTAVAEEVNAILKALC	FCA FIA	4183, 4091 4556
HSP60/ human	p124/pHSP60	ALLRCIPALDSLTPANEDC	FCA	124, 134

Proteins	code	expression and purification	adjuvant	antisera code
CBV4/VP0	GST-VP0	*E.coli,* SDS-gel extraction	FIA/denatured	N25, N125
	GST-VP0	*E.coli* affinity-purified	FIA/native	N48, N60
CBV4/2C	GST-2C	*E.coli* SDS-gel extraction	FIA/denatured	N85, N95
GST	GST	*E.coli* affinity-purified	FIA/native	N115
CBV4/VP0	His-VP0	*E.coli* SDS-gel extraction	FIA/denatured	k3898
CAV9/VP1	His-VP1	*E.coli* SDS-gel extraction	FIA/denatured	k3944
Mycobacterium bovis HSP65	HSP65/*Myco*	*E.coli* affinity purified		13, 19
Chlamydia pneumoniae HSP60	HSP60/*Cpn*	*Bacillus* in denaturated form	FCA/FIA	1504, 1505

virus	code	propagation	adjuvant	antisera code
coxsackievirus B4/J.V.B	CBV4	GMK	FIA	265, 275
coxsackievirus A9/Griggs	CAV9	GMK	FCA	861, 3896
coxsackievirus A9/Griggs	CAV9	GMK	FIA	4952
coxsackievirus B5/Faulkner	CBV5	GMK	-	-
poliovirus type 3/Sabin	polio, PV3	WHO-Hep2c	FCA	808
human parechovirus 1/Harris	HPE-1	A-549	FCA	3912

5. RESULTS AND DISCUSSION

One mechanism by which enteroviruses could induce or accelerate β-cell destruction, eventually resulting in clinical type 1 diabetes is through immunological cross-reactivity of enterovirus proteins and islet cell autoantigens. This 'molecular mimicry' principle is largely based on sequence similarity between diabetes associated region of glutamic acid decarboxylase (GAD65) and the nonstructural 2C protein of enteroviruses (Atkinson *et al.*, 1994; Hovi, 1998; Kaufman *et al.*, 1992b).

By sequence comparison (II, III) we found additional homologies between enteroviruses and diabetogenic epitopes in islet cell autoantigens IA-2 (Honeyman *et al.*, 1998), and in HSP60 (Elias & Cohen, 1994; Elias *et al.*, 1991; Raz *et al.*, 2001) (Table 5). An immunogenic diabetes associated HSP peptide (p277) had sequence similarity with VP0 of enterovirus capsid protein and with conservative region of the VP1 protein (Table 4, 5). It has been indicated that this peptide p277 (amino acids 437-460) in HSP60 is an important autoantigen in NOD-mice: autoreactive T-cells are directed to it, and when attenuated *in vitro*, these T-cells can protect from diabetes (Elias *et al.*, 1990). This peptide has been used as therapeutic agent both in NOD-mice and recently also in humans (Abulafia-Lapid *et al.*, 1999; Elias *et al.*, 1991). An immunogenic, conservative epitope of VP1 PALTAVET G/AT had also sequence homology with a region in the C terminal end of IA-2 (ALTAV), which has been shown to be part of diabetogenic T-cell epitope (Honeyman *et al.*, 1998; Peakman *et al.*, 1999). A high percent of patients with type 1 diabetes also have antibodies directed against the C terminal end of IA-2 (Leslie *et al.*, 1999).

These observations prompted us to investigate immune responses induced in animals and humans by these proteins and homologous peptides derived from them using various immunological assays. As cross-reactive epitopes can be differently exposed in different tests, antiserum was considered cross-reactive if it gave positive results by either EIA or immunoblotting, or both.

Table 5. Sequence comparison of CAV9 VP1 and VP0 regions with HSP60 (**a**). Sequence comparison of pIA-2a (tyrosine phosphatase) and pVP1a (coxsackievirus- derived) peptides (**b**). Identical amino acids are shown in bold.

a

CAV9 VP0 VMIKSL**PAL**N**S**P**T**VEECG
HSP60 ALLRCI**PAL**D**S**L**T**PANED
CAV9 VP1 SNSASV**PAL**TAVETGHTS

b

pVP1a -**E**AIP-**ALTAV**ETGHTSQVC
pIA-2 K**E**QFEF**ALTAV**AEEVC

5.1. Preparation and characterization of tools (I, II)

5.1.1. Expression of viral proteins in *E.coli* and production of antisera

Coxsackievirus B4 (CBV4) capsid protein VP0 and nonstructural 2C proteins were expressed as GST-fusion proteins in *E.coli* using CBV4 cDNA as template and expression vector pGEX-2T. Initially used induction conditions (37 °C, 0.5 mM IPTG) gave high expression levels, but insoluble fusion proteins. The solubility of proteins was significantly increased by lowering temperature (RT) and inducer concentration (0.05mM), that allowed the purification of both fusion proteins, GST-VP0 and GST-2C by glutathione sepharose column in a soluble form. Attempts to cleave off the GST-part by thrombin resulted in degradation of the viral part of fusion proteins and therefore the fusion proteins as such were used in rabbit immunizations (Table 4). Rabbits were also immunized with denatured fusion proteins, obtained by extraction from insoluble pellet fraction with detergents (Triton X-100, Tween-20 and SDS), which solubilized most of the bacterial proteins, but not the fusion proteins, which were then excised from SDS gels for immunization.

Fusion protein specific antisera recognized the corresponding recombinant protein in a dilution of 1:50 000. Reactivity was observed between fusion proteins and GST, only with weaker intensity. Antisera raised against denaturated and native GST-VP0 recognized VP0 and the cleavage products of VP0, VP2 and VP4 of CBV4 in immunoblotting at 1:20 000 dilution. Immunoblotting also revealed antigenic cross-reactivity with other enteroviruses CAV9 and CBV5, which is consistent with previous results showing high degree of cross-reactivity between enteroviruses (Hohenadl *et al.*, 1994). That reacting antibodies are directed to the viral domain of fusion protein was confirmed by preincubation of GST-VP0 antiserum with GST, which abolished the reaction with GST but not with virus.

As purified virus preparations do not contain nonstructural 2C, CBV4 infected GMK cell lysates were used to verify 2C reaction by immunoprecipitation. Antisera raised against denatured GST-2C fusion protein precipitated a protein corresponding to molecular size of 2C.

The purity of fusion protein preparations was confirmed by immunoblotting with VP0-pepide, MBP-2C (from Dr. L. Carrasco), GST-VP0, GST-2C and GST induced antisera. In addition to full size expression proteins, immunoblotting confirmed that smaller molecular weight by-products in the preparations were of fusion protein origin, not impurities from *E.coli*. These fusion protein preparations have been successfully used in lymphocyte stimulation experiments in determination of T-cell epitopes of enteroviruses and in the characterization of proliferative responses in diabetes patients and and healthy subjects (Juhela *et al.*, 2000; Marttila *et al.*, 2002; Marttila *et al.*, 2001).

Attempts to express CAV9 as soluble GST-fusion protein were unsuccessful. We later expressed VP1 and VP3 of CAV9 and VP0 of CBV4 in His-tagged form (His-VP1, His-VP3, His-VP0), but this did not overcome the solubility problem for any of these proteins.

However, the proteins could be purified to high degree by Ni-NTA agarose under denaturing conditions. To avoid urea in eluates in rabbit immunization these fusion proteins were excised from SDS gels. Polyclonal antisera raised against His-fusion proteins recognized the corresponding immunogen and purified virus protein in immunoblotting, and both antisera and fusion protein preparations were used in studies II and IV.

5.1.2. Peptides and peptide induced rabbit antisera (1, II, III, IV)

Peptides corresponding to immunogenic regions of VP1 (coxsackivirus- and poliovirus derived pVP1, and pVP1b, pVP1c, respectively), HSP60 (pHSP) and IA-2/ (pIA-2a) and a longer partially overlapping pIA-2b (Table 4) were used in EIA and immunoblotting either as free molecules or coupled to BSA and used in rabbit immunizations (Table 4). The sera recognized the corresponding peptide in EIA and preimmune were negative. Because sera from parallel immunizations with these peptides behaved highly similarly not all reactions are shown in this section or in original manuscripts (I, II, III, IV, V).

5.2. Islet cell autoantigens, HSP60 (II) and IAR-2 (III) share antigenic epitopes with enterovirus capsid proteins

5.2.1. Cross-reactions detected with viral and tyrosine phosphatase derived peptides

Possible cross- reactivities were tested by EIA and/or immunoblotting. Antisera raised against immunogenic epitope of tyrosine phosphatase IA-2/IA-2β (pIA-2a) (KEQFEFALTAVAEEVC) recognized coxsackievirus derived VP1 and VP0 peptides EAIPALTAVETGHTSQVC and VMIKSPALNSPTVEEC (pVP1a and pVP0, respectively). All of the pVP1a (coxsackievirus derived) induced antisera tested cross-reacted with pIA-2a, while none of nine poliovirus derived KEVPALTAVETGATC and KEVPALTAVETGATNPLVC (pVP1b and pVP1c, respectively) induced antisera recognized this peptide. The result is somewhat unexpected, as the sequences in polio-and coxsackieviruses are very similar. Indeed, when used as an antigen in EIA, poliovirus derived peptide was recognized by IA-2a-peptide-induced antisera. Similarly, both poliovirus peptide induced antisera recognized coxsackievirus pVP1a peptide as well purified CAV9 and CBV4 in EIA (not shown). Possibly amino acid(s) outside the homologous motif influence the reactivity of antibodies produced. On the other hand, pVP1b and pVP1c peptides may have formed conformations in which the homologous region is not exposed for recognition in rabbits. Similar discordance with antibody responses was observed with two tyrosine phosphatase peptides, pIA-2a and a partly overlapping, longer peptide of the same region in tyrosine phosphatase pIA-2b EFALTAVAEEVNAILKALC. While pIA-2a induced antisera recognized both the corresponding immunogen (pIA-2a), the longer peptide pIA-2b and both viral peptides, antisera raised against the longer tyrosine phosphatase peptide pIA-2b basically recognized only the corresponding peptide.

The specificity of the reactions between tyrosine phosphatase peptide pIA-2a and viral peptides was tested with blocking experiments. Preincubation of pIA-2a induced antiserum with the corresponding immunogen or with pVP1a abolished the recognition of VP1 peptide. The reaction of the same antiserum with pVP0 could only be blocked by preincubation with corresponding peptide (pIA-2a). Recognition of IA-2a peptide by the two viral peptide-induced antisera could be blocked both with corresponding viral peptide and with pIA-2a. No cross-reactivity was observed between viral VP1 and VP0 peptides.
The summary of cross-reactive immune responses by EIA is presented in Table 6. As a whole, parallel peptide induced antisera gave similar results, and not all results are shown in original manuscript (III). All of the tested rabbit preimmune sera were negative.

Table 6. Cross-reactive immune responses observed between PTP-like peptides (pIA-2a and pIA-2b), coxsackievirus derived (pVP1a), poliovirus derived (pVP1b, pVP1c) and CBV derived VP0 peptides. The data shows positive reaction/tested antisera. NT, not tested.

			antiserum 1:50			
peptide	αpIA-2/ IA-2a	αIA-2 / IA-2b	αpVP1 (a)	αpVP1 (b)	αpVP1 (c)	pVP0
pIA-2a	3/3	1/3	4/4	0/6	0/3	1/3
pIA-2b	3/3	3/3	0/4	NT	0/3	0/3
pVP1a	3/3	0/3	4/4	6/6	3/3	0/3
pVP1b	0/3	0/3	4/4	6/6	3/3	0/3
pVP1c	NT	NT	NT	NT	NT	NT
pVP0	3/3	0/3	0/4	0/6	0/3	3/3

5.2.2. Cross-reactivity observed with proteins

Characterization of possible immunological cross-reactions between enterovirus proteins and islet cell antigens were further studied using viral expression proteins, human HSP60, HSP65/ *M.bovis,* HSP60/*Cpn,* intracellular part of IAR and purified virus preparations.

5.2.2.1. Immune responses induced by enteroviruses and heat shock proteins (HSPs) (II)

The current study was prompted by the discovery of a moderate degree of homology beteen enterovirus capsid proteins and an immunogenic, diabetes-associated epitope in human HSP60 and HSP65 of *M. bovis*, an epitope named p277 in literature (Elias *et al.*, 1990, 1991). Heat-shock proteins are phylogenetically highly conserved proteins, still microbial HSPs are known to be highly immunogenic in humans and induce immune responses in many bacterial and parasitic infections (Young, 1990). Furthermore, numerous different regions of human HSP60 have been shown to possess sequence similarity with many autoantigens (Jones *et al.*,

1993) (Table 2) and they have been associated with a series autoimmune diseases, e.g type 1 diabetes (Abulafia-Lapid *et al.*, 1999; Elias *et al.*, 1991; Horvath *et al.*, 2002).

In order to evaluate whether enteroviruses share antigenic determinants with HSP60/65, two polyclonal antisera raised to *Mycobacterium bovis HSP65* (HSP65/*Myco*) (Table 4) were tested for reactivity with CBV4 and CAV9 in immoblot and in EIA. Both of these were shown to cross-react with *Chlamydia pneumoniae* (HSP60/*Cpn*), and one of the two, which strongly reacted with human HSP, also recognized purified CAV9 (VP1) and CBV4 (either VP1or VP0 proteins). Both of the two antisera raised against HSP60/*Cpn* also recognized human HSP60 and mycobacterial HSP65, and one of two antisera also recognized purified CAV9 and the recombinant VP1 protein of CAV9 in EIA. In immunoblotting both antisera did, however give positive reaction with purified viruses (CAV9, CBV4). The authenticity of virus recognition was confirmed with blocking experiments using corresponding HSP immunogens and virus preparations. CBV4 capsid proteins VP1 and VP2 are not separated in SDS-PAGE, but the results obtained with recombinant VP1 and VP0 proteins and in blocking experiments with purified VP1 indicated that the reacting protein is VP1, not VP2.

Cross-reactive immune responses were less clear the other way around. Antisera raised against purified enteroviruses and recombinant viral proteins did not cross-react with human HSP60, while some reactivity was seen against bacterial HSPs. Among numerous virus antisera screened, antiserum to human parechovirus 1 (HPE-1), a virus formerly classified to enteroviruses, reacted with human HSP60 but not with bacterial HSPs. The motif inducing this cross-reaction remains unclear, as the homologous region in VP1 does not exist in HPE-1. Some cross-reactivity was seen to VP1a and HSP peptides in immunoblotting, but compared to IA-2a peptides this was not so clear.

In the present study, immunological cross-reactivity was shown in antisera raised HSPs and VP1 protein of coxsackie viruses. The importance of immune response to HSP60 and especially to its p277 peptide has been clearly demonstrated in NOD-mice (Elias & Cohen, 1994; Elias *et al.*, 1990, 1991). In NOD-mice antibodies against HSP60 occured spontaneously before the onset of clinical disease, declining at the time point of manifested clinical diabetes (Elias *et al.*, 1990). Recently, by epitope mapping antibodies specific for p277, present both to in human HSP60 and *M.Bovis*, were shown to be significantly higher in diabetic patients compared to healthy children (Horvarth *et al.*, 2002). No significant difference was, however, found in the antibody levels to whole HSP proteins. The signicance of these epitope-specific antibodies in the pathogenesis T1D is unclear. They may indicate that these antibodies have a role in the autoimmune process against β-cells or they could be a secondary phenomenon resulting from cell damage.

Our results indicate that immunological cross-reactivity exist between HSPs and enterovirus VP1 protein, which can be induced by peptides carrying a homologous motif between the pro-

teins. However, our results do not exclude the possibily that there are other motifs as well. For example conformational structures are capable of inducing the observed cross-reactions.

5.2.2.2. Immune responses induced by enteroviruses and tyrosine phosphatase IA-2/IA-2β (III, IV)

The results obtained with virus and IA-2 peptide-induced rabbit antisera clearly showed that immunizations with these peptides are able to induce cross-reacting antibody responses. The cross-reactivity was not, however, limited to peptides. When purified viruses were used as an antigen in EIA, antisera raised against IA-2a peptide recognized purified CAV9, CBV4-E2 and CBV5 with no obvious difference in recognition in used assay conditions.
On the contrary, when antisera raised against purified virus were tested for reactivity, a phenomenon similar to that previously observed with peptide antisera reoccured. Both of CBV4, and one of three CAV9 induced rabbit antisera reacted with IA-2a peptide, but the poliovirus antiserum did not react with the pIA-2a peptide. The intracellular part of IAR was recognized by both VP1a and IA-2a induced antisera, while antisera against VP0 peptide did not recognize the protein.

Epitope mapping data further confirmed the results obtained by EIA. Synthetic peptides comprising IA-2 protein from 7-979, immobilized on membrane, were incubated with peptide- and CBV4- induced antisera. Antiserum raised against IA-2a-peptide recognized four peptide spots corresponding to peptide sequence aa 955-975 in IA-2 (SKDQFEF**ALTAV**AEEVNAILK), three of the peptide spots containing the ALTAV sequence. The pVP1a induced antiserum, used at a dilution as high as 1:70 000, recognized these three peptides, all containing the whole ALTAV sequence. Likewise, the antiserum to purified CBV4, at dilution of 1:50 000, gave a definite reaction with all ALTAV- motif containing peptide spots. In addition, this serum reacted also with other peptides in the protein, one very strong reaction was seen in the extracellular part of the protein and one in the intracellular part, earlier shown be be diabetes associated T-cell epitope (Honeyman *et al.*, 1998; Peakman *et al.*, 1999). In concordance with the EIA results, antiserum raised against poliovirus derived peptide (pVP1b) did not react with the ALTAV-motif in IA-2. At a dilution of 1:10 000, two other regions in IA-2 was recognized, both of which have been shown to activate T-cell proliferation in diabetic patients (Honeyman *et al.*, 1998; Peakman *et al.*, 1999).
Epitope mapping with CBV4 induced antiserum gave a definite reaction with three ALTAV containing peptides in the C-terminal end of IA-2. This part of IA-2, showing amino acid similarity of 10 amino acid with CBV4 VP1 protein, has been shown by many groups to be the major antigenic site to which autoantibodies in IA-2 positive diabetic patients are directed. Besides being a B cell epitope, this ALTAV containing seguence has been identified also as diabetogenic T-cell epitope (Honeyman *et al.*, 1998; Peakman *et al.*, 1999). Interestingly, the

cross-reactive amino terminal region of VP1 (21-35) contains a T cell epitope, and immune responses to this region has been suggested to be important in the pathogenesis of CBV3-induced myocarditis (Huber *et al.*, 1993). In addition to the ALTAV sequence, CBV4-induced antiserum gave strong reactions also with other parts of IA-2, some of which are also part of identified diabetogenic epitope (Figure 2). The significance of these immunological cross-reactions between motifs in IA-2 and virus remains unknown.

5.3. Characterization of viral protein induced epitopes in NOD-mice (IV)

The NOD-mice is widely used animal for characterizing effects of different proteins in development of diabetes. It has been shown that autoimmune process against islet cells can be modulated by administration of different β-cell specific autoantigens to NOD-mice (Elias *et al.*, 1991; Zhang *et al.*, 1991; Tisch *et al.*, 1993; Trembleau *et al.* 2000). We were interested in finding out the immune response induced in NOD-mice by CBV4 immunzation and the effect of this in the development of diabetes in NOD-mice. Female NOD-mice were immunized with purified inactivated CBV4-E2, and the individual sera tested for antibodies to CBV4-E2 virus, His-tagged fusion proteins VP0, VP1 and VP3, GAD65, insulin, and peptides corresponding to observed homology motifs in VP1, VP0, IA-2 and HSP60. All immunized mice had antibodies to purified CBV4 -E2 in EIA. Recombinant His-tagged VP1 protein (His-VP1) was also recognized by most immunized sera, while more variation was seen with individual sera in reactivity to recombinant proteins His-VP0 and His-VP3. In capsid proteins VP1 and VP0 special interest was in the two regions previously shown to cross-react with two islet cell autoantigens, IA-2 and HSP60 (II, III). The highly immunogenic N terminal VP1 region (PALTAVETGHT) was recognized by sera from all immunized NOD-mice, while there was poor reaction towards VP0-peptide. In contrast, many of the immunized NOD-mice had antibodies to pIA-2, confirming our earlier results of cross-reactivity obtained with peptides and virus-immunized rabbit antisera (III).

Virus-induced humoral responses in NOD-mice were further characterized by epitope mapping with partially overlapping peptides in CAV9 capsid proteins VP1-VP4, and islet cell autoantigens IA-2, insuli, GAD54 and HSP60 (Fig. 2). CBV4-E2 immunization in NOD-mice resulted in recognition of epitopes covering the whole capsid, the most intensive reaction directed to VP1, VP3 and VP4 proteins. Recognition of both pVP0 and pVP1 sequences (Table 4) was prominent. Control mice did not respond to viral peptides.

Spontaneous humoral responses in NOD-mice have been observed to GAD65, IA-2, HSPs and to insulin (Elias & Cohen, 1994; Myers *et al.*, 1998; Tisch *et al.*, 1993) although the results are somewhat controversial (Bonifacio *et al.*, 2001; DeSilva *et al.*, 1996). The data presented by the International Workshop on the standardization of autoantibody assays in animal

models, however, gives strong evidence that insulin antibodies are markers of autoimmunity in NOD-mice (Bonifacio *et al.*, 2001). In our experiments, insulin autoantibodies predominated in control NOD-mice at age of 12-15 weeks, while limited reactivity was seen to HSP and to IA-2 peptides. CBV4 immunization resulted in increased antibody responses to GAD65, insulin and IA-2 peptides in EIA.

Virus immunization induced humoral responses to islet cells were further characterized by epitope mapping (Fig. 2). ALTAV and PALLDSPANED (p277) motifs in IA-2 and HSP60, respectively, were both recognized by CBV4-E2 immunized NOD-mice. In addition, some other sequences in these proteins were recognized. The virus induced immune response to preproinsulin was targeted to one epitope located at C-terminal half of the leader peptide, aa 10-26 in protein (LLALWGPDPPAAAFVN). This region was recognized to some extent also by unimmunized control mice suggesting that this motif is a natural humoral epitope in NOD-mice. The spontaneous recognition of this preproinsulin peptide and a strong immune response induced against it in virus immunization is intriguing, since this epitope is present only in immature insulin and the same peptide sequence has been shown to be one of the naturally processed, DRB1*0401-restricted T-cell epitopes in preproinsulin (Congia *et al.*, 1998). The significance and mechanism of this CBV4-E2 immunization induced PP1 peptide recognition remain to be studied.

In general, CBV4-E2 enhanced B-cell immune responses to certain islet cell epitopes. Whether this is due to cross-reactive immune responses induced by immunogenic virus proteins and islet cell autoantigens or immunization induced shift from TH1 to TH2 reactivity with concomitant antibody production, is not resolved.

5.4. Development of diabetes in CBV4-E2 immunized NOD-mice (IV)

Coxsackievirus (CBV4-E2) immunization protected NOD-mice from spontaneous diabetes and the effect was, at least partially, due to activation of splenocytes by the virus, as shown by splenocyte transfer. According to our results, concomitant transfer of splenocytes from CBV4-E2 immunized mice together with lymphocytes obtained from diabetic mice partly protected NOD-*scid* mice from diabetes.

CBV4 immunization resulted in strong humoral response to purified virus preparation, many CBV peptides and to several islet cell autoantigens. On the contrary, only few immunized mice showed T-cell proliferation in response to virus immunization, but IFN-γ and IL-10 secretion was often detected in response to inactivated CBV4. In NOD-mice, high cytokine response to autoantigen without T-cell proliferation has been observed also by other groups (Trembleau *et al.*, 2000). In our study, T-cell reactivity upon autoantigen stimulation was low in control NOD-mice, and the responses did not change remarkably in immunized mice.

Female NOD-mice develop diabetes spontaneously and, in contrast to human studies, pathogen and islet cell antigen immunizations have been shown to prevent or delay diabetes in

NOD-mice (Cooke *et al.*, 2001; Cooke *et al.*, 1999; Elias *et al.*, 1995; Kaufman *et al.*, 1993). Developmental and functional defects have been reported in antigen presenting cells (APC) of NOD-mice and these defects appear to perturb the presentation of self antigens in the course of tolerance induction (Lund & Strid, 2000). Some of these defects are associated with defective secretion of endogenous cytokines. It is probable that the infection and immunization of autoantigens in NOD-mice stimulates of cytokine release and of APC function (Lund & Strid, 2000). This then leads to generation of functional immunoregulatory cells capable of suppressing the diabetogenic process. Several β-cell autoantigens have been reported to postpone or prevent diabetes in NOD-mice (Daniel & Wegmann, 1996; Elias *et al.*, 1991; Hutchings & Cooke, 1998; Kaufman *et al.*, 1993). It has been shown, however, that the route of antigen administration and timing of immunization have an effect on the type of Th response generated (Cooke *et al.*, 2001; Hutchings & Cooke, 1998). In mice, oral immunization with an antigen results in Th2 immunity, while both Th2 and Th1 are induced after intravenous administration (Hutchings & Cooke, 1998). In our study, both Th1 and Th2 immune responses were induced after intraperitoneal admistration of CBV4-E2.

5.5. Cross-reactivity associated with immunization and infections in humans (V)

Enteroviruses may reach pancreatic islets and destroy β-cells by virus-induced cytolysis (Roivainen *et al.*, 2000; Vuorinen *et al.*, 1992; Yoon *et al.*, 1978). On the other hand, virus-induced inflammatory reactions may result in β-cell destrucion (Chehadeh *et al.*, 2000; See & Tilles, 1995; Vreugdenhil *et al.*, 2000b). Alternatively, β-cell destruction may be induced by cross-reactive immune responses between enteroviruses and islet cell autoantigens (Kaufman *et al.*, 1992; Lönnrot *et al.*, 1996; Tian *et al.*, 1994). This later aspect was further studied in humans. We were especially interested in whether enterovirus infection or poliovirus vaccination can induce immune responses that could cross-react with islet cell autoantigens. Furthermore, diabetic children and healthy subjects were assayed for islet cell and enterovirus antibodies.

The study showed that during serologically confirmed enterovirus infections antibodies to known diabetes associated epitopes in pIA-2 or pHSP60 (Table 4) appeared in 10% and in 1% of cases, respectively. In addition, some patients showed increased antibody levels to human HSP60 and HSP65/*M.bovis* proteins. CAV9-infection induced immune responses were investigated in detail in humans using epitope mapping with overlapping peptides of CAV9 capsid proteins VP1-VP4 and CBV4 2C, GAD65, IA-2, HSP60 and preproinsulin immobilized in peptide spots. We chose CAV9-infected patients based on previous results that CAV9 is a diabetes-associated virus (Roivainen *et al.*, 1998) and infected human β cells *in vitro* (Roivainen *et al.*, 2000; Roivainen *et al*., 2002 in press).

According to our results, humoral immune responses were targeted to two N terminal and one C-terminal epitope of the capsid protein VP1. These regions were also found by CAV9 immu-

nized rabbit sera (Pulli *et al.*, 1998), but only PALTAVETGHT-sequence of VP1 was recognized by CBV4 immunized NOD-mice. Out of the tested autoantigens by epitope mapping, the most remarkable change in antibody specificity during CAV9 infection was the emergence of response against HSP60 SIQSIVPALEIA peptide, not found in acute infection. The same epitope was also recognized by CVB4-E2 immunized NOD-mice. The search for viral sequence (Blast sequence comparison-program), possibly inducing this cross-reaction, did not provide any good candidate. CAV9 infection was not shown to change the reactivity pattern against other autoantigens.

The individual sera of diabetic patients and healthy controls were assayed for antibodies to VP1-, VP0-, IA-2- and HSP60-peptides (Table 4). Most diabetic patients and controls had high levels of antibodies to both coxsackievirus-and poliovirus- derived VP1 peptides and some had a definite reaction with the diabetogenic epitope in IA-2 (pIA-2a), while very low antibody levels, if any, were directed to HSP60 peptide (pHSP).

Since epitope scanning worked out in recognition of the main B-cell epitopes induced by CAV9, we proceeded with our investigation to islet cell autoantigens. The diabetes sera were divided according to their reactivity to different autoantigens. Sera of control subjects were further divided to those with high reactivity to VP1 peptides (pVP1a, pVP1b) and to those with low levels of antibodies to these peptides.

The amino terminal region of VP1 representing the SASPALTAVETGHTSQV region was recognized by all serum pools from diabetic patient (GADA, IA-2A, IAA and positive for all three autoantibodies). In many cases, the serum pools originally positive for a certain autoantibody did not react with the peptides derived from the corresponding autoantigens. This emphasizes the importance of conformational epitopes in β-cell autoantigens. We identified some linear epitopes derived from autoantigens, which were recognized by serum pools from autoantibody positive patients but not by serum pools from healthy children. The reactivity to these linear epitopes was associated with the presence of multiple autoantibodies in patients with type 1 diabetes. Many of these epitopes have not been reported to be diabetes associated B-cell epitopes and the significance of this finding remains open.

We also studied poliovirus-induced responses in poliovirus-vaccinated Finnish children. This was prompted by our previous results, which indicated that coxsackievirus-derived peptide antiserum was more cross-reactive with the diabetes-associated IA-2 epitope (ALTAV) than the corresponding poliovirus-derived peptide antiserum (III). In the present study, children immunized with three doses of IPV rarely had antibodies to the conserved VP1 epitope of enteroviruses. A dose of live poliovirus vaccine increased the reactivity with VP1 peptides but did not result in cross-reactivity with the diabetes-associated epitope in IA-2 as did natural enterovirus infection.

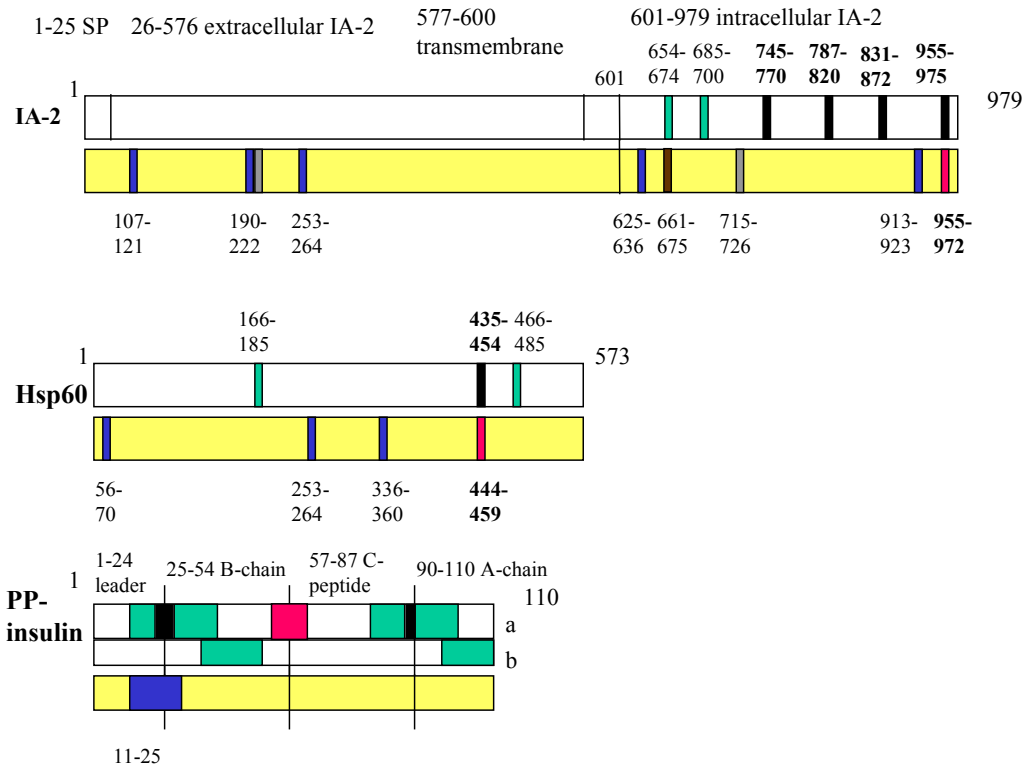

Figure 2. Positions of identified epitopes IA-2, HSP60 and preproinsulin (PP-insulin) according to literatures, and recognized in this study (II, III, IV, V).

IA-2. T-cell epitopes of IA-2 recognized by diabetic patients (Hawkes *et al.*, 2000; Honeyman *et al.*, 1998; Peakman *et al.*, 1999), upper panel with white background. Epitopes, identified by more than one group are shown in black and amino acid numbers shown in bold. Lower panel, with yellow background represents regions identified by us. Blue bars: epitopes of CBV4-E2 immunized NOD-mice; brown bar: epitope recognized by poliovirus derived VP1 peptide (pVP1b) induced rabbit antiserum, epitopes recognized by CBV4 induced rabbit antiserum are shown in grey; red bar: epitope recognized by coxsackievirus derived VP1 peptide (pVP1a) and CBV4 immunized rabbit sera, CBV4-E2 immunized NOD-mice, and by some human sera. Humoral resonses to IA-2 are directed mainly to C terminal end of intracellular proteins, but also to N-terminal and middle part of of the molecule (Leslie *et al.*, 1999).

HSP60: Diabetes associated epitopes in HSP60 (Bockova *et al.*, 1997; Elias *et al.*, 1990; Horvath *et al.*, 2002; Raz *et al.*, 2001) (upper panel). The epitopes, identified by more than one group is shown in black and aa numbers bolded. Lower panel: blue bars: epitopes recognized by sera from CBV4-E2 immunized NOD-mice. The region recognized both by CBV4-E2 immunized NOD mice and by pVP1a induced rabbit serum is shown in red. B-cell epitopes recognized by sera from diabetic patients are aa 437-460 (p277) and aa 394-413, the latter showing sequence homology with GAD65 aa 520-534 (Horvath *et al.*, 2002).

Preproinsulin (PP-insulin) Panel a: Diabetes associated T-cell epitopes in preproinsulin (Congia *et al.*, 1998) (aa 11-26, 20-36, 73-90, 85-101 in preproinsulin) shown in green and in proinsulin p24-33 spanning the B chain/C peptide junction shown in red (Chen *et al.*, 2001). The overlapping region of epitopes are shown in black. Panel **b:** Insulin specific epitopes, B-chain aa 9-23, A-chain aa 7-21 (Abiru *et al.*, 2001; Daniel & Wegmann, 1996). Region 24-36 in proinsulin has sequence similarity with GAD65 aa 506-518 (Rudy *et al.*, 1995). Panel **c:** an epitope 11-25 in PP-insulin recognized by CBV4-E2 immunized NOD-mice in our study (IV)

6. GENERAL DISCUSSION AND CONCLUSIONS

Antigenic molecular mimicry is defined by cross-reactive immunity due to structural homologies shared by molecules encoded by distinct genes. Shared epitope can be based on either linear amino acid sequence of the molecules or their conformation. Data base searches of protein sequences derived from mammals and microorganisms reveal many potential epitopes shared between numerous microbes and host self proteins and which could have relevance to autoimmune disease (Jones *et al.*, 1993; Oldstone, 1998; Wucherpfennig & Strominger, 1995; Wucherpfennig & Eisenbarth, 2001). Sequence similarity does not necessarily induce cross-reacting immune responses. On the other hand, foreign antigen may not necessarily share identical amino acid sequences with self protein to drive autoreactive responses, and such epitopes would not have been identified by sequence alignment alone (Wucherpfennig & Strominger, 1995). Out of a panel of 129 peptides that matched the molecular motif reguired for the MHC class II binding and TCR recognition of immunodominant myelin basic protein (MBP) peptide, only seven viral and on bacterial peptide stimulated T-cell clones of multiple sclerosis patients. Of these, only one would have been found by sequence alignment (Wucherpfennig & Strominger, 1995). To be immunologically important, shared epitopes must be immunogenic, become processed by antigen presenting cells and induce antibody and T-cell responses. Moreover, to induce T-cell responses peptides must be bound to HLA molecules, some of which have been shown to be either protective or susceptible in e.g. type 1 diabetes (Awata *et al.*, 1992; Ilonen *et al.*, 1997).
We found sequence similarity between enteroviruses capsid protein VP1 and procapsid protein VP0 with islet autoantigens IA-2/IA-2β and HSP60. These autoantigen epitopes were previously reported to be important diabetes-associated epitopes both in humans and NOD-mice (Elias *et al.*, 1991; Honeyman *et al.*, 1998; Horvath *et al.*, 2002; Peakman *et al.*, 1999; Raz *et al.*, 2001). Furthermore, both of these enterovirus epitopes have been shown to be immunogenic and capable to induce both humoral and cellular immune responses during infection (Huber *et al.*, 1993; Marttila *et al.*, 2002; Roivainen *et al.*, 1991).

This study demonstrates that apart from the well documented sequence homology (PEVKEK) between coxsackievirus 2C protein and GAD65, there are also other homologies between islet cell autoantigens and enteroviruses. Among the homologous and potentially cross-reactive motifs in enteroviruses and different cell autoantigens studied, the cross-reactive immune response to pIA-2 and coxsackievirus-derived VP1 peptide, sharing ALTAV sequence, was most convincing. Peptides derived from homologous motifs were shown to induce cross-reactive immune responses frequently. Moreover, this cross-reaction was not limited to the hyperimmune sera in rabbits and NOD-mice, but was also observed in humans.
It is important to understand the potential mechanism by which enterovirus infections could be involved in the etiology of type 1 diabetes. Especially, in order to develop a safe enterovirus

vaccine the mechanisms by which enteroviruses possibly are involved in β-cell destruction should be known. If the mechanism is direct β-cell cytolysis (Roivainen *et al.*, 2000; Vuorinen *et al.*, 1992; Yoon *et al.*, 1978), virus induced inflammatory reactions (Chehadeh *et al.*, 2000; See & Tilles, 1995) or bystander damage (Horwitz *et al.*, 1998), then vaccination might prevent some enterovirus infections and accordingly possibly some of the diabetes cases. However, if the mechanism is based on molecular mimicry, then the vaccine itself might increase the risk developing T1D.

Poliovirus vaccination is considered safe, and it did not induce cross-reactive immune responses with diabetogenic epitopes studied here. This potential of cross-reactivity between enteroviruses and islet cell autoantigens should, however, to be taken into account if vaccines to enteroviruses are to developed.

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