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Enterovirus Infections
in the Pathogenesis of Type 1 Diabetes

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ACADEMIC DISSERTATION

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ACADEMIC DISSERTATION

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ORIGINAL PUBLICATIONS

I   Lönnrot M, Hyöty H, Knip M, Roivainen M, Kulmala P, Leinikki P, Åkerblom HK and the Childhood Diabetes in Finland Study Group (1996): Antibody cross-reactivity induced by the homologous regions in glutamic acid decarboxylase (GAD65) and 2C protein of coxsackievirus B4. Clinical and Experimental Immunology, 104:398-405.

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III   Lönnrot M, Knip M, Roivainen M, Koskela P, Åkerblom HK and Hyöty H (1998): Onset of insulin-dependent diabetes mellitus in infancy after enterovirus infections. Diabetic Medicine, 15:431-434.

IV   Lönnrot M, Korpela K, Knip M, Ilonen J, Korhonen S, Savola K, Koskela P, Simell O and Hyöty H: Enterovirus infections as a risk factor for beta-cell autoimmunity in a prospectively observed birth-cohort – the Finnish Diabetes Prediction and Prevention (DIPP) Study. Submitted

V   Lönnrot M, Marciulionyte D, Rahko J, Gale E, Urbonaite B, Vilja P, Knip M, and Hyöty H: Enterovirus antibodies in relation to signs of beta-cell autoimmunity in two populations with high and low incidence of Type I diabetes. Diabetes Care, in press

VI   Lönnrot M, Sjöroos M, Salminen K, Maaronen M, Hyypiä T and Hyöty H (1999): Diagnosis of entero- and rhinovirus infections by RT-PCR and time-resolved fluorometry with Lanthanide chelate labeled probes. Journal of Medical Virology, 59:378-384

ABBREVIATIONS

CAR	coxsackievirus-adenovirus receptor
CAV	coxsackie A virus
CBV	coxsackie B virus
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CSF	cerebrospinal fluid
DAF	decay-accelerating factor
DiMe	the Childhood Diabetes in Finland study
DIPP	the Finnish Diabetes Prediction and Prevention study
EIA	enzyme immunoassay
EIU	enzyme immunoassay unit
EMCV	encephalomyocarditis virus
GAD	glutamic acid decarboxylase
GADA	glutamic acid decarboxylase antibody
HLA	human leukocyte antigen
IAA	insulin autoantibody
IA-2	tyrosine phosphatase-like protein
IA-2A	tyrosine phosphatase-like protein antibody
ICA	islet cell antibody
ICAM	intercellular adhesion molecule
IDDM	insulin-dependent diabetes mellitus
Ig	immunoglobulin
IL	interleukin
JDF-U	Juvenile Diabetes Foundation unit
MHC	major histocompatibility complex
NOD mouse	non-obese diabetic mouse
NPA	nasopharyngeal aspirate
OR	Odd's ratio
PCR	polymerase chain reaction
RIA	radioimmunoassay
RNA	ribonucleic acid
RT	reverse transcriptase
RU	relative unit

INTRODUCTION

Type 1 diabetes is a chronic disease caused by a selective destruction of pancreatic beta cells by the immune system resulting in insulin deficiency and hyperglycemia (Atkinson and Maclaren 1994). In spite of improvements in therapy and prognosis, the disease is still associated with considerable morbidity and reduced life-expectancy. Finland has the highest incidence of type 1 diabetes in the world, and for unknown reasons the incidence appears to be continuously increasing (Tuomilehto et al. 1995).

The pathogenesis of human type 1 diabetes is incompletely understood, but both genetic and environmental factors are involved (Atkinson and Maclaren 1994). It is widely held that the effect of some environmental factor(s) is essential for the induction of the beta-cell-damaging process in genetically susceptible individuals. Virus infections, especially enterovirus infections, are among the most seriously suspect environmental factors. The purpose of this work was to evaluate the possible role of enterovirus infections in the pathogenesis of type 1 diabetes.

LITERATURE REVIEW

HUMAN ENTEROVIRUS INFECTIONS

Enteroviruses are small nonenveloped viruses with a single-stranded RNA genome of positive polarity and an icosahedral capsid composed of four polypeptides. Enteroviruses belong together with aphto-, cardio-, hepato-, parecho- and rhinoviruses to the family of picornaviruses (Table 1). They are grouped on the basis of their pathogenicity in humans and in laboratory animals. Human enteroviruses currently comprise 64 serotypes, which have been identified by neutralization assays using specific antisera. The serotypes include polio viruses, coxsackie A viruses (CAV), coxsackie B viruses (CBV), echoviruses and more recently identified enterovirus serotypes numbered in order of identification.

Table 1. The picornavirus family.

Genus	Number of serotypes	Members
Aphthovirus	7	Foot-and-mouth disease viruses
	1	Equine rhinitis A virus
Cardiovirus	1	Encephalomyocarditis virus
	1	Theiler's murine encephalomyelitis virus
	1	Vilyuisk human encephalomyelitis virus
	1	Rat encephalomyelitis virus
Enterovirus	3	Human polioviruses 1-3
	23	coxsackie A viruses 1-22, 24
	6	coxsackie B viruses 1-6
	28	echoviruses 1-7,9,11-21,24-27,29-33
	4	enteroviruses 68-71
	2	Bovine enteroviruses 1, 2
	3	Porcine enteroviruses 8-10
	20	Simian enteroviruses 1-18, N125, N203
Hepatovirus	1	Hepatitis A virus
	6	Avian encephalomyelitis-like virus
Parechovirus	2	Human parechoviruses 1, 2
Rhinovirus	>100	Human rhinoviruses
	3	Bovine rhinoviruses

(King et al. 1999)

Epidemiology

Surveillance data from the United States show that more than half of the clinical enterovirus isolates belong to the echovirus group and approximately one fourth to the group of CBVs. The nonpolio serotypes most frequently isolated have been echovirus 11 (12%), echovirus 9 (11%), CBV5 (9%), echovirus 4 (6%) and echovirus 6 (6%) (Strikas et al. 1986). According to Finnish surveillance data the most frequent clinical isolates have been CBV5 (15%), echovirus 11 (12%), CAV9 (9%), CBV3 (7%) and echovirus 30 (7%) (Hovi et al. 1996).

In most cases close human contact is required for the spread of human enteroviruses, as humans are the only known reservoir for these viruses (Feachem et al. 1981). In transmission from person to person a fecal-oral route is the most common way of spread, although some enterovirus serotypes are known to spread mainly by inoculation acquisition (Kono 1975). In addition, aerosols from coughing or sneezing may transmit the virus, and vertical transmission from mother to fetus via the placenta is also assumed to occur (Morens and Pallansch 1995). Enteroviruses are found in surface and ground waters throughout the world and are abundant in sewage (Feachem et al. 1981). Indirect transmission via contaminated water or food is thus possible, but it has rarely been described.

Age is one of the principal host-related factors affecting the incidence and outcome of enterovirus infections, and since these infections are more frequent in young children than in adolescents or adults, the young are considered to be the most important transmitters (Irvine et al. 1967, Melnick 1996). A high frequency of enterovirus infections already in early infancy has been suggested (Juhela et al. 1998). The host's age also influences the severity of the infection: coxsackie B virus infections are usually more severe in newborns than in older children or adults , but in the case of poliovirus infections as well as in most infections caused by coxsackie A and echoviruses adults are more likely to be severely affected than children (Mackay-Scollay et al. 1973, Melnick 1996). Gender is another host-related factor influencing the epidemiologic pattern of enterovirus infections. Males appear to be more susceptible to severe enteroviral diseases such as meningitis or carditis, although it is not certain whether subclinical or mild infections occur more frequently in males (Haynes et al. 1969, Moore 1982).

Several environmental factors are known to influence the epidemiology of enteroviruses. In tropical climates, the circulation of enteroviruses is more or less constant around the year (Melnick 1996), whereas in temperate climates, infections are typical for late summer and fall (Moore 1982). Frequent and close person-to-person contacts facilitate the circulation of enteroviruses: intrafamiliar transmission is common (Kogon et al. 1969, Lagercrantz et al. 1973), and spreading in day-care centers and nurseries has also been described (Gear and Measroch 1973, Helfand et al. 1994). Crowding, low socio-economic status and poor sanitation sometimes go hand in hand, and they have all been shown to be connected with an increased incidence of enterovirus infections. Paralytic poliomyelitis, a well-known complication of poliovirus infection, follows a different epidemiological pattern from the infection itself, as it tends to be a disease of development. Improvement in socio-economic and hygienic conditions at the beginning of the 20th century led to a decrease in the rate of poliovirus infections, while at the same time the incidence of paralytic disease paradoxically increased. The basis for this phenomenon was a delay in age at first poliovirus exposure due to the decreased rate of transmission; children and adults are more susceptible to the paralytic disease than infants (Melnick 1996).

Clinical manifestations

Enteroviruses cause a wide range of diseases (Grist et al. 1978), although a high frequency of subclinical infections is characteristic for most serotypes (Kogon et al. 1969). Paralytic poliomyelitis, caused by lower motor neuron damage, was the first enterovirus disease described. Poliovirus infections are mostly asymptomatic, and even in overt cases the symptoms are usually mild: upper respiratory tract infection, gastroenteris or influenza-like illness. The more severe forms, polioencephalitis and flaccid paralysis, occur in only 1-2 % of all poliovirus infections. In addition to polioviruses enteroviruses of other serotypes also cause diseases of the central nervous system, being among the most frequent causes of aseptic meningitis and encephalitis (Rotbart 1990a, Berlin et al. 1993a). They can also, although less commonly, cause paralytic disease (Kopel et al. 1965, Roden et al. 1975).

Viral myopericarditis is most commonly caused by enteroviruses, especially CBVs (Grist 1972, Woodruff 1980). The disease course is usually acute and mild, but complications such as arrythmias, cardiomegaly and congestive heart failure may occur (Woodruff 1980). Even dilated cardiomyopathy has been suggested to result from enteroviral myocarditis (Why 1995).

Neonates are most susceptible to serious consequences of enterovirus infections, including fulminant sepsis-like illness, multiorgan involvement and death. The infections are most often caused by CBVs (Eichenwald et al. 1967).

Acute hemorrhagic conjunctivitis emerged in Africa in the late 1960s and spread in a pandemic way to the Middle East, Asia and Oceania. A new enterovirus, designated enterovirus 70, was recognized as the cause for the syndrome (Kono et al. 1972). The symptoms may be severe, but recovery is usually complete. A variant of CAV 24 has also caused widespread epidemics of conjunctivitis (Chou and Malison 1988), and sporadic cases of conjunctivitis caused by enteroviruses of other serotypes have been described (Sandelin et al. 1977).

Respiratory tract diseases as well as nonspecific febrile illnesses caused by enteroviruses are very common and typically mild, with excellent prognosis. Pleurodynia, herpangina and various exanthems are also fairly common and benign forms of enterovirus diseases (Melnick 1996).

Illnesses caused by enterovirus infections are classically regarded as acute. However, evidence has accumulated suggesting that they may also be persistent and lead to chronic diseases, such as chronic heart diseases or chronic fatigue syndrome (Woodruff 1980, Gow et al. 1991, Why 1995). Some of the chronic diseases with enteroviral association, e.g. myocarditis and type 1 diabetes, have an autoimmune component (Why 1995, Åkerblom and Knip 1998).

Pathogenesis

It is widely held that the primary site of enterovirus replication is the local lymphoid tissue of the respiratory and gastrointestinal tract, including the tonsils, Peyer's patches and mesenteric and deep cervical lymph nodes (Bodian 1955), although mucosal cells of the respiratory tract and intestine have also been suggested as main primary replication sites (Sabin 1956). The infection may remain local or lead to viremia, which allows the spread of the virus to susceptible secondary replication sites such as the spinal cord and brain, meninges, myocardium or skin (Dagan et al. 1985, Melnick 1996).

Expression of a cellular receptor is a prerequisite for infection, but this is not the only factor determining tissue susceptibility (Freistadt et al. 1990, Ren et al. 1990). Some enteroviral receptors have been identified. Polioviruses share a common receptor which belongs to the immunoglobulin superfamily (Minor et al. 1984, Mendelsohn et al. 1989). Decay-accelerating factor (DAF), a membrane protein protecting cells from complement lysis (Lublin and Atkinson 1989), has been reported to be the receptor for several echoviruses (Bergelson et al. 1994) as well as for CBV 1, 3 and 5 (Shafren et al. 1995). CBVs also use a recently identified coxsackievirus-adenovirus receptor (CAR), and also other receptor molecules have been proposed, implying that CBVs may use multiple receptor proteins (Bergelson et al. 1997, Selinka et al. 1998). Echoviruses 1 and 8 use $\alpha2\beta1$ integrin as receptor (Bergelson et al. 1992), and $\alpha v\beta3$ integrin is used by CAV 9 (Roivainen et al. 1994). Several other CAV serotypes use the same receptor as human rhinoviruses, namely the intercellular adhesion molecule ICAM-1, which is a member of the immunoglobulin superfamily (Colonno et al. 1986, Greve et al. 1989, Pulli et al. 1995).

Binding of the virus to its receptor initiates the viral replicative cycle. The virus enters the cell, and the viral RNA is released into the cellular cytoplasm, where the replication occurs (Rueckert 1996). The viral genome acts as messenger RNA for the synthesis of a large polyprotein which is cleaved to generate the structural and nonstructural proteins of the virion. The viral RNA is copied into negative sense RNA, which acts as template for the synthesis of viral genomic RNA. Enterovirus replication induces various morphological changes in the host cell, broadly termed the cytopathic effect: shrinkage of the nucleus and accumulation of membranous vesicles in the

cytoplasm are followed by shrivelling of the entire cell. The metabolic alterations mediating this cytopathic effect include inhibition of host cell protein synthesis and redistribution of lysosomal enzymes, and changes in plasma membrane permeability (Schneider and Shenk 1987, Joachims and Etchison 1992). Finally the cell lyses, releasing mature virions. Lytic infection is the usual form of enterovirus infection, but also non-lytic and chronic infections in specific cell lines are known (Colbere-Garapin et al. 1989). The replication cycle takes 5 to 10 hours depending on the virus serotype and host cell type (Rueckert 1996).

Enterovirus infections induce both humoral and cell-mediated immune responses. The humoral immune response includes rapid production of specific IgM, followed by production of IgA and IgG. The IgM levels decline within 6 months, whereas the IgA and IgG can persist for years. The response can be homotypic or heterotypic (Katze and Crowell 1980). The frequency of the latter seems to increase with age, suggesting an influence of anamnestic enterovirus infections (King et al. 1983). Protection against enterovirus infection and restriction of the spread of infection is largely dependent on neutralizing antibodies in the mucosa and circulation (Dagan et al. 1983, Melnick 1996). Transplacentally transported maternal IgG as well as maternal IgA in breast milk can provide passive humoral immunity in the offspring (Zaman et al. 1993). The cellular immune response is but poorly characterized, but its main role probably resides in the elimination of primary enterovirus infection and regulation of the immune response (Juhela et al. 1998).

Diagnosis

Enterovirus infections can not be diagnosed on clinical grounds alone due to the high frequency of subclinical infections and the wide variety of clinical manifestations, few of which are unique to enterovirus infections. No specific treatment is available for enterovirus diseases, and therefore the importance of enteroviral diagnosis lies mainly in distinguishing between enterovirus-induced disease and clinically similar treatable diseases. Laboratory diagnosis also serves for enterovirus infection surveillance.

Conventional methods for the diagnosis of enterovirus infection include virus isolation with subsequent serotyping and measurement of enterovirus antibodies in

the serum. Enteroviruses are most often isolated from stool samples, but this provides only circumstantial evidence for the disease etiology, since enteroviruses can be isolated from the alimentary tract of asymptomatic individuals (Kogon et al. 1969). Isolation of an enterovirus from an affected organ or associated body fluids, e.g. myocardium of cerebrospinal fluid, provides the strongest evidence of enteroviral disease, and isolation from blood is also a sign of systemic spread of the virus (Dagan et al. 1985). Isolated enteroviruses are classically identified by neutralization of infectivity with serotype-specific antisera (Melnick et al. 1973). Although virus isolation is often considered the "golden standard" for enterovirus identification, it has several weaknesses. The most prominent drawback is a poor diagnostic sensitivity, which can be due to low viral titer, inadequate collection, handling and processing of the samples, or insusceptibility of the cell line used (Chonmaitree et al. 1982). In addition, the method is time-consuming, labor-intensive and costly.

Enterovirus serology is complicated by the large number of serotypes; antibody responses are often heterotypic, and on the other hand no uniformly cross-reactive enterovirus group antigen has been found. A four-fold increase in titer of neutralizing or complement-fixing antibody is a classical diagnostic criterion for serological test. The neutralization assay is considered accurate and serotype-specific, but due to the large number of enterovirus serotypes the test is laborious as long as the virus type is not known. Serological assays with broad enterovirus reactivity are usually more convenient, and type-specific diagnosis is usually not required, since differentiation between viral and bacterial disease is often sufficient from the clinical point of view (Wildin and Chonmaitree 1987). Several enzyme immunoassays (EIA) for the detection of enteroviral IgM, IgG and IgA antibodies have been described (Frisk et al. 1989, Samuelson et al. 1993, Swanink et al. 1993, Cello and Svennerholm 1994, Bendig and Molyneaux 1996). In these reports the highest assay sensitivities were seen with broadly reactive antigens, i.e. heat-treated viruses, viral procapsids and widely shared synthetic peptide antigens. The sensitivity of serological assays can also be enhanced by combining the results of several assays (Samuelson et al. 1993, Swanink et al. 1993). The sensitivity of different EIA assays varies considerably (23% - 83%) when virus isolation is used as reference, depending on the antibody measured, the antigen used and the time of serum collection (Samuelson et al. 1993, Swanink et al. 1993, Bendig and Molyneaux 1996). However, the sensitivity of the

enterovirus IgM assay has also been reported to surpass that of virus isolation (Bell et al. 1986). The specificity of the serological assays is good, although some false-positive reactions have been reported to occur especially in the IgM assays (Bell et al. 1986, Samuelson et al. 1993, Swanink et al. 1993). EIA tests are less laborious than virus isolation or other serological assays, but the requirement of paired serum samples limits their utility in clinical work. However, in the case of IgM a positive finding in a single serum sample provides evidence of a recent infection, and by merit of this rapid assay outcome IgM assay is more convenient for diagnostic purpose.

Methods for detecting an enteroviral nucleic acids in clinical specimens have been introduced during recent years. Nucleic acid hybridization assays utilizing cDNA or RNA probes have been successfully used, but their low sensitivity as well as laborious technology are regarded as disadvantages (Muir et al. 1998). Methods based on amplification of enteroviral genomic sequences by RT-PCR have proved useful diagnostic tools, and have therefore become extremely popular during recent years. These assays can detect most or all enteroviruses when the primer sequences are located in highly conserved genomic regions, which have been identified in the 5' noncoding region of the enterovirus genome (Hyypiä et al. 1989b, Chapman et al. 1990b, Rotbart 1990b). RT-PCR assays with these and other primers located in the 5' noncoding region have been tested in clinical settings, and they have been shown to be more sensitive than virus isolation and specific, although PCR contamination leading to false-positive results is possible (Rotbart 1990a). Type-specific identification of the RT-PCR amplification products requires subsequent sequence analysis or hybridization with a specific probe. With advances in molecular technology enteroviral sequence information is accumulating; the complete nucleotide sequence of at least 19 human enterovirus serotypes is currently known, and partial sequence data are available on virtually all enterovirus serotypes (Hyypiä et al. 1997). This information provides for classification of enteroviruses according to genotypic characteristics, which opens up new diagnostic prospects (Hyypiä et al. 1997).

ENTEROVIRUS-INDUCED TYPE 1 DIABETES

Overview of the pathogenesis of type 1 diabetes

Type 1 diabetes is caused by a selective destruction of pancreatic beta cells by the immune system, resulting in insulin deficiency and hyperglycemia (Atkinson and Maclaren 1994). The basis for the specific breakdown of self-tolerance leading to beta-cell autoimmunity is not known. The autoimmune process is underway for months or years in a preclinical phase, and the clinical manifestations occur late in the course of the disease, after most beta cells –about 80 % according to histologic studies - have been destroyed (Foulis et al. 1986, Castano and Eisenbarth 1990, Atkinson and Maclaren 1994). According to histologic findings islet regeneration is uncommon (Foulis et al. 1986).

Chronic inflammatory infiltrate in the Langerhans islets, comprising cytotoxic T-cells (CD8+), helper T-cells (CD4+), B lymphocytes, macrophages and natural killer cells, is characteristic for the disease (Bottazzo et al. 1985, Foulis et al. 1991). It is not known which component of the immune system has the main role in beta-cell destruction, but most evidence suggests that autoreactive T-lymphocytes are major contributors to the process (Castano and Eisenbarth 1990, Atkinson and Maclaren 1994, Roep 1996). The present understanding of T-cell responses in human type 1 diabetes is limited. One major limitation in T-cell studies is the fact that only peripheral blood lymphocytes are available, although the presence of islet reactive T-cells in peripheral blood has been shown (Atkinson and Maclaren 1994). Peripheral-blood mononuclear cells from patients with type 1 diabetes have been reported to proliferate e.g. to islet cells, a 38 kDa islet-cell antigen, insulin and glutamate decarboxylase (GAD) (Atkinson and Maclaren 1994), but the accuracy of T-cell responses measured in proliferation assays has been questioned (Bonifacio and Christie 1997).

Antibodies against islet cell antigens were discovered about 25 years ago (Bottazzo et al. 1974), and these islet cell antibodies (ICA) have been a subject of extensive research ever since. ICA consists of a heterogenic pool of antibodies against multiple and variable target molecules (Timsit et al. 1992), including e.g. GAD, tyrosine phosphatase-like proteins IA-2 and IA-2 beta as well as insulin, which is the

only beta-cell-specific autoantigen so far identified (Bonifacio and Christie 1997). The emergence of beta-cell autoantibodies is the first known marker of ongoing beta-cell damage in humans. They can be detected years before the clinical manifestation of diabetes, making possible the identification of individuals with increased risk of type 1 diabetes (Bingley et al. 1993, Knip et al. 1994, Knip 1997). ICA have been detected in approximately 0.5 % of the general population - although in Finland a prevalence as high as 4.1% has been reported (Karjalainen 1990) - 3 to 4 % of nondiabetic first-degree relatives of patients with type 1 diabetes and 70 - 90 % of patients with newly diagnosed type 1 diabetes. Not all subjects with detectable beta-cell autoantibodies progresses to type 1 diabetes; the probability of this is related to the autoantibody titer and the variety of autoantibodies present (Bonifacio and Christie 1997, Kulmala et al. 1998).

Type 1 diabetes has a strong genetic component. The disease is more prevalent among first-degree relatives of patients with type 1 diabetes (5%) as compared to the general population (0.5%) (Wagener et al. 1982, Tillil and Kobberling 1987). The disease concordance rate is 20 - 50 % in monozygotic twins and approximately 5 % in dizygotic twins (Barnett et al. 1981, Kaprio et al. 1992). The genetic component of type 1 diabetes is mainly associated with genes in the HLA complex on chromosome 6, although other genes are also involved, e.g. the insulin gene on chromosome 11 (Davies et al. 1994). The strongest genetic susceptibility or resistance to type 1 diabetes is associated with HLA class II molecules HLA-DR and DQ, which are expressed on the surface of antigen-presenting cells, and present antigenic peptides to CD4 T lymphocytes (Todd and Bain 1992).

It is widely recognized that in addition to genetic factors also environmental factors contribute to the pathogenesis of type 1 diabetes. The fairly low concordance rate among monozygotic twins indicates that genetic factors are important but not sufficient for the disease to occur. The very considerable geographic variation in the incidence of type 1 diabetes is probably partly but not exclusively due to differences in genetic susceptibility (Diabetes Epidemiology Research International Group 1988, Åkerblom and Knip 1998). Such a conception is further supported by migrant studies showing a significant increase in the incidence of type 1 diabetes among immigrants who have moved from a low-incidence area to a region with higher incidence (Bodansky et al. 1992). A temporal variation in the incidence is a conspicuous feature

of type 1 diabetes, and the rapid temporal increase observed particularly in some European countries - including Finland - during recent decades suggests the influence of environmental factors (Bingley and Gale 1989, Tuomilehto et al. 1995, Åkerblom and Knip 1998). In addition to the epidemiological evidence, experimental studies in animals have shown that several dietary components, virus infections and chemicals and toxins can cause diabetes (Åkerblom and Knip 1998). In human studies viral infections and dietary components are the environmental factors most frequently implicated. The former include retrovirus, mumps virus, rubella virus, cytomegalovirus, Epstein-Barr virus and enteroviruses (Yoon 1995). Among the putative dietary factors cow's milk proteins and N-nitroso compounds are the most likely candidates so far (Åkerblom and Knip 1998). It has been postulated that a number of environmental factors may be diabetogenic in certain combinations, a conception supported by some animal models (Blay et al. 1985, Haverkos 1997). Moreover, interaction between genetic and environmental factors is probable, rendering the pathogenetic picture even more complicated (Åkerblom et al. 1997). According to the general view the effect of environmental factors is essential for the induction of the autoimmune process in genetically susceptible individuals.

Epidemiological evidence suggesting involvement of enteroviruses

Numerous retrospective case-control studies have demonstrated an increased prevalence or a higher level of enterovirus antibodies in patients with newly diagnosed type 1 diabetes as compared to nondiabetic controls (Gamble et al. 1969, Barrett-Connor 1985a, Banatvala 1987a, Frisk et al. 1992a), although conflicting findings have also been reported (Palmer et al. 1982, Mertens et al. 1983). Most of these studies have concentrated on CBV serology, but in some of them also antibodies against other serotypes have been reported to be more frequent in diabetes patients (Frisk et al. 1992b, Helfand et al. 1995b). Elevated levels of enterovirus-IgM antibodies have been a frequent finding, suggesting that the infection has occurred not long before clinical diabetes. This in turn implies that enterovirus infections could have a precipitating role in the pathogenesis of type 1 diabetes. In more recent case-control studies enterovirus RNA has been found more frequently in the serum and blood of subjects with newly diagnosed diabetes than in control subjects (Clements et al. 1995, Andreoletti et al. 1997). As viremia is usually a sign of an acute infection - although the possibility of viremia as a sign of a chronic enterovirus infection has been proposed - these findings support the hypothesis of enterovirus infections as precipitators of the clinical manifestation of type 1 diabetes.

Retrospective study settings usually involve patients with clinical diabetes together with control subjects. Such a study design is not optimal for detection of infections which have occurred long before clinical manifestation of diabetes, and does not allow for analysis of the temporal relationship between infections and induction of the autoimmune process. Prospective studies are seen to be of utmost importance, in that they offer an opportunity to obtain information also on the early stages of the autoimmune process and on the possible environmental factors involved in the induction and progression of the condition.

Several prospective studies are currently under way to study the natural course of the beta-cell damage and to test various intervention strategies for the prevention of type 1 diabetes. Such projects include the Finnish Diabetes Prediction and Prevention (DIPP) study, the Nutritional Prevention of IDDM Intervention Trial, the European Nicotinamide Intervention Trial (ENDIT) and the Diabetes Autoimmunity Study in the Young (DAISY). The Childhood Diabetes in Finland (DiMe) study, which was carried

out in Finland in 1986 - 1989, was the first in the world to include virological analyses of a prospective series; the prospective sibling cohort comprised initially healthy siblings of type 1 diabetes patients, and the intrauterine exposure series included pregnant women whose child manifested type 1 diabetes before 7 years of age. Serological signs of enterovirus infections were almost twice as frequent in the siblings who progressed to diabetes as in those who remained non-diabetic (Hyöty et al. 1995). Interestingly, the excess of infections was observed both closely prior to the clinical diagnosis as well as several years before overt diabetes. This finding suggested that, in addition to their previously assumed precipitating role, enterovirus infections could also operate during the early phases of the pathogenesis. Furthermore, serological signs of enterovirus infections were temporally associated with increases in the levels of ICA, implying that enterovirus infections may enhance beta-cell autoimmunity (Hiltunen et al. 1997). Several enterovirus serotypes appeared to be involved (Roivainen et al. 1998), but the diabetes risk effect seemed to be specific for enteroviruses, as it was not associated with other virus infections documented in the same subjects (Hiltunen et al. 1995). In the intrauterine exposure series of the DiMe study, maternal enterovirus infections during pregnancy were shown to entail a risk of diabetes in the offspring (Hyöty et al. 1995). The risk effect was associated with clinical diabetes manifesting before the age of 3 years. Maternal enterovirus infections during pregnancy have also been studied in Sweden, where a similar serological finding was reported: an excess of enterovirus infections was found during pregnangy in the mothers of diabetic children (Dahlquist et al. 1995).

HLA type is known to influence the host's susceptibility or resistance to various infectious diseases, e.g. malaria, AIDS and hepatitis (Hill 1998). Accordingly, HLA type may also influence susceptibility to enterovirus infections and to their possible diabetogenic actions. This conception is supported by animal studies, and likewise in human studies enterovirus antibody levels have been reported to be higher in diabetic children with HLA DR3 and/or HLA DR4 than in other diabetic children, and in one study an association between CBV infections and clinical onset of the disease was observed only in HLA DR3-positive persons (Fohlman et al. 1987, D'Alessio 1992). In addition, the HLA-DR4 allele as well as the HLA-DQB1*02 allele have been reported to be associated with high enterovirus T-cell responses (Bruserud et al. 1985, Juhela et al. 1999). Accordingly, the fact that HLA type may influence not only susceptibility to

type 1 diabetes but also immune responses against enteroviruses may give rise to bias in studies without matching for HLA type between case and control groups.

Possible mechanisms of enterovirus-induced type 1 diabetes

The numerous hypotheses as to the role of enteroviruses in the pathogenesis of type 1 diabetes can be categorized into the following scenarios: 1) Pancreatic beta cells are destroyed by an acute lytic infection without an autoimmune component. 2) A chronic non-lytic infection of beta cells impairs insulin secretion without an autoimmune component. 3) Infection of peri-insular tissue leads to beta-cell damage. 4) An acute infection triggers beta-cell autoimmunity. 5) A chronic infection triggers and supports beta-cell autoimmunity (Szopa et al. 1993, Yoon 1995, See and Tilles 1998).

Some enterovirus strains are known to infect and lyse beta cells in vitro (Szopa et al. 1993, See and Tilles 1995). Autopsy case reports have documented insulitis in newborns and young children who died of fulminant CBV infection, and CBV antigens have been found in their beta cells, suggesting that CBVs are able to infect beta cells also in vivo (Yoon et al. 1979, Jenson et al. 1980, Nigro et al. 1986). Chronic enterovirus infections have also been described, although not convincingly, in human beta cells, and persistent enterovirus infections have been associated with other autoimmune diseases such as myocarditis (McKinney, Jr. et al. 1987, Colbere-Garapin et al. 1989). Accordingly, the first two hypotheses, suggesting either acute lytic beta-cell destruction or a chronic infection leading to gradual impairment of insulin secretion, are both basically possible pathogenetic models. However, both hypotheses assume that no autoimmunity is required for progression to type 1 diabetes. A case report has indeed demonstrated progression to type 1 diabetes without preceding autoimmunity (Orchard et al. 1983), but in the vast majority of patients an autoimmune component is evident (Atkinson and Maclaren 1994). It is therefore likely that the first two hypotheses can explain the pathogenesis in very few cases or alternatively, the infection of beta cells represents a final insult in the beta-cell-damaging process leading to clinical manifestation of the disease.

According to the third hypothesis, also known as the "innocent bystander" theory, enteroviruses infect pancreatic cells other than beta cells, leading to local release of interleukin-1α (IL-1α) and IL-1β. IL-1β would then act on IL-1 receptors of beta cells, leading to an excess of free radicals in the beta cells, which in turn causes oxidative beta-cell damage (Rewers and Atkinson 1995). Another innocent bystander theory proposes that peri-insular inflammation makes beta cells more susceptible to enterovirus infections by inducing the expression of beta-cell surface molecules used by enteroviruses as receptors (Craighead et al. 1990).

Beta-cell autoimmunity is a key element in the pathogenesis of type 1 diabetes. Accordingly, some kind of interplay between enterovirus infections and autoimmunity would seem likely if enterovirus infections are major pathogenetic factors. The actual mechanism of the specific breakdown in self-tolerance leading to beta-cell autoimmunity is not known. It has been proposed that a virus may induce alterations in the host cells which in turn trigger an autoimmune attack against the cells (Yoon 1995). Such possible alterations include induction of new surface antigens or modification of the existing surface antigens into immunogenic forms, induction of HLA class I or class II molecules on the target tissue, and release of intracellular antigens during host cell lysis. The infection may also induce alterations in the host's immune system by polyclonal activation of B or T lymphocytes, or by release of cytokines (Yoon 1995). The infection may also give rise to anti-idiotypic antibodies reacting with the viral cell receptors, or to lymphocyte cross-reactivity between viral and host antigens due to molecular mimicry (Yoon 1995). It is worth noting, that many of these hypotheses do not require beta-cell infection or even a local infection of adjacent cells.

Animal models

Encephalomyocarditis virus (EMCV) infection in mice is the best characterized model of virus-induced diabetes. EMCV is a murine picornavirus, i.e. not an enterovirus, but a member of the same virus family (Picornaviridae). The virus produces acute lytic infection of beta cells and subsequent diabetes (Yoon 1995). The occurrence of beta-cell destruction depends on the virus strain as well as on the genetic background of the host. The genetic susceptibility is not associated with the MHC, but is possibly related to genes modulating the expression of viral receptors on beta cells (Boucher et al. 1975, Yoon 1995). The diabetogenicity of EMCV strains appears to be related to their beta-cell tropism; a single amino acid change influencing the efficiency of viral attachment to beta cells has been shown to be critical in this context (Eun et al. 1988, Bae and Yoon 1993). Interestingly, induction of diabetes by a diabetogenic EMCV strain has been prevented by prior immunization with a nondiabetogenic EMCV variant (Notkins and Yoon 1982). Macrophages have been shown to be involved in the beta-cell damage of the EMCV model, but T lymphocytes or B lymphocytes do not seem to play an important role (Yoon et al. 1985, Baek and Yoon 1991). Thus, the relevance of this model to human type 1 diabetes can be questioned, as in human type 1 diabetes T-cell mediated as well as humoral autoimmunity is present.

CBV 4 has been shown to infect and destroy beta cells in mouse models. Induction of diabetes in mice with CBV 4 isolated from the pancreas of a child with fatal diabetic ketoacidosis is one of the strongest pieces of evidence for enteroviral involvement in the pathogenesis of diabetes (Yoon et al. 1979). As in the case of EMCV, not all CBV strains are diabetogenic and only certain strains of mice develop diabetes after infection. Sequence analyses have revealed amino acid differences between the diabetogenic and non-diabetogenic CBV 4 strains, but the critical sites responsible for the potential have not yet been identified (Kang et al. 1994, Titchener et al. 1994). In general, mouse strains susceptible to EMCV-induced diabetes are also susceptible to CBV4-induced diabetes, and vice versa, mouse strains resistant to EMCV-induced diabetes are also resistant to CBV 4 -induced diabetes (Yoon 1995). Thus, CBV-induced diabetes is likely to share some of the characteristics of the EMCV model. The candidate gene responsible for susceptibility to CBV 4 has been

mapped to chromosome 4, and it is associated with impaired humoral response to CBV 4 infection (Loria et al. 1984, Montgomery and Loria 1986). The pathomechanism of CBV-induced diabetes is still largely unknown, but the diabetogenic virus has been shown to infect pancreatic islets, leading to infiltration of lymphocytes, increased expression of GAD_{65} and appearance of ICA and GAD antibodies (See and Tilles 1995, Yoon 1995). Long persistence of the virus in the islets appears to be associated with progression to diabetes (See and Tilles 1995). Molecular mimicry between the GAD molecule and the CBV 2C protein has been proposed to play a role in the induction of beta-cell autoimmunity (Figure 1.) At least two molecular forms of GAD exist, i.e. GAD_{65} and GAD_{67}, the former isoform being the principle target of immunity in type 1 diabetes (Atkinson et al. 1994).

CBV 2C protein 28 | F I | E | W L K V | K | I L | P E V K E K | H E F | - | L | S | R L | 50

Human GAD_{65} 250 | AM | M | I A R F | K | M F | P E V K E K | GM | A | A | L | P | R L | 273
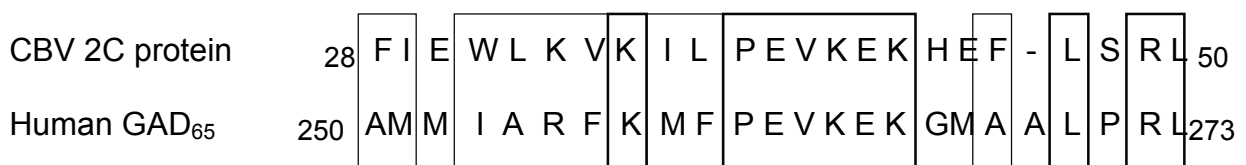
Figure 1. Sequence homology between CBV 2C protein and human GAD_{65}. Identical acid residues are indicated with thick lines, and thin lines enclose amino acid residues with similar charge, polarity or hydrophobicity. Numbers refer to the number of amino acid residues (Modified from Atkinson et al. 1994).

In the non-obese diabetic (NOD) mouse model T-cell responses to GAD represent a key event in the induction and propagation of beta-cell autoimmunity (Atkinson 1997). Indeed, cross-reacting T-cell responses have been induced in these mice by immunization with the 2C protein or a viral peptide containing the homology region (Atkinson 1997), but CBV 4 infection would not appear to be associated with enhanced GAD immunity or accelerated progression to diabetes in the NOD mouse model (Horwitz et al. 1998).

OBJECTIVES OF THE PRESENT STUDY

The objectives of the present study were the following:

1. To evaluate the impact of enterovirus infections on the risk of beta-cell autoimmunity and clinical type 1 diabetes by determining the occurrence of these infections in prospective observation series.

2. To test the hypothesis that the marked international variation in the incidence of type 1 diabetes may reflect differences in enterovirus epidemiology.

3. To study antibody cross-reactivity between CBV4 2C protein and human GAD, and to evaluate its possible role in beta-cell autoimmunity.

4. To facilitate enterovirus RNA detection in large study series by developing an applicable hybridization assay for enterovirus RT-PCR amplification products.

SUBJECTS AND METHODS

DiMe subjects (Reports I and II)

The recruitment phase in the nationwide "Childhood Diabetes in Finland" (DiMe) study was carried out between September 1986 and April 1989 in all hospitals in Finland treating children with diabetes. Serum samples were obtained at the diagnosis of type 1 diabetes from a total of 780 index cases and 765 siblings (Tuomilehto et al. 1992, Hyöty et al. 1995). The prospective follow-up sera were collected every 6 months among a cohort of altogether 765 originally non-diabetic siblings of the index cases with diabetes.

Antibodies against synthetic peptides containing the homology regions between CBV4 2C protein and human GAD molecule were measured in follow-up samples of seven siblings who had manifested type 1 diabetes and had serologically documented enterovirus infections during the follow-up (Hyöty et al. 1995). Peptide antibodies were also analysed in 15 non-diabetic control siblings who were negative for ICA, GAD antibodies and insulin antibodies during the follow-up. In addition, peptide antibodies were analysed in a case-control series of samples: serum samples were taken from 90 under-7-year old children within 14 days after the diagnosis of type 1 diabetes, and from age- and sex-matched unrelated control children selected from the Finnish population register. The mean age of the children was 4.5 years and 48 (53%) of them were males.

Ninety-three serum samples from 11 siblings who progressed to type 1 diabetes during the follow-up were analysed for enterovirus RNA using reverse transcription and polymerase chain reaction (RT-PCR). The samples had been stored at -20°C for 3 to 10 years. Forty-nine of these samples had never previously been thawed, making them optimal for RT-PCR analysis, whereas 44 specimens had previously been subjected to three to six freezings and thawings. The control group comprised 108 follow-up serum samples from 34 siblings who belonged to the same follow-up cohort but did not develop beta-cell autoimmunity or type 1 diabetes (105 of

these samples had never been thawed). The groups of prediabetic and control children were comparable in regard to average age at sample drawing as well as gender distribution. The mean age was 8.4 (range 2.6 – 17) and 8.9 (range 2.5 – 20) years and the proportion of males 61% and 60%, respectively. The mean observation period was 4 years in the prediabetic children and 2 years in the controls. The distribution of the observation periods was similar in the two groups within the follow-up years 1987 – 1993. In addition, serum samples from 47 children with newly diagnosed type I diabetes belonging to the case-control series were analysed for enterovirus RNA. The mean age in this group was 4.4 years and 53% of them were males. None of these samples had been previously thawed.

Subjects from the Nutritional Prevention of IDDM Intervention Trial (Report III)

The prospective Nutritional Prevention of IDDM Intervention Trial was designed to evaluate the possible effect of elimination of cow's milk proteins in early infancy on the risk of manifesting type 1 diabetes. The first pilot trial involved 20 infants of mothers with type 1 diabetes. One of the infants participating progressed to type 1 diabetes during an observation period of 4 years. Serum samples for virological analyses were taken from the infants at the age of 3, 6, 9, 12 and 14 months. Maternal serum samples taken at the end of the first trimester were also available. The occurrence of enterovirus infections in the infants was studied by measuring IgG antibodies against an enteroviral peptide antigen, and IgG and IgM antibodies against CAV 9 and heat-treated CBV 4. The maternal sera were tested for antibodies against the peptide antigen and for neutralizing antibodies against all CBV serotypes.

DIPP subjects (Report IV)

The Finnish Diabetes Prediction and Prevention (DIPP) Study was initiated in Finland in 1994. In this trial all newborn infants in the University Hospitals of Turku, Oulu and Tampere are screened after parental permission for HLA-DQB1 alleles associated with type 1 diabetes. Infants carrying either the HLA-DQB1*02/*0302 or the *0302/x genotype (x stands for alleles other than *02, *0301 or *0602) are regularly observed from birth over the first 2 years at intervals of 3-6 months and subsequently with an interval of 6-12 months.

Twenty-one children, 10 boys and 11 girls, who were identified as the first in the DIPP cohort with signs of continuous beta-cell damage, comprised the group of cases. Clinical type 1 diabetes has hitherto been diagnosed in three of these children. Of the remaining 18 cases 16 have tested positive for a minimum of two autoantibody types associated with type 1 diabetes on at least two occasions. Two cases were positive for islet cell antibodies (ICA) only; in both cases the ICA have remained positive for at least two years. The mean age at seroconversion to autoantibody positivity was 13 months (range 6 to 21 months). The cases were born between November 1994 and June 1997, and all were followed from birth, the mean follow-up time being 20 months (range 9 to 29 months). Samples were taken at birth (cord blood) and subsequently at intervals of 3-6 months. Altogether 20 cord blood samples and 125 follow-up serum samples from the case children were analysed. Nine cases had the HLA-DQB1*02/*0302 genotype, 12 had the HLA-DQB1*0302/x genotype.

Three to six control children (mean five), matched for time of birth, gender and HLA-DQB1 alleles, were chosen from the DIPP follow-up cohort for each case. The control group comprised altogether 104 children from whom altogether 98 cord blood samples and 567 follow-up serum samples were analysed. The controls were followed from birth according to the same protocol as the cases. Their mean follow-up period was 20 months (range 9 – 31 months). All control children have constantly tested negative for ICA. The same enteroviral analyses as in the case children's samples were carried out on the control sera.

In addition to the follow-up samples we also had access to sera obtained from the mothers of 20 cases and 103 control children at the end of the first trimester of

pregnancy. These maternal samples were also analysed for the presence of enterovirus antibodies and enterovirus RNA.

All serum samples were analysed for IgG and IgA antibodies against purified CBV 4, purified echovirus 11, an enteroviral peptide antigen and adenovirus. Enterovirus antibodies of the IgM class were measured against a mixture of three enterovirus antigens (coxsackie B virus 3, coxsackie A virus 16 and echovirus 11). Antibodies against adenovirus were analysed as control. The sera were also studied for the presence of enterovirus RNA.

Non-diabetic Finnish and Lithuanian schoolchildren (Report V)

In Lithuania 1049 serum samples were collected during the year 1994 among non-diabetic schoolchildren living within the region of Kaunas. The Finnish serum samples were derived from a study on beta-cell autoimmunity in which sera were collected during the year 1994 from 3662 unaffected schoolchildren living in Northern Finland. ICA negative sera were available from 200 Lithuanian and 200 Finnish children matched for age and sex. These matched groups comprised 76 (38%) males and 124 (62%) females with a mean age of 10.8 years. ICA-positive samples were available from 104 Finnish and 23 Lithuanian children. The mean ages in these groups were 11.8 and 11.6 years and the proportions of males were 50% and 48%, respectively.

The sera were analysed for IgG antibody levels against an enteroviral peptide antigen and purified CBV 4 , as well as for neutralizing antibodies against CBV 4, CBV 5, poliovirus 1 and poliovirus 3. Antibodies against hepatitis A virus were also measured.

Antibody assays

Antibodies against viruses and viral peptides

Heavy-chain capture radioimmunoassay (RIA) was used to detect IgG, IgM and IgA antibodies against enterovirus antigens (CBV 4, CBV 5, CAV 9, echovirus 1, and procapsid antigens of CBV 3 and CBV 5) as previously described (Reports I and III) (Hyöty et al. 1995).

Enzyme immunoassay (EIA) was used to measure IgG and IgA antibodies against purified CBV 4, purified echovirus 11 and adenovirus (Reports IV and V). The CBV 4 and echovirus 11 antigens were incubated for 15 min at +56 °C to expose antigenic determinants common to various enterovirus serotypes. IgM antibodies were measured (Report IV) against a mixture of three enterovirus antigens (CBV 3, CAV 16 and echovirus 11) using a capture EIA method which is a modification of our previously employed capture RIA (Hyöty et al. 1995, Hiltunen et al. 1997). The performance of these EIA tests is described in greater detail in report IV. IgG and IgA antibodies against a synthetic peptide antigen (amino acid sequence KEVPALTAVETGAT-C, reports I, III, IV and V) were measured using EIA as described elsewhere (Hyöty et al. 1995). The enterovirus peptide is derived from an immunodominant region of capsid protein VP1 (Roivainen et al. 1991), and is an epitope common for several enteroviruses (Hovi and Roivainen 1993). In all the aforementioned EIA assays a twofold or greater increase in the antibody level, which was observed between two consecutive serum samples and exceeded the cut-off level for seropositivity (three times the level of conjugate controls in the capture EIA; 15 EIU in the other EIAs), was considered significant, indicating a recent infection. EIA was also used in the detection of antibodies against synthetic peptides carrying various modifications of the homology region between CBV 2C and human GAD proteins (see report I for a detailed account of assay performance). In this EIA assay 50 EIU was used as a cut-off limit for antibody positivity, and an increase of 20 EIU or more in antibody level was considered significant.

Antibodies against hepatitis A virus were measured using Enzygnost Anti HAV kit (Behringwekle AG, Marburg, Germany) according to the manufacturer's protocol (Report V). Commercial EIA kits were also used in study I in the measurement of antibodies against Epstein-Barr virus (Du Pont Company, Billerica, MA, USA) and cytomegalovirus (CMV IgG/IgM EIA, Labsystems, Helsinki, Finland).

Neutralizing antibodies against CBV serotypes 1-6 and poliovirus serotypes 1 and 3 (Reports II, III, V) were measured using a classical plaque neutralization test (Roivainen et al. 1998). The viruses were treated with serial fourfold dilutions of sera for 1 hour at 36°C and overnight at room temperature. The serum-treated virus was administered to monolayers of Green monkey kidney cells, and the amount of infectious virus measured by counting the plaques after 46 hours of incubation at 36°C. The reciprocal of the last serum dilution able to block virus infectivity by 80% was taken as the neutralization titer, in which a fourfold or higher increase was considered significant.

Antibodies against beta-cell antigens (Reports I - V)

Antibodies against islet cells (ICA), glutamic acid decarboxylase (GADA), insulin (IAA) and the protein tyrosine phosphatase-related IA-2 protein (IA-2A) were analysed as previously described (Kulmala et al. 1998, Savola et al. 1998a, Savola et al. 1998b). In study IV a recently described microassay was used in the measurement of IAA (Williams et al. 1997). The detection limits were 2.5 Juvenile Diabetes Foundation units (JDF-U) for ICA, 6.6 relative units (RU) for GADA, 54 nU/ml (Reports I, II and III) and 1.56 RU (Report IV) for IAA and 0.43 RU for IA-2A. In study II seroconversion to autoantibody positivity and a significant increase in autoantibody level were taken as markers of autoimmune activation. Tripling of the ICA level was considered significant, whereas in GADA, IAA and IA-2A a doubling of the antibody level was considered significant; however, in the case of IA-2A the doubled antibody level had to be at least 1.54 RU.

Binding of rabbit antibodies raised against peptides carrying CBV and GAD sequences to GAD was studied by immunoprecipitation (for a detailed assay description see report I) according to principles previously described (Baekkeskov et al. 1990, Petersen et al. 1994).

Detection of enterovirus RNA (Reports II, IV and VI)

RNA was extracted from 140 µl serum using a commercial kit (QIAamp viral RNA kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. A primer pair from the highly conserved 5' noncoding region of the enterovirus genome was used (Hyypiä et al. 1989, Santti et al. 1997) for the RT-PCR. The primer sequences as well as a detailed description of the RT and PCR methods are to be found in reports II, IV and VI. The RT-PCR assay is highly sensitive, as it can detect less than 0.1 fg of enterovirus RNA (Santti et al. 1997). In study II a southern blot hybridization with a digoxigenin-labeled probe was used for specific detection of the amplification products. In addition, some of the amplification products were sequenced (for a more detailed description of the hybridization and sequence analysis see report II). In study IV the RT-PCR amplification products were hybridized with an europium-labeled enterovirus-specific probe in liquid phase on microtiter plates. The development and performance of this hybridization assay is described in report VI. Briefly, biotinylated amplification product (one of the primers carries a biotin label) is captured on a streptavidin-coated microtiter well. After denaturation hybridization is performed with europium- and samarium-labeled probes specific for entero- and rhinoviral sequences, respectively, and europium and samarium fluorescences are measured in a time-resolved manner.

HLA-DQ typing (Reports II, III and IV)

The HLA-DQB1 alleles associated with an increased or decreased risk of type 1 diabetes were determined as previously described (Ilonen et al. 1996). High risk is associated with the HLA-DQB1*02/*0302 genotype, and the HLA-DQB1*0302/x genotype entails a moderate risk (x stands for a neutral allele or homozygoticity). Low risk is associated with genotypes DQB1*0301/*0302, DQB1*02/*0301, DQB1*02/x and DQB1*0302/*0602-3, whereas genotypes DQB1*0301/0602-3, DQB1*02/*0602-3, DQB1*0602-3/x, DQB1*0301/x and x/x are associated with lesser risk.

Statistical analyses (Reports I, II, IV and V)

The difference in infection frequency during follow-up (Report IV) as well as the difference in antibody levels (Reports I and V) between case and control subjects was tested using two-tailed Student's t-test (paired test in analyses of paired data) and McNemar's test. The Odd's ratio (OR) with 95% confidence intervals (CI) was determined when the occurrence of enterovirus RNA in serum was compared between case and control subjects, and the association between viremia and enhancement of autoimmunity was analysed by Fisher's exact test and chi-square test (Report II). The difference in the occurrence of infections between case and control children during a 6-month observation period prior to the first detection of autoantibodies, and the difference in the occurrence of in utero infections between case and control children was assessed by calculating the Mantel-Haenszel Odd's ratio (multiple controls/case, varying number of controls/case) using Stata statistical software (Report IV). The difference in the number of infections among cases prior to seroconversion to autoantibody positivity vs. the other sample intervals during the follow-up was tested using the chi-square test or Fisher's exact test (Report IV).

Other methods (Reports I and VI)

Soluble peptides carrying different modifications of the homology sequence in the CBV4 2C, GAD_{65} and GAD_{67} proteins were synthesised using FMOC chemistry and Zinsser Analytical SMPS 350 multiple peptide synthesiser (Frankfurt, Germany). The precise amino acid sequences synthesised are presented in Report I.

Peptide antisera (Report I) were prepared by immunizing New Zealand white rabbits with BSA-coupled peptides using four subcutaneous injections at 2-week intervals, the first in Freund's complete adjuvant and then in Freund's incomplete adjuvant. Blood samples were drawn before the first injection and 1 week after the last.

Prototype entero- and rhinoviruses (Report VI) were obtained from the American Type Culture Collection, and virus isolates from the collection at the Department of Virology in the University of Turku. Enteroviruses were grown in susceptible cells and typed using WHO serum pools A to H. Rhinoviruses were grown in HeLa cells and identified on basis of acid lability.

Eighty-one cerebrospinal fluid (CSF) samples and 69 nasopharyngeal aspirates (NPA) from patients with signs and symptoms of meningitis/encephalitis or respiratory infection, respectively, were analysed in study VI. These specimens were collected from patients living within the district of Turku University Hospital and sent for testing as part of the daily virological diagnostic routine during the years 1996 and 1997.

RESULTS

Immunological cross-reactivity between CBV4 2C and GAD proteins (Report I)

Rabbit antibodies against synthetic peptides carrying modifications of the homology sequence in CBV4 2C and human GAD proteins were raised to study antibody cross-reactivity between these proteins, and to evaluate the possible role of cross-reacting antibodies in beta-cell autoimmunity. The immunized rabbits produced high titers of antibodies against all seven peptides. Six of the antibodies cross-reacted with matching peptides from the other molecules. Antibody cross-reactivity between 2C peptides and GAD_{65} peptides was observed: two of the four sera raised against 2C peptides cross-reacted with high intensity with matching GAD_{65} peptides, and one of the two GAD_{65} peptide specific sera bound to the matching 2C peptide. Binding of the antibodies could be blocked by preincubation of the sera with the homologous peptides. Two rabbit sera against 2C peptides bound to GAD_{65} in immunoprecipitation. One of these sera, as well as one serum against a GAD_{65} peptide, stained CBV4-infected cells in indirect immunofluorescence.

The presence of antibodies against the peptides deduced from the homology region was also studied in human subjects (Table 2.). Antibodies against the peptides were produced during enterovirus infections in five of the seven prediabetic siblings who later manifested clinical type 1 diabetes. These five siblings were all positive for ICA, IAA and GADA. In two siblings an increase in peptide antibody and autoantibody levels (IAA in one child, ICA, IAA and GADA in the other) coincided with an enterovirus infection. In one child peptide antibody responses were seen during two successive enterovirus infections, which were immediately followed by the appearance of IAA. GADA and ICA appeared a few months later. In one child the peptide antibody response was followed by an increase in ICA level after 6 months, while in the remaining case no alterations were observed in autoantibody levels. Increases in peptide antibody levels during enterovirus infections were also observed in the 15

control siblings who were constantly negative for autoantibodies and did not progress to diabetes during the follow-up.

Serum samples taken from 90 children with newly diagnosed type 1 diabetes and their matched non-diabetic controls were also analysed for peptide antibody levels. Peptide antibody levels (one 2C peptide, one GAD65 peptide and one GAD67 peptide) did not differ between these groups.

Table 2. Main findings in report I with human subjects.

Specimens (DiMe study)	Findings
Follow-up serum samples from 7 prediabetic children and 15 controls	Increases in peptide antibody levels did occur during enterovirus infections; no difference in peptide antibody responses between the prediabetic and control children
Serum samples from 90 children with newly diagnosed type 1 diabetes and 90 matched controls	No difference in the peptide antibody levels between the patients and controls

Enterovirus infections during prospective observation of prediabetic children

DiMe Study *(Report II)*

The occurrence of viremic enterovirus infections in prospectively followed children was studied. Enterovirus RNA was found in 11 (12%) of the 93 serum samples taken from prediabetic children compared to only two (2%) out of the 108 serum samples from the matched controls (OR 7.1, p<0.01). The RNA-positive samples were taken 0-9.5 years before diagnosis from altogether six prediabetic children, while five prediabetic children were RNA-negative during the follow-up. None of the RNA-positive children was constantly RNA-positive.

The occurrence of enterovirus RNA was statistically associated with the appearance of or significant increases in ICA and GADA, but not with changes in IAA or IA-2A levels. According to serological neutralization tests only one of the 11 RNA-positive infections was caused by CBV. This CBV3 infection was not associated with induction of the autoantibodies, but clinical type 1 diabetes appeared at the time of the infection.

Previous thawings of the sera seemed to impair the sensitivity of the RT-PCR, being associated with a negative result: no enterovirus RNA was found in the 47 previously thawed samples, while 13 out of the 154 samples without previous thawings gave a positive result (p<0.05). Accordingly, in the optimally stored samples enterovirus RNA was found in 11 of the 49 samples from prediabetic children (22%) and in two of the 105 samples from the control children (2%) (OR 14.9, p<0.001).

A complete set of follow-up sera was available from one sibling pair. Both of these children had the high-risk-associated genotype HLA-DQB1*02/*0302. They were twice concomitantly positive for enterovirus RNA, and at the time of the second infection the level of GAD antibodies exceeded the cut-off limit for autoantibody positivity in both of them. The children were 3.7 and 12.1 years old at the time of this infection. The autoantibody levels were high only in the younger child, who later manifested clinical type 1 diabetes. Enterovirus RNA was found in altogether four serum samples of this prediabetic child. These four PCR amplification products, as well as the one associated with GADA positivity in her sibling, were chosen for

sequence analysis to evaluate the possibility of chronic infection. Judging from the sequencing results, the infections were caused by various non-polio enteroviruses.

In addition to the sera from prediabetic children, 47 serum samples taken from children with newly diagnosed type 1 diabetes were analysed, but none or them contained enterovirus RNA.

The Nutritional Prevention   of IDDM Intervention Trial (Report III)

Enterovirus infections were detected during the first year of life in three of the 20 infants participating in the first pilot of the Nutritional Prevention of IDDM Intervention Trial. One boy had two infections: the first occurred before the age of 3 months and the other between 9 and 12 months of age. One other boy had an infection between 6 and 9 months, and one girl between 9 and 12 months of age.

The boy with two enterovirus infections was the only child who progressed to clinical type 1 diabetes during an observation period of 4 years. This boy was born 4 weeks prematurely in August 1992, birth weight being 3.245 kg and length 48 cm. He carried the HLA-DQB1 allele *0302 but no protective alleles, indicating an increased genetic susceptibility to type 1 diabetes. He was suspected of having a respiratory infection at birth. The infant had high levels of IgM and IgG enterovirus antibodies in the first serum sample taken at the age of 3 months, indicating an enterovirus infection during the first months of life. Elevated IgM and IgG enterovirus antibody levels at the age of 12 months suggested another infection between 9 and 12 months of age. The first signs of beta-cell autoimmunity appeared soon after the first enterovirus infection: IAA was detected at 6 months of age, and ICA and GADA at the age of 9 months. The appearance of IA-2A and the second enterovirus infection occurred within the same time interval, i.e. between 9 and 12 months of age. Clinical type 1 diabetes was diagnosed at the age of 14 months. The infant had been breast-fed for 35 days and exclusively breast-fed for 2 days. He belonged to the dietary intervention group receiving a casein hydrolysate formula (Nutramigen, Mead Johnston & Company, Evansville, IN, USA) devoid of intact cow's milk proteins as supplementary milk over his first 9 months of life. The mother of this infant had a low IgG antibody level (9 EIU) against the enteroviral peptide antigen, and neutralizing antibodies against only one of the six CBV serotypes.

DIPP study *(Report IV)*

The frequency of enterovirus infections was studied during a prospective follow-up of children with an increased genetic risk of type 1 diabetes. The frequency of enterovirus infections was compared between children who had turned positive for autoantibodies and their matched autoantibody negative controls. Enterovirus infections were observed in 26 % of the follow-up sample intervals in the 21 autoantibody-positive cases and in 18 % of the follow-up sample intervals in the autoantibody-negative control children (p = 0.03). Only 8% of all observed enterovirus infections occurred before the age of 6 months, i.e. 2/33 infections in cases and 9/103 infections in controls. Enterovirus RNA was found in 4% of the samples from the cases and in 3% of the samples from the control children (p = 0.8). Gender had no effect on the frequency of enterovirus infections among cases (p = 0.5) or controls (p = 0.8), but the difference in the frequency of enterovirus infections between the case and control groups was more marked among boys (28% vs. 18%, p = 0.02) than girls (23% vs. 18%, p = 0.3). The frequency of enterovirus infections did not differ between children with the HLA-DQB1*02/*0302 genotype and those with the *0302/x genotype (p = 0.5 among cases, p = 0.3 among controls).

A 6-month follow-up period preceding the first appearance of autoantibodies in the cases was analysed separately in order to evaluate a possible temporal relationship between enterovirus infections and seroconversion to autoantibody positivity. A matching 6-month period for each control child was observed. Enterovirus infections were detected 0-6 months prior to seroconversion to autoantibody positivity in 12 (57%) out of the 21 case children and in 32 (31%) of the 104 matching follow-up periods among the control children (OR 3.7, 95 % CI 1.2 - 11.4). The difference was even more marked when enterovirus RNA detection in the cases (29%) was compared to that in the control children (6%) during this 6-month period (OR 8.4, 95 % CI 1.7 - 40.2). Enterovirus RNA was detected more frequently especially in male cases (OR 8.3, 95%, CI 1.3 - 53.4) and on the other hand in cases with the HLA-DQB1*0302/x genotype (OR 8.8, 95% CI 1.2 - 65.3) as compared to their matched controls. In most cases (17/21) the first autoantibody exceeding the limit for positivity was IAA or a combination of IAA and other antibodies.

Among the case children enterovirus infections were detected in 36% of the sample intervals during this 6-month period compared to 23% of the intervals during

the rest of the follow-up (p = 0.2). Enterovirus RNA was detected more frequently in case sample intervals during this period than in intervals during the rest of the follow-up (18% vs. 0%, p = 0.0002). Among the control children the total frequency of enterovirus infections as well as detection of viremic enterovirus infections did not differ between the sample intervals of this period and the rest of the follow-up (enterovirus infections: 20% vs. 17%, p = 0.5 ; detection of enterovirus RNA: 4% vs. 4%, p = 0.9).

The occurrence of enterovirus infections during pregnancy was evaluated using maternal serum samples as well as cord blood samples. Two out of the 21 mothers of case children (10 %) versus 17 among the 104 control children (16 %) had had an enterovirus infection during pregnancy, showing no significant difference between the groups (OR = 0.5, 95% CI 0.1-2.5).

Adenovirus infections were analysed as control in order to evaluate whether the difference in the frequency of infections between the cases and controls is a general phenomenon observed in the case of any infectious agent or applies more specifically to enteroviruses. Adenovirus infection was detected in 7 % of the sample intervals of both case and control children (p = 0.9). Gender or HLA-DQB1 genotypes had no effect on the frequency of adenovirus infections. No clustering of adenovirus infections was observed in the case children at the time of seroconversion (p = 0.5), and the frequency of adenovirus infections did not differ between the cases and controls 0-6 months prior to seroconversion to autoantibody positivity in case children (p = 0.9). Adenovirus infections were detected during pregnancy in none of the 21 cases and in three of the 104 controls (p = 0.5).

Table 3. Main findings in the prospective study series (Reports II, III and IV)

Subjects / Specimens	Findings
DiMe study 98 follow-up samples from 11 prediabetic children and 108 follow-up samples from 34 control children	Enterovirus RNA was detected more frequently in the prediabetic children than in the controls. Detectioin of enterovirus RNA was temporally associated with increases in autoantibody levels in the case children.
The Nutritional Prevention of IDDM Intervention Trial Follow-up samples from 20 infants over their first year of life; One of the infants progressed to type 1 diabetes at the age of 13 months.	The infant who progressed to diabetes had the strongest enteroviral exposure (serological evidence of two infections) in the series.
DIPP study Follow-up samples from 21 autoantibody-positive children and 104 matched autoantibody-negative controls.	Enterovirus infections were observed, using serology and enterovirus RNA detection, more frequently among the children who turned autoantibody-positive. Enterovirus infections were associated with seroconversion to autoantibody positivity in the case children.
DIPP study Sera obtained at the end of the first trimester of pregnancy from the mothers of the case- and control children	No difference in the occurrence of enterovirus infections during pregnancy between the case- and control groups

Enterovirus antibodies in non-diabetic schoolchildren in Finland and Lithuania (Report V)

In the ICA-negative group the Lithuanian children had higher IgG antibody levels against the common enteroviral peptide antigen ($p < 0.001$) and purified CVB 4 virions ($p < 0.001$) than the Finnish children. The Lithuanian children also had a higher frequency of neutralizing antibodies against CVB4 ($p < 0.01$) and against CVB5 ($p < 0.001$). The Finnish and Lithuanian children had similar titers of neutralizing antibodies against poliovirus 1, but the Finnish children had higher levels of neutralizing antibodies against poliovirus 3 ($p < 0.01$). Seropositivity in the hepatitis A virus antibody assay was more common in the Lithuanian group than the Finnish (20% vs. 1%, respectively, $p < 0.001$).

Similar differences were observed between the ICA-positive Lithuanian and Finnish children: the former had higher IgG antibody levels against the enteroviral peptide antigen ($p < 0.05$) and CVB4 ($p < 0.001$). They also had a higher frequency of neutralizing antibodies against CVB4 ($p < 0.05$) and CVB5 ($p = 0.06$).

The Finnish ICA-positive children had higher levels of IgG antibodies against the enterovirus peptide antigen ($p < 0.05$) than the ICA-negative Finnish children, but they did not differ in IgG antibody levels against CVB4 or in the frequency of neutralizing antibodies against CVB4 and CVB5. The Lithuanian ICA-positive children had higher levels of IgG antibodies against purified CVB 4 virions than the Lithuanian ICA-negative children, but the difference was statistically non-significant ($p = 0.15$). The levels of antibodies against the enterovirus peptide as well as the frequency of neutralizing antibodies against CVB4 and CVB5 did not differ between ICA-positive and -negative Lithuanian children. The children with and without ICA also had equal frequencies of antibodies against hepatitis A virus.

New hybridization method for entero and rhinoviral RT-PCR amplification products (Report VI)

RT-PCR amplification products from reference viruses and virus isolates were used in optimization of the hybridization assay. Out of 12 different candidate probes, three were selected for the final optimization experiments. One of these was designed to detect enterovirus sequences (755), one rhinovirus sequences (204) and one both entero- and rhinovirus sequences (795). The primer and probe sequences are shown in figure 2.The conditions for each step in the hybridization assay were optimized. The optimal hybridization conditions differed considerably between the three probes with regard to temperatures and subsequent washing. Probe 755 required a hybridization temperature as high as 35 –40°C for optimal reactivity and the optimal temperature in the subsequent washing was 45°C, whereas the signal obtained with probe 795 decreased markedly at hybridization temperatures above 30°C. Probe 204 was less temperature-sensitive, as it worked moderately well in temperatures between 20°C and 45°C, the optimal hybridization temperature being 35-40°C. Thus, probe 204 can be combined with either 755 or 795. The signal intensity of 130 clinical samples negative both in agarose gel electrophoresis and in hybridization was as low as the background signal intensity obtained with negative control samples, in which water was used as template (mean signal-to-background ratio approximately 1, range 0.3 – 2.2). The cut-off value for positivity was determined to be five times the background value obtained with negative controls included in each assay.

Amplification in RT-PCR was demonstrated by the presence of a fragment of expected molecular weight in agarose gel electrophoresis. All available enterovirus and rhinovirus serotypes were found to be amplified by the primers. Binding of the three probes was tested using RT-PCR products from prototype strains and clinical isolates representing altogether 30 different enterovirus serotypes, nine different rhinovirus serotypes and 20 clinical rhinovirus isolates of unknown serotypes. Probe 755 hybridized with all enterovirus sequences and one rhinovirus sequence. The binding of probe 204 was specific for rhinovirus sequences, but it failed to detect five out of the 20 untyped rhinoviruses. However, these sequences were detected by the third probe 795, which bound to 52 of the altogether 59 entero- or rhinovirus sequences. One amplification product, which originated from a rhinovirus of an

unknown serotype, was not detected by any of the probes. Different intensity in the binding of probes to sequences from entero- and rhinoviruses allowed clear distinction between these virus groups.

The sensitivity of the RT-PCR hybridization assay was tested by a dilution series of CBV 4 RNA. In the hybridization assay, the RT-PCR amplification product corresponding to 0.015 fg of RNA gave a positive signal. In gel electrophoresis, a very weak band corresponding to 0.15 fg RNA could be discerned, but practically only RT-PCR products corresponding to 1.5 fg RNA were visible.

The sensitivity of the RT-PCR hybridization assay was also compared to that of virus isolation in the analysis of 81 CSF and 69 NPA specimens. Enterovirus was isolated from seven CSF specimens and from two NPA specimens. All these specimens and additionally 19 CSF and 8 NPA specimens gave a positive RT-PCR hybridization result with the enterovirus-specific probe 755. Rhinovirus was isolated from six NPA specimens. These and an additional 10 NPA samples gave a positive RT-PCR hybridization result with the rhinovirus-specific probe 204. Furthermore, four NPA specimens, which were negative in virus isolation, proved to be positive in hybridization only with the entero-rhinovirus probe 795, indicating either entero- or rhinovirus infection.
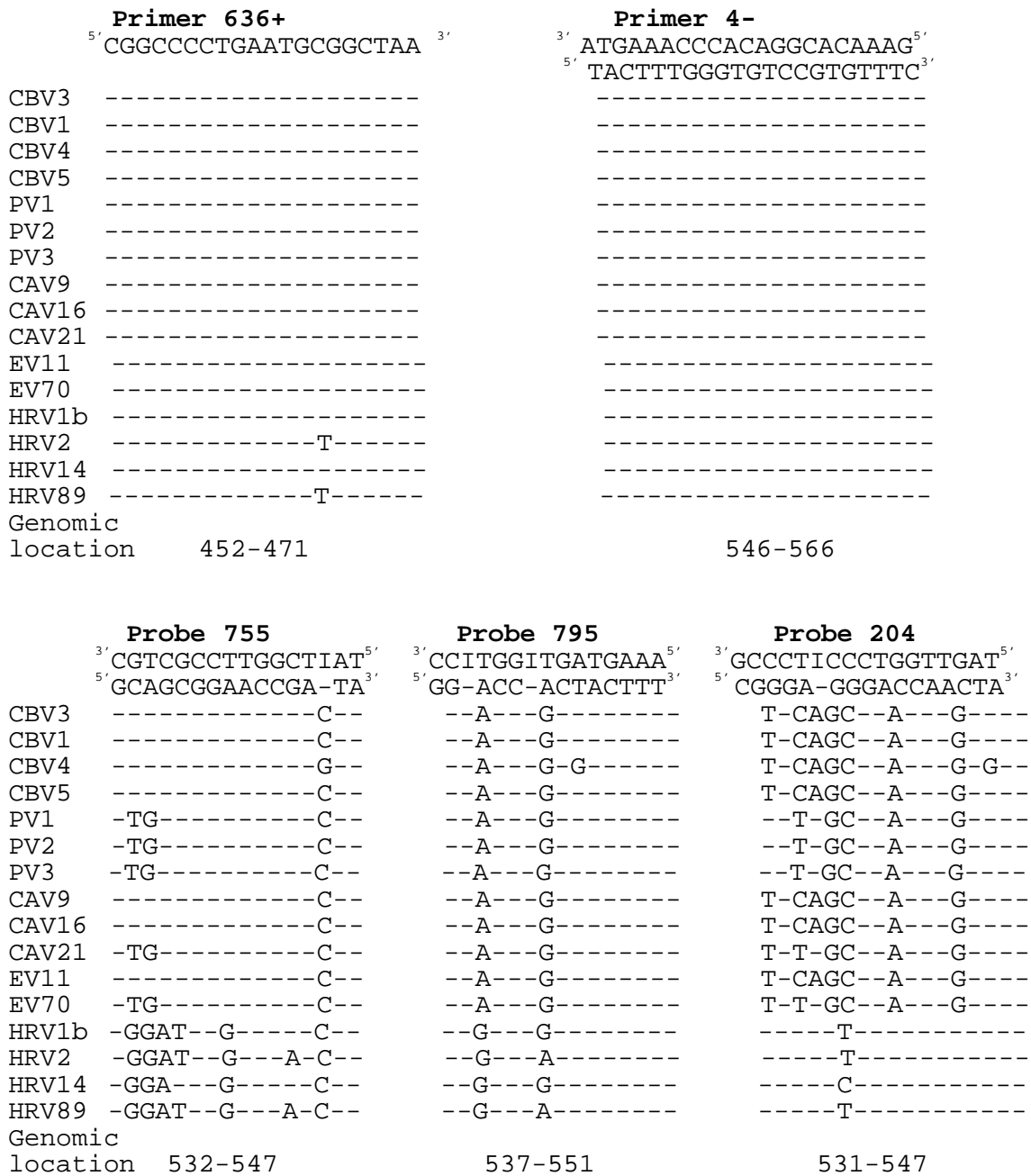
```
          Primer 636+                        Primer 4-
      5′CGGCCCCTGAATGCGGCTAA 3′          3′ATGAAACCCACAGGCACAAAG5′
                                          5′TACTTTGGGTGTCCGTGTTTC3′
CBV3    --------------------             --------------------
CBV1    --------------------             --------------------
CBV4    --------------------             --------------------
CBV5    --------------------             --------------------
PV1     --------------------             --------------------
PV2     --------------------             --------------------
PV3     --------------------             --------------------
CAV9    --------------------             --------------------
CAV16   --------------------             --------------------
CAV21   --------------------             --------------------
EV11    --------------------             --------------------
EV70    --------------------             --------------------
HRV1b   --------------------             --------------------
HRV2    -------------T------             --------------------
HRV14   --------------------             --------------------
HRV89   -------------T------             --------------------
Genomic
location       452-471                          546-566


          Probe 755              Probe 795               Probe 204
     3′CGTCGCCTTGGCTIAT5′    3′CCITGGITGATGAAA5′    3′GCCCTICCCTGGTTGAT5′
     5′GCAGCGGAACCGA-TA3′    5′GG-ACC-ACTACTTT3′    5′CGGGA-GGGACCAACTA3′
CBV3   -------------C--        --A---G--------       T-CAGC--A---G----
CBV1   -------------C--        --A---G--------       T-CAGC--A---G----
CBV4   -------------G--        --A---G-G------       T-CAGC--A---G-G--
CBV5   -------------C--        --A---G--------       T-CAGC--A---G----
PV1    -TG----------C--        --A---G--------       --T-GC--A---G----
PV2    -TG----------C--        --A---G--------       --T-GC--A---G----
PV3    -TG----------C--        --A---G--------       --T-GC--A---G----
CAV9   -------------C--        --A---G--------       T-CAGC--A---G----
CAV16  -------------C--        --A---G--------       T-CAGC--A---G----
CAV21  -TG----------C--        --A---G--------       T-T-GC--A---G----
EV11   -------------C--        --A---G--------       T-CAGC--A---G----
EV70   -TG----------C--        --A---G--------       T-T-GC--A---G----
HRV1b  -GGAT--G-----C--        --G---G--------       -----T----------
HRV2   -GGAT--G---A-C--        --G---A--------       -----T----------
HRV14  -GGA---G-----C--        --G---G--------       -----C----------
HRV89  -GGAT--G---A-C--        --G---A--------       -----T----------
Genomic
location  532-547              537-551               531-547
```

Figure 2. Alignment and genomic location (CBV3) of primer and probe sequences used in the study with representatives of entero- and rhinoviruses. Nucleotides differing from the primer or probe sequences are indicated. CBV, coxsackie B virus; PV, poliovirus; CAV, coxsackie A virus; EV, echovirus; HRV, human rhinovirus.

DISCUSSION

Detection of enterovirus infections in prospective study series

Importance of enteroviral analyses

The Finnish DiMe study initiated a new era in the investigation of the pathogenesis of type 1 diabetes, as it was the first in the world to include virological analyses of a prospective study series. One of its most important findings was the high frequency of serologically verified enterovirus infections in initially healthy children who progressed to clinical diabetes during follow-up (Hyöty et al. 1995). The risk effect of enterovirus infections was observed already years before the clinically overt disease - even in utero – and the infections were temporally associated with increases in ICA levels (Hyöty et al. 1995, Hiltunen et al. 1997). The risk effect of enterovirus infections occurring during pregnancy has also been reported in Swedish case-control studies (Dahlquist et al. 1995, Dahlquist et al. 1999). These findings suggested that enterovirus infections could already operate in the early phases of the autoimmune process.

The serological findings of Hyöty and coworkers (Hyöty et al. 1995) were confirmed in study II by enterovirus RNA detection in the same prospective serum samples of the DiMe study; the occurrence of enterovirus RNA proved to be a risk factor for later manifestation of type 1 diabetes (OR 7.1, 95 % CI 1.5 - 33.0), and RNA was detected years before the clinical manifestation of the disease, suggesting that the infections already play a role in the early phases of the pathogenesis. In the DIPP study (Report IV) it was found that enterovirus infections were more frequent among children who turned permanently positive for type 1 diabetes-associated autoantibodies than in matched controls, implying that the infections are a risk factor for beta-cell autoimmunity (p = 0.03). These findings consolidate the hypothesis that enteroviruses play a role in the autoimmune process leading to diabetes, warranting further enteroviral analyses in the context of prospective studies.

The main aim of the Nutritional Prevention of IDDM Intervention Trial is to study the effect of the elimination of cow's milk proteins in early infancy on the risk of type 1 diabetes. In the first pilot study the only infant who progressed to diabetes belonged to the intervention group, i.e. intact cow's milk proteins were eliminated from his diet. Enteroviral analyses were also included in the study, and the results were interesting: The infant who progressed to diabetes had two enterovirus infections during the first 12 months of life, which appeared to be the strongest enteroviral exposure observed in the study series. The first infection was followed by beta-cell autoimmunity, and diabetes was diagnosed shortly after the second. These findings suggest that in this infant's case the enterovirus infections may have contributed to the process leading to diabetes. This study also shows, that simultaneous evaluation of several possible risk factors, e.g. dietary factors and enterovirus infections in the Nutritional Prevention of IDDM Intervention Trial, may increase the sensitivity of the study to detect protective effects of an intervention protocol. Such a study design would also allow for evaluation of possible interactions between different environmental factors.

Demand for high sensitivity in the detection of enterovirus infections

Detection of enterovirus infections in prospectively followed subjects is not a simple task. Enteroviruses are among the most difficult groups of viruses when it comes to serological detection, because this group comprises as many as 64 serotypes. The sensitivity of serological assays can be enhanced by using broadly reacting enteroviral antigens and by combining the results of several assays (Samuelson et al. 1993, Swanink et al. 1993). Accordingly, several serological assays were used in our studies, most of them designed to detect antibodies against as many enterovirus serotypes as possible. It is nevertheless probable that not all enterovirus infections were detected in the serological assays used.

Enterovirus RNA detection was used to further improve the diagnostic sensitivity. One advantage of our RT-PCR method is that the assay detects RNA of all known enteroviruses, as the amplified sequence is common to all. However, not all enterovirus infections necessarily cause viremia, and the assay result is dependent on the moment of sampling, since the viremic period is believed to last only a few weeks

at most. It is thus evident that only a fraction of enterovirus infections can be detected by RT-PCR study of serum. The RT-PCR method used here detects all enteroviruses, but also all rhinoviruses (Hyypiä et al. 1989). Hence, hybridization of the amplification products is required for specific detection of enterovirus RNA. Gel electrophoresis of the amplification products followed by southern-blot hybridization - a method used in study II – is rather laborious and cumbersome, and therefore not particulary suitable for analysing large series. In report VI we describe a new hybridization method applicable in large-scale screening due to easy performance on a microtiter plate: entero- and rhinoviral amplification products are specifically identified using liquid-phase hybridization with lanthanide chelate-labeled probes and time-resolved fluorescence. This RT-PCR hybridization assay is highly sensitive, since it was able to detect an amplification product corresponding to 0.015 fg of RNA. The method was used in study IV, and has been adopted in the routine diagnosis of entero- and rhinovirus infections in three university hospital laboratories in Finland.

In conclusion, assessment of the role of enterovirus infections in the pathogenesis of type 1 diabetes is difficult or even impossible if diagnostic sensitivity is low. The sensitivity can be improved by short sample intervals and a combination of various assays. In addition to serological assays and RNA detection, virus isolation would also be useful, as the isolated virus would allow evaluation of the possible diabetogenic properties of the virus strain detected.

Elimination of possible HLA-related bias

The same HLA alleles may mediate susceptibility to both diabetes and enterovirus infections. If so, then results of case-control studies without HLA-matching can be questioned: they may have been biased by the influence of HLA type on the immunological response against enteroviruses or on the severity of enterovirus infections.

In study II the prospectively followed case and control children were not matched for HLA. However, even if children with diabetes-related HLA susceptibility alleles were more prone to viremic enterovirus infections than other children, the temporal relationship between the infections and increases in autoantibody levels can hardly be explained by HLA-related bias. In study IV the possible confounding effect of

the diabetes-associated HLA-DQB1 risk alleles was eliminated by matching, and yet the frequency of enterovirus infections was higher in the autoantibody-positive cases than in the autoantibody-negative controls. This suggests a true relationship between enterovirus infections and beta-cell autoimmunity.

The idea of matching is to eliminate possible confounding factors. Study groups are most frequently matched for age and gender, and in study IV we used additional matching for the time of birth and HLA-DQB1 alleles. However, the pathomechanism of type 1 diabetes appears to be complex and multifactorial, and the possible interplay between various risk factors is poorly understood. In this kind of situation extensive matching may lead to overmatching, which weakens the sensitivity of the study to detect risk effects associated with the variable tested (Rothman 1986). The study protocol should always be carefully considered in order to avoid both insufficient matching and overmatching, and this is often problematic in the case of type 1 diabetes.

Pathogenetic mechanisms underlying enterovirus-induced type 1 diabetes

Cross-reactivity between CBV4 2C and GAD proteins

One possible mechanism whereby enteroviruses could promote the destructive process of beta cells is immunological cross-reactivity between viral and beta-cell proteins. One potential target for such cross-reactivity is the homologous sequence identified in the CBV4 2C protein and human GAD_{65} (Kaufman et al. 1992). We showed in study I that rabbit antibodies raised against CBV4 2C-derived peptides cross-react with GAD_{65} peptides derived from the homology region and *vice versa*. Two 2C peptide antisera bound to the GAD_{65} molecule, and one of these sera also bound to CBV-infected cells. These results demonstrate that the region in CBV4 2C and GAD_{65} with sequence homology can induce cross-reactive antibodies in peptide immunization.

Antibodies against the peptides derived from the region with sequence homology were induced during enterovirus infections in prediabetic subjects, and were also shown to be present in patients with newly diagnosed type 1 diabetes (Report I). However, similar peptide antibody responses were seen during enterovirus infections in control children who did not progress to diabetes, and the diabetic patients' matched non-diabetic control children had levels of peptide antibodies equal to those in the patients. Accordingly, antibody reactivity against the homology region was not associated with an increased risk of type 1 diabetes. This is in line with a report by Richter and coworkers (Richter et al. 1994) showing an equal frequency of antibodies against CBV4 2C protein in GADA-positive patients with type 1 diabetes and GADA-negative non-diabetic subjects, but in conflict with another report showing that antibodies against peptides carrying the homology sequence were more frequent in type 1 diabetes patients than in non-diabetic controls (Hou et al. 1994).

In study II detection of enterovirus RNA was temporally associated with increases in ICA and GADA levels but not in IAA or IA-2A levels. On the other hand, in study V enterovirus infections were associated mostly with induction of IAA. Accordingly, the possible enterovirus-induced beta-cell autoimmunity does not seem to be targeted exclusively against GAD.

Our findings do not confirm the role of antibody cross-reactivity targeted to the homologous region in 2C and GAD as an important pathomechanism in type 1 diabetes. This does not exclude the possibility that the homology sequence may induce T-cell cross-reactivity, as suggested both in human studies (Atkinson et al. 1994) and studies in NOD mice (Kaufman et al. 1993, Tisch et al. 1993), although conflicting T-cell study results have also been reported (Atkinson 1997).

Risk associated with high frequency of enterovirus infections

In studies II and IV enterovirus infections were more frequent during the prospective follow-up of children who progressed to type 1 diabetes and permanent beta-cell autoimmunity, respectively, as compared to their controls. In study III the child who progressed to diabetes had been more frequently exposed to enterovirus infections than other children in the cohort. Thus, all these studies consistently suggest that

frequent occurrence of enterovirus infections is associated with beta-cell autoimmunity and clinical type 1 diabetes.

The occurrence of enterovirus infections and beta-cell autoimmunity and/or diabetes in the same individuals does not automatically imply a causal relationship between the infections and the pathogenesis of type 1 diabetes. However, in all the aforementioned studies (II, III and IV) a temporal association between the infections and enhancement of beta-cell autoimmunity was observed: in II the infections were temporally associated with increases in the levels of ICA and GADA, in III beta-cell autoantibodies appeared soon after the first enterovirus infection, and in IV enterovirus infections were associated with seroconversion to autoantibody positivity within 6 months from the infection. These findings are in line with a previous report indicating a temporal association between enterovirus infections and the appearance of ICA (Hiltunen et al. 1997). Temporal association is one of the key characteristics of a causal relationship, and hence the findings support the hypothesis that enterovirus infections may induce beta-cell autoimmunity.

Enteroviruses are among the most frequent pathogens in humans. It is thus evident that most enterovirus infections are not associated with progression to diabetes. The risk associated with a high frequency of enterovirus infections can derive from at least two sources. Firstly, a high infection frequency includes an increased probability of exposure to a diabetogenic enterovirus strain. Secondly, multiple infections may be required for induction and/or progression of beta-cell autoimmunity.

Viremic enterovirus infections

Detection of enteroviral RNA in serum is a marker of viremia, which allows systemic spread of the virus to susceptible target organs, including the pancreas. In study II detection of enterovirus RNA was associated with coinciding increases in autoantibody levels as well as later manifestation of clinical diabetes. In study IV seroconversion to autoantibody positivity was more prominently associated with viremic than serologically documented enterovirus infections. These results may indicate that viremia is an important risk factor underlying the induction of beta-cell autoimmunity. This might further imply that local enterovirus infection in beta cells or other pancreatic cells mediates beta-cell autoimmunity. On the other hand, the

primary site of enterovirus replication is the local lymphoid tissue of the respiratory and gastrointestinal tracts, and the frequent viremia might reflect an impaired or aberrantly functioning gut immune system which allows the systemic spread of the virus from the primary replication site. According to this hypothesis the key events in the induction of autoimmunity may already take place in the gut, and viremia would be a mere reflection of these events without pathogenetic significance.

It has been suggested that detection of enterovirus RNA in serum may be a marker of a chronic enterovirus infection, e.g. in pancreatic cells. The findings in studies II and IV do not support such a hypothesis, as enterovirus RNA persistence in serum was not observed, and the sequence analyses indicated that in a repeatedly RNA-positive child (Report II) the infections were caused by genetically distinct enterovirus strains. However, our findings do not rule out the possibility of a local chronic beta-cell infection, as it is not known whether such an infection could produce sufficient quantities of viruses in the circulation to be detected by RT-PCR.

Intrauterine enterovirus exposure

Enterovirus infections during pregnancy have been suggested to be risk factors for type 1 diabetes in the offspring (Dahlquist et al. 1995, Hyöty et al. 1995, Dahlquist et al. 1999). In study IV no difference was observed between the case and control groups in the frequency of enterovirus infections during pregnancy. However, the number of subjects in study IV was fairly small, and the end-point was autoantibody-positivity, whereas in the aforementioned reports the end-point was type 1 diabetes. Further studies are needed to evaluate the hypothesis.

Enterovirus epidemiology in Finland and Lithuania

One of the characteristics of type 1 diabetes is a marked country-to-country variation in incidence and prevalence which may be due to both differences in the genetic predisposition to the disease in various populations and variations in the influence of environmental factors. Finland has the highest incidence of type 1 diabetes in the world, whereas in Lithuania the incidence is substantially lower (35/100 000 vs. 7/100 000 in 0-14-year-old children in 1983-1992, respectively) (Padaiga et al. 1997). If

enterovirus infections were a major etiological factor in the pathogenesis, one could expect to see differences in the enterovirus epidemiology between the two countries.

In study V we found significantly higher enterovirus antibody levels in Lithuanian children than in their age- and sex-matched Finnish counterparts, which implies a higher frequency of enterovirus infections in Lithuania. Accordingly, the results suggest an inverse relationship between the frequency of enterovirus infections and the incidence of type 1 diabetes in Lithuania and Finland. On the other hand, enterovirus IgG antibody levels were higher in the ICA-positive than in the ICA-negative Finnish children, suggesting that enterovirus infections are associated with ICA positivity. Whatever the explanation for this discrepancy is, the results show that at population level the association between enterovirus infections and type 1 diabetes is not straightforwardly dose-dependent, *i.e.* a high frequency of enterovirus infections does not necessarily lead to an increased incidence of type 1 diabetes.

The Lithuanian children were also significantly more often seropositive for hepatitis A virus than the Finnish children (Report V). The epidemiology of viruses with fecal-oral transmission such as enteroviruses and hepatitis A virus is highly dependent on climatic and socio-economic factors. Thus, the climatic differences between Finland and Lithuania may partly explain the different epidemiology of these infections. In addition, the socio-economic status differs considerably between the two countries, as e.g. the gross national products of Lithuania and Finland were $1,350 *vs.* $18,850 *per capita*, respectively, at the time of serum collection. Improvement in general socio-economic conditions has led to a decreased incidence of hepatitis A virus infections in many countries; in Finland these infections have been almost nonexistent since the 1970s (Pohjanpelto and Lahdensivu 1984, Melnick 1995). It is probable that the same factors have changed not only the epidemiology of hepatitis A virus, but also that of other viruses transmitted via the fecal-oral route, including enteroviruses.

Epidemiological changes can sometimes have unexpected consequences, as is known from one particular enterovirus group, namely polioviruses. The improvement in hygienic conditions at the end of the 19th century resulted in a new epidemiological situation: the total frequency of poliovirus infections decreased, but the clinically overt polio, i.e. paralytic disease, became more common. Thus, secular changes in the

epidemiology of non-polio enteroviruses may have an impact on the occurrence of beta-cell damage possibly mediated by these viruses.

In prospective studies enterovirus infections have been shown to increase the risk of type 1 diabetes especially when occurring in utero or in infancy (Dahlquist et al. 1995, Hiltunen et al. 1997). During these periods of life maternal antibodies are needed to compensate for the immaturity of the fetal and neonatal immune system. However, if the mother lacks an antibody repertoire against enteroviruses, the infant will be poorly protected against these infections. This may be the case in Finland and in other countries with a high socio-economic standard: a low frequency of enterovirus infections has led to poor maternal immunity against these infections. Due to the high socio-economic standard the total frequency of enterovirus infections may have also decreased among infants, but the occurrence of severe enteroviral disease may have increased since the infants lack maternal enterovirus antibodies. This may partly explain why the most prominent increase in the incidence of IDDM during the last decade has been observed in the youngest group of children under 5 years of age (Tuomilehto et al. 1995).

SUMMARY

Enteroviruses are common pathogens causing a wide range of diseases in humans. The present study series was designed to evaluate the possible involvement of enterovirus infections in the pathogenesis of type 1 diabetes.

The occurrence of enterovirus infections was studied during a prospective follow-up of children who progressed to permanent beta-cell autoimmunity or type 1 diabetes and of their control subjects. A consistent finding in these studies was an association between frequent occurrence of enterovirus infections and progression to beta-cell autoimmunity or clinical type 1 diabetes. In addition, a temporal association between enterovirus infections and induction of beta-cell autoimmunity was observed.

Enterovirus RNA detection by RT-PCR was included in the assay repertoire to increase its sensitivity to detect enterovirus infections, and also to study the occurrence of viremia. A highly sensitive and specific hybridization assay for the detection of the amplified enteroviral sequences was developed. The assay is well suited for large-scale screening by merit of its easy performance on a microtiter plate format. Detection of enterovirus RNA in serum was associated with increased risk of diabetes.

Enterovirus antibody levels were measured in Finnish and Lithuanian schoolchildren to elucidate the epidemiology of enterovirus infections in these populations. The Lithuanian children had higher levels of enterovirus antibodies, suggesting a higher frequency of enterovirus infections in Lithuania as compared to Finland. This demonstrates that at population level a high frequency of enterovirus infections does not necessarily lead to a high incidence of type 1 diabetes. The low frequency of enterovirus infections in Finland may increase the risk of enterovirus infections in utero and in infancy due to a lack of transplacentally transferred maternal antibodies.

Antibody cross-reactivity between CBV4 2C protein and human GAD is one possible pathogenetic mechanism in type 1 diabetes. Cross-reactive antibodies were induced in peptide immunization, and antibodies against these peptides were induced during enterovirus infections, but antibody reactivity against the peptides was not

associated with an increased risk of type 1 diabetes. Our findings do not confirm the role of antibody cross-reactivity targeted to this homology region as an important pathomechanism in type 1 diabetes, but the results do not exclude the possibility of T-cell cross-reactivity.

In conclusion, the findings in the present series consolidate the hypothesis that enteroviruses can play a role in the autoimmune process leading to diabetes. Further enteroviral analyses in the context of prospective studies are warranted to shed light on the still obscure pathomechanism of enterovirus-induced diabetes, and to assess whether vaccination against enterovirus infections would be an applicable approach in the prevention of type 1 diabetes.

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