Diabetologia (1998) 41: 40--46

Diabetologia

Molecular mimicry in diabetes mellitus: the homologous domain in coxsackie B virus protein 2C and islet autoantigen GAD_{65} is highly conserved in the coxsackie B-like enteroviruses and binds to the diabetes associated HLA-DR3 molecule

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Summary It has been proposed that molecular mimicry between protein 2C (p2C) of coxsackie virus B4 and the autoantigen glutamic acid decarboxylase (GAD_{65}) plays a role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM). In this study we show that the amino acid sequence of p2C which shares homology with a sequence in GAD_{65} (PEVKEK), is highly conserved in coxsackie virus B4 isolates as well as in different viruses of the subgroup of coxsackie B-like enteroviruses. These are the most prevalent enteroviruses and therefore exposure to the mimicry motif will be a frequent event throughout life. Presentation of the homologous peptides by HLA molecules is essential for T-cell reactivity. Therefore, we tested whether the PEVKEK motif can bind to the IDDM-associated HLA-DR1, -DR3 and -DR4 molecules. Synthetic peptides with sequences derived from p2C and GAD_{65} did bind to HLA-DR3 but not to HLA-DR1 or -DR4. Replacement of amino acids within the motif showed that the PEVKEK motif binds specifically to HLA-DR3. Moreover, both p2C and GAD_{65} peptides bind in the same position within the peptide binding groove of the DR3 molecule which is an essential requirement for T-cell cross-reactivity. The results support molecular mimicry between p2C of coxsackie B-like enteroviruses and GAD_{65}. However, this molecular mimicry may be limited to the HLA-DR3 positive subpopulation of IDDM patients. [Diabetologia (1998) 41: 40--46]

Keywords Coxsackie B virus, peptide binding, HLA-DR, molecular mimicry, IDDM.

Despite a growing knowledge of insulin-dependent diabetes mellitus (IDDM), its aetiology is still obscure. Both, genetic and environmental factors are involved, and autoimmune mechanisms play a crucial role in the pathogenesis [1, 2]. Studies in transgenic mice have renewed interest in the potential role of viruses in causing autoimmunity in diabetes [3, 4]. Strong candidates are the enteroviruses, particularly the coxsackie B viruses (CVB). The association of these viruses with IDDM is based on isolation of CVB from the pancreas in fatal cases of diabetes [5, 6] and on epidemiological studies that showed an increased incidence of IDDM after epidemics caused by enteroviruses [7, 8]. It has also been reported that antibodies to CVB are more prevalent in patients at onset of IDDM, as compared to matched control subjects [9, 10]. Serological case-control studies indicated intrauterine exposure to enteroviruses to be a risk factor for childhood onset of IDDM [11, 12]. Furthermore it has been shown that patients who acquired IDDM had a higher rate of enteroviral infections in the years before onset of the disease as compared to non-affected siblings or unrelated control subjects [12].

The mechanism by which enteroviruses may contribute to destruction of pancreatic beta cells remains to be established. The virus may destruct beta cells

Received: 24 June 1997 and in revised form: 9 September 1997

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Abbreviations: p2C, Protein 2C; GAD_{65}, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes mellitus; CVB, coxsackie virus B, PV, poliovirus.

either directly by a lytic infection, or indirectly by a virus-directed immune response against infected beta cells [13]. Beta cells may also specifically be damaged by nitric oxide radicals and cytokines that are produced as a consequence of viral infection and subsequent inflammation, a process of so called "innocent bystander killing"" [14]. Alternatively, a mechanism, called molecular mimicry, may play a role in induction of autoimmunity, ultimately leading to destruction of pancreatic beta cells [15]. Molecular mimicry is based on a sequence homology between a foreign antigen, e.g. a viral protein, and a host protein. It is postulated that immune reactivity against the virus can lead to a cross-reactive response to the homologous sequence of the host protein [3, 4, 15]. In the concept of molecular mimicry both genetic factors such as HLA association and viruses as environmental factors, are integrated in a single aetiologic concept.

In 1992 it was reported that a homology exists between the 65kDa isoform of glutamic acid decarboxylase (GAD_{65}) and p2C, a non-structural protein of CVB4 [16, 17]. A complete homology was reported for 10 amino acids, and similarity for 9 residues. GAD_{65} is mainly expressed in the pancreas and central nervous system [18], and is a dominant autoantigen that has a predictive value for progression to IDDM [16, 17, 19]. Autoantibodies against GAD_{65} precede the onset of diabetes and are found in up to 80% of newly diagnosed patients, but rarely in healthy control subjects [19, 20]. In a study on antibody reactivity to GAD_{65} and to synthetic p2C peptides, evidence was obtained in support of a cross-reactive antibody response [21]. In two other studies, however, cross-reactivity could not be demonstrated [22, 23]. T-cell responses to GAD_{65} peptides that share homology with p2C, were found in newly diagnosed diabetic patients [24] but also in healthy unrelated control subjects [25]. In one of these studies, T-cell reactivity was also found against the corresponding p2C peptides [24]. Formal evidence for T-cell cross-reactivity was not obtained, however, because the studies were performed with bulk cultures of peripheral blood mononuclear cells and not with T-cell clones [24]. Cross-reactivity between GAD_{65} and p2C has furthermore been demonstrated in the non-obese diabetes (NOD) mouse where it appeared to be linked with an MHC allele that is associated with susceptibility for diabetes [26]. Presentation of the mimicry motif by MHC molecules is an essential requirement for T-cell cross-reactivity. In humans, binding of the mimicry motif to HLA molecules that are associated with IDDM, has not been reported so far.

Enteroviruses are RNA viruses which replicate at a high mutation rate [27] and show a considerable genetic variation [28]. As a consequence, the mimicry motif might be found only in a few isolates, thus restraining the frequency of exposure to this sequence. To address this question we analysed conservation of the mimicry sequence among different CVB-like enteroviruses. Based on the sequences that were obtained, binding of the viral peptides as well as the GAD_{65} peptide to HLA-DR1, -DR3, and DR4 alleles was studied, which are associated with IDDM.

Materials and methods

Virus isolates. Ten CVB4 virus strains, isolated in the Netherlands between 1974 and 1990 were obtained from the National Institute of Public Health and Environmental Protection, Bilthoven (RIVM), The Netherlands. Another 12 enteroviruses of different strains were isolated in the Academic Hospital Nijmegen between 1981 and 1994 and were routinely serotyped with a set of typing sera from the RIVM [29]. The enteroviruses were subdivided into the group of polio-like (PV-like) viruses and CVB-like viruses as classified by Hyypiä et al. [30]. The clinical isolates belonged to the CVB-like group, including 3 CVB1 isolates, 2 CVB2, 2 CVB3, 1 CVB5, 3 CVA9 and 1 echovirus 9 isolate. The isolates were randomly selected. The echovirus 9 isolate was obtained from a patient with clinical symptoms of IDDM.

Reverse transcription-polymerase chain reaction (RT-PCR) and sequence analysis. Viruses were propagated in Buffalo Green Monkey kidney cells. After complete cytopathic effect was reached the cultures were frozen and thawed three times. RNA was extracted by using a single extraction procedure with guanidinium thiocyanate-phenol-chloroform according to the method of Chomczynski and Sacchi [31]. Reverse transcriptase reactions and PCR were performed as previously described [32]. Briefly, cDNA was synthesised in a 20 µl reaction mixture containing 75 mmol/l KCl, 50 mmol/l Tris-HCl pH 8.3, 3 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol, 0.2 mmol/l of each deoxynucleoside triphosphate (Boehringer Mannheim, Mannheim, Germany), 50 pmol of the degenerated antisense primer (5′TCGTCTCCGTGGCGATGGCTNAANCA 3′), 5 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wisconsin, USA) and extracted RNA. After incubation at 37 °C for 60 min, 80 µl of the PCR mixture was added. The PCR mixture contained 50 mmol/l KCl, 10 mmol Tris-HCl pH 8.9, 3.6 mmol/l $MgCl_2$, 0.2 mmol/l of each deoxynucleoside triphosphate, 100 µg of bovine serum albumin/ml, 80 pmol of sense primer (5′GCATTTGGACTTGAACTGTATG 3′), 40 pmol of antisense primer and 0.2 units of SuperTaq DNA polymerase (HT Biotechnology, Cambridge, UK). RNA-cDNA hybrids were denatured at 94 °C for 5 min. The amplification was performed in 40 cycles consisting of denaturation for 1 min at 94 °C, primer annealing for 1 min at 42 °C and elongation for 1 min at 72 °C. The reactions were analysed by electrophoresis in 1.5% agarose gels, and the product was purified from low melting point agarose with the Wizard Clean Up DNA purification system (Promega) according to the instructions of the manufacturer. DNA sequence analysis was performed using the Ampli Cycle sequencing kit according to the manufacturer's instructions (Perkin-Elmer, Foster City, California, USA). The sequencing primer was selected in p2C position 4248-4230 (5′AACCCTACCGCGCGAGACGAG 3′).

Sequence comparisons. Sequences obtained from the virus isolates and published enteroviral sequences were analysed for presentation and conservation of the sequence that encodes for the PEVKEK motif. Analysis and alignments of the

sequences were performed using the sequence analysis software package of the Genetic Computer Group at the University of Wisconsin, implemented on a UNIX computer. Amino acid sequences were deduced from the nucleic acid sequences and pairwise compared to the CVB4 sequence described by Jenkins et al. [33].

Peptide synthesis. Peptides were made on an ABIMED422 synthesiser (ABIMED, Langenfeld, Germany) using the simultaneous multiple peptide synthesis method according to Gausepohl et al. [34]. The purity of the peptides was checked on an Rp C18 HPLC (LiChrosper, 60RP-select B 5 mm, 250 × 4 mm; Merck, Darmstadt, Germany) and shown to be routinely over 75 %.

Generation of purified HLA-DR molecules. As a source of HLA-DR molecules Epstein-Barr virus-transformed B-lymphoblastoid cell lines homozygous for HLA-DR were used: LG2.1 (DRB1*0101, DR1), HAR (DRB1*0301, DR3), and BSM (DRB1*0401, DR4). Cells were cultured in RPMI 1640 (GIBCO, Paisley, UK), supplemented with 2 mmol/l L-glutamine (GIBCO), 100 U100 mg/ml penicillin/streptomycin solution (GIBCO), and 10 % heat-inactivated fetal calf serum (GIBCO). Cells were lysed at a concentration of 10^8 cells/ml in 50 mmol/l Tris-HCl, pH 8.5, containing 2 % Renex (Accurate Chemicals and Scientific Corp., Westbury, N. Y., USA), 150 mmol/l NaCl, 5 mmol/l EDTA, and 2 mmol/l phenylmethylsulphanylfluoride. The lysates were cleared of nuclear and other debris by centrifugation at 10 000 × *g* for 20 min. HLA-DR molecules were purified by affinity chromatography as described previously [35].

HLA-DR-peptide binding assay. The analysis of peptide binding to purified HLA-DR molecules was performed using N-terminally fluorescence-labelled standard peptides [35]. For each of the HLA molecules different fluorescent-labelled standard peptides were used: HA p307--319 (PKYVKQNTLKLAT, DR1), hsp65 p3--13 (KTIAYDEEARR, DR3) and HA p307--319 Y→F (PKFVKQNTLKLAT, DR4). In preliminary experiments, each DR preparation was titrated in the presence of 100 fmol standard peptide to determine the DR-concentration necessary to bind 10--20 % of the total fluorescent signal. All subsequent inhibition assays were then performed at this concentration. Peptides, of which the DR binding capacity was to be determined, were added to HLA-DR molecules simultaneously with the standard peptide. The HLA-DR-peptide complexes were separated from free peptide by gel filtration on a Synchropak GPC 100 column (250 × 4.6 mm; Synchrom, Inc., Lafayette, Ind., USA). Fluorescence emission was measured at 528 nm on a Jasco FP-920 fluorescence detector (B & L Systems, Zoetermeer, The Netherlands). The percentage of labelled peptide bound was calculated as the amount of fluorescence bound to MHC divided by the total fluorescence. Peptides were typically tested at concentrations ranging from 66 µmol/l--6.6 nmol/l. The concentration of peptide yielding 50 % inhibition of fluorescence (IC_{50}) was deduced from a dose-response curve. A ratio was calculated by dividing the IC_{50} obtained with the test peptide by the IC_{50} of the corresponding standard peptide. The IC_{50} of the different standard peptides were: HA p307--319 (HLA-DR1): 0.01 mmol/l, heat shock protein 65p3--13 (HLA-DR3): 0.06 mmol/l; HA p307--319 YrF (HLA-DR4): 0.2 mmol/l. Binding was determined in at least three separate experiments, for each peptide-HLA-DR combination.

Results

The *PEVKEK sequence is conserved among CVB-like enteroviruses.* To determine the conservation of the CVB sequence that shares similarity with GAD_{65}, we determined the amino acid sequence 1129--1154 of 10 different CVB4 and 11 different CVB-like enteroviruses. The sequences were compared to the published CVB4 sequence [33]. The homology of 100 nucleotides in the p2C gene was 79 % (74 to 85 %). The p2C sequences of the CVB4 strains had the same degree of homology as the sequences of different CVB-like strains. The deduced amino acid sequence in this p2C region turned out to be highly conserved among the CVB-like enteroviruses. A homology was observed varying from 88 to 100 % (mean 94 %). The polio-like enteroviruses however, share less homology with the CVB4 sequence. The amino acid sequence homology in the PEVKEK region was 59 %, and no significant sequence homology exists with GAD_{65}. In Table 1 amino acid alignments of GAD_{65} and the CVB isolates as well as the polio-like sequences are shown. The PEVKEK motif was found in all isolated viruses. Within the PEVKEK motif a single substitution of lysine into arginine (K→R) was observed in two out of ten CVB4 isolates. This amino acid substitution could also be found in the CVB3, CVA9 and echo 11 isolates.

The *PEVKEK-motif binds to HLA-DR3.* To test whether the homologous sequences bind to HLA-DR molecules, peptides with a length of 20 amino acids, deduced from the GAD_{65} protein and from the corresponding sequences of CVB4 p2C, CVB3 p2C, and from poliovirus 3 p2C (Table 2) were generated. The peptides were tested for binding to HLA-DR1, -DR3, and -DR4. Binding capacities for the different peptides are given as ratio of the IC_{50} of the test peptide compared to the IC_{50} of the standard peptide. Both GAD_{65} and CVB p2C 20-mer peptides bound to HLA-DR1, -DR3, and DR4 (Table 2). The poliovirus p2C peptide, which was included for comparison, bound to all three HLA-DR molecules. Peptide binding to HLA-DR alleles was confirmed by evaluation of HLA-DR binding-motifs using a computer model [36] adapted for HLA-DR3 and HLA-DR4 [37, 38]. An HLA-DR3 binding motif was found that includes the PEVKEK sequence and some amino acids N-terminal of both GAD_{65} and p2C. An HLA-DR4 motif was found adjacent to the PEVKEK motif. The PEVKEK motif as such however, does not fit with the HLA-DR4 motif. The PEVKEK motif was not suspected to bind to HLA-DR1 either [37]. To test whether the PEVKEK motif, rather than adjacent sequences, binds to HLA-DR1, -DR3, and -DR4, smaller peptides of 12 amino acids containing PEVKEK were generated and tested for binding. In contrast to the 20-mer peptides, the smaller peptides

Table 1. Amino acid sequence comparisons of enterovirus isolates.

GAD_{65}	AMMIARF**K**MF**PEVKEK**GMAA**L**P**RL**
Coxsackie B-like enteroviruses	
CVB4	FIEWLKV**K**IL**PEVKEK**HEF**L**S**RL**KQL
CVB4 '74	.....N...H.
CVB4 '85	.....R.....
CVB4 '88	.....R.....N.....
CVB4 '88	.....N.....
CVB4 '89	.....N.....
CVB4 '89	.....M...
CVB4 '89	.....N.....
CVB4 '90	.....M...
CVB4 '90	.....N.....
CVB4 '90	.....N.K...
CVB1	.....N.....
CVB1 '81	.....N.....
CVB1 '84	.....N.....
CVB1 '94	.....N.....
CVB2 '93	.....I.....N.....
CVB2 '95	.....NK....
CVB3	.....R.....N.....
CVB3 '94	....F.....R.....N.....
CVB3 '94	.....N.....
CVB5	..D.....N.....
CVB5 '92	..D.....N.....
CVA9	.....R.....N.....
CVA9 '94	L.....R.....N.....
CVA9 '94	.....R.....N.....
CVA9 '94	LV.....N.....
Echo 11	.....R.....N.....
Echo 9 '97	..D.....V.....T.....
Polio-like enteroviruses	
CVA4	..D...ER.I..A.D.V..ITK....
PV2	..D...E..I.QARD.L..VTK....
PV1	..D...E..I.QARD.L..VT..R..
PV3	..D..RE..I.QARD.L..VTK....
CVA2	.VD...C..I..A.D.V...TK....

Deduced amino acid sequences of protein 2C 28--53 compared to the published CVB4 strain [33]. Sequences of the various clinical enterovirus isolates are indicated by year of isolation. Non-specified sequences are obtained from the European Molecular Biology library (EMBL) database. Identical amino acid residues indicated by '.'; non-homologous amino acid residues are indicated by their single letter code. Amino acids identical with the corresponding human GAD_{65} sequence (amino acid 250--277) are depicted in bold

did not bind to HLA-DR4 and -DR1. However, binding affinity to HLA-DR3 was found with comparable affinity as was found with the 20-mers (Table 2). In order to investigate whether PEVKEK actually bound in the peptide binding groove of HLA-DR3, the suspected anchor residue glutamic acid on position 2 in the PEVKEK motif (E_2) was replaced by residues that influence binding affinity. Replacement of E_2 to aspartic acid ($E_2 \rightarrow D$), which is predicted to improve binding with HLA-DR3 indeed resulted in a 25-fold increase of binding affinity, whereas binding was abolished after replacement to valine ($E_2 \rightarrow V$). These results prove that E_2 functions as anchor residue in HLA-DR3 binding and confirmed the computer predictions. From the HLA-DR3 binding motif in PEVKEK it could be ascertained that residue K_4 also functions as an anchor residue in binding to HLA-DR3. In different CVB-like strains the K_4 was mutated to R (PEV**R**EK). Both, K_4 and R_4 are allowed for binding in the pocket of HLA-DR3 [38]. Therefore, this mutation was predicted not to change binding affinity, which was experimentally confirmed (Table 2). The PEVKEK motif contains two of the anchor residues needed for binding to HLA-DR3. In addition the GAD_{65} and p2C sequences contain a large hydrophobic residue N-terminally to PEVKEK that fits in the first pocket of HLA-DR3.

Discussion

In this study it is shown that the viral p2C sequence with homology with GAD_{65} is highly conserved in the group of CVB-like enteroviruses. In about 20 % of these viruses a single amino acid substitution ($K \rightarrow R$) within the PEVKEK motif could be found. Based on computer predictions both K and R are permitted as anchor residues in HLA-DR3 binding which could be confirmed by binding experiments (Table 2). Anchor residues are usually buried inside the pockets of the peptide binding groove and not exposed to the T-cell receptor. Therefore this sequence variation may even be without consequences for T-cell recognition. The binding ratio of the poliovirus p2C peptide appeared to be significant. The possibility of cross reactivity, however, is not likely since homology with GAD_{65} is very limited. The CVB-like enteroviruses are highly prevalent comprising over 90 % of the clinical isolates that are serotyped in the Netherlands. This implies that exposure to the mimicry motif will occur frequently throughout life.

In spite of frequent exposure to the mimicry motif, antibody responses to GAD_{65} are rarely found in subjects with no risk of developing IDDM [19, 20]. Apparently, other factors such as immune regulatory mechanisms and a genetic background may restrict cross-reactive responses. With regard to susceptibility to diabetes, the genetic association of HLA with IDDM might be important. HLA molecules may play a role in susceptibility or protection by presentation of peptides that either induce or prevent autoimmune responses. For the concept of molecular mimicry the homologous sequences are required to be presented by HLA molecules in a way that cross-reaction can occur. As discussed below, the mimicry sequence bound only to HLA-DR3 and not to HLA-DR1 or -DR4, which suggests that a putative role of enteroviral infections by means of molecular mimicry will indeed be restricted by HLA-DR alleles. In support of this finding an association has been reported of diabetes with CVB-like virus infections in HLA-DR3 positive, rather than in HLA-DR3 negative patients [39].

Table 2. Binding capacities of a GAD_{65} peptide and the homologous enteroviral p2C peptides to HLA-DR1, -DR3 and -DR4

20-mer peptides		HLA-DR1	HLA-DR3	HLA-DR4
GAD_{65}	AMMIARFKMFPEVKEKGMAA	259	267	100
CVB4 p2C	FIEWLKVKILPEVKEKHEFL	187	275	99
CVB3 p2C	FIEWLKVKILPEVREKHEFL	136	98	83
PV3 p2C	FIDWLRERIIPQARDKLEFV	240	11	55
12-mer peptides				
GAD_{65}	FK**M**FP**E**V**K**EKGM	>	300	>
CVB4 p2C	VK**I**LP**E**V**K**EKHE	>	250	>
CVB3 p2C	VKILPEVREKHE	>	280	>
PV3 p2C	ERIIPQARDKLE	>	80	>
p2C $E_2 \rightarrow D$	VKILPDVKEKHE	>	10	>
p2C $E_2 \rightarrow V$	VKILPVVKEKHE	>	>	>

Binding is expressed as a ratio of the IC_{50} of the test peptide to the standard peptide. A ratio over 3000 is represented by > and indicates no binding. Values shown are the means of at least 3 separate experiments. Standard errors of the mean were < 20 %. Peptide sequences are depicted by the single letter amino acid code. Amino acids suspected to be anchor molecules in HLA-DR3 binding are depicted in bold

Both, the GAD_{65} peptide containing PEVKEK as well as the viral p2C homologue bound to HLA-DR3. It is furthermore shown that the PEVKEK motif as such is involved in the specific binding to the peptide-binding groove and can be part of a T-cell epitope. Moreover, the GAD_{65} and p2C peptides seemed to be located in an identical position within the peptide binding groove, a condition required for T-cell cross-reactivity. Thus essential conditions for a putative cross-reaction between p2C and GAD_{65} are fulfilled. Our data therefore support the hypothesis of molecular mimicry as a mechanism involved in autoimmunity to GAD_{65}. It remains to be proven, however, whether these epitopes can be generated by naturally processing and subsequent presentation by HLA-DR3 positive cells.

In previous studies it has been shown that the GAD_{65} sequence with homology to p2C is immunogenic and induces T-cell reactivity to GAD_{65} in vitro [24, 25]. Controversy exists as to whether the PEVKEK motif itself is recognised, or flanking sequences in the peptides. Schloot et al. [25] found a positive correlation for reactivity to the GAD_{65} peptides designated p17 and p18 that overlapped for the PEVKEK sequence. This suggests that the PEVKEK motif is recognised. In the study of Atkinson et al. [24], however, reactivity was observed to only one of the peptides, suggesting that epitopes adjacent to PEVKEK were recognised. In their study, T-cell reactivity to p18 was only observed in persons positive for HLA-DR4. In support of that observation, we found that the small peptide containing PEVKEK did not bind to HLA-DR4 whereas the extended 20-mer GAD_{65} peptide did. This suggests that a GAD_{65} sequence adjacent to PEVKEK binds to HLA-DR4. Taken together, these data imply that HLA-DR4 cannot be involved in a cross-reactive immune response to a sequence with homology to p2C. However, this does not rule out that HLA-DR4 positive subjects may elicit a response to the homologous sequence through presentation by HLA-DQ.

The strongest HLA association with IDDM exists for HLA-DQ3.2, for which Kwok et al. [40] recently described the peptide binding motif. They demonstrated that a GAD_{65} peptide encoded by the region with sequence similarity to coxsackievirus p2C binds to HLA-DQ3.2. However, large peptides were used, and the binding-motif of HLA-DQ3.2 was found in a sequence that shares only three amino acids with p2C. Binding of the viral p2C peptide was not mentioned, but even when the corresponding p2C sequence binds to HLA-DQ3.2, the homology is limited and the possibility of T-cell cross-reactivity is therefore questionable.

However, it should be noted that IDDM is a multifactorial disease in which several autoantigens and different environmental factors are suggested to be involved [2]. HLA-DR4 and DQ3.2 molecules might be involved in other mechanisms in the pathogenesis of IDDM.

In animal studies it has been shown that reactivity to GAD_{65} is a key event in induction and propagation of beta-cell autoimmunity in the NOD mouse [41, 42]. In humans however, it is not clear whether autoimmunity to GAD_{65} is associated with induction of IDDM. Individuals, who produce solely anti-GAD_{65} antibodies, do not seem to progress to IDDM [43, 44]. Furthermore, T-cell reactivity to GAD_{65} can be found in healthy control subjects as well as recent-onset IDDM patients [25]. Additional factors seem to be involved in the process leading to IDDM.

From studies in transgenic mice it appeared that sequence homology alone may not be sufficient to break immunological non-responsiveness to autoantigens [3, 4]. According to these studies a natural infection can break T-cell non-responsiveness, whereas immunisation with the homologous viral antigen did not, suggesting that non-specific factors such as local inflammation will contribute to the pathological process. It was shown that a combination of infectious virus, local MHC expression and a tumour necrosis factor (TNF)-α response in the pancreas were required

for onset of diabetes in the transgenic mouse [43]. Recently other experiments confirmed the important role of local inflammation [46]. With regard to diabetes in humans, it might be important that a virus infection can reach the pancreas or even infect beta cells, in order to give rise to a local inflammation. Probably only few enteroviral infections will do so, particularly early in life when immune competence has still to mature. It has been shown that about 30% of CVB-like enteroviruses can damage pancreatic bet cells [47]. Furthermore, repeated exposure to the mimicry motif might be required to break immunological tolerance. This is in agreement with the observation of a higher rate of enteroviral infections during the years before onset of IDDM as compared to non-affected control subjects [12].

Although the evidence remains indirect, our study demonstrates that essential requirements for cross-reactivity between GAD_{65} and protein 2C of CVB-like viruses are met, but only in the HLA-DR3 positive subpopulation of IDDM patients. It is concluded that, besides the epidemiological and serological evidence for their role in IDDM, the CVB-like enteroviruses also fit conditions for molecular mimicry and therefore remain important candidates to induce autoreactivity to GAD_{65}.

Acknowledgements. This study was financially supported by grant 94.120 from the Dutch Diabetes Foundation (DFN)

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