Brief Definitive Report

Sequence Homology of the Diabetes-associated Autoantigen Glutamate Decarboxylase with Coxsackie B4-2C Protein and Heat Shock Protein 60 Mediates No Molecular Mimicry of Autoantibodies

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Summary

Molecular mimicry between viral antigens and host proteins was often suggested to be involved in induction of autoimmune diseases. In type 1 diabetes where pancreatic β cells are destroyed by autoimmune phenomena, a linear sequence homology between a major autoantigen, glutamate decarboxylase (GAD), and the 2C protein of coxsackie B4 was identified. In addition, a sequence homology between GAD and the mycobacterial heat shock protein 60 was described and the suggestions were made that molecular mimicry between GAD, coxsackievirus B4-2C protein, and/or heat shock protein 60 (hsp60) may be actively involved in an autoimmune reaction towards the pancreatic β-cells. Our group was the first to isolate human monoclonal autoantibodies to GAD (MICA 1–6) from a patient with newly diagnosed type 1 diabetes. The MICA allowed a detailed characterization of the diabetes associated self-epitopes in GAD and represent a set of GAD autoantibodies present in sera from patients with type 1 diabetes. Using deletion mutants of GAD we demonstrated that the regions of GAD covering the homology sequences to coxsackievirus B4 and to the hsp60 were absolutely required for binding of the MICA to GAD. We now designed an antibody-based analysis to ask whether molecular mimicry between GAD and coxsackie B4-2C or hsp60 is relevant in type 1 diabetes. Since part of the MICA recognize conformational epitopes, they allow to test for conformational molecular mimicry in viruses that have been incriminated in the development of type 1 diabetes. Our data reveal no crossreactivity between the diabetes associated GAD epitopes defined by the MICA and hsp60, rubellavirus, cytomegalovirus, and coxsackie B1-B6 virus antigens. Neither coxsackie B4–specific antibodies in sera from normal individuals nor GAD-positive sera from patients with type 1 diabetes indicated a crossreactivity between coxsackie B4-2C and GAD. Although the regions in GAD homologous to coxsackie B4-2C and hsp60 represented parts of GAD indispensible for binding of diabetes associated autoantibodies they did not mediate a crossreactivity of autoantibodies between GAD and these two proteins. No evidence for molecular mimicry between GAD and a whole panel of foreign antigens was detected by autoantibodies in type 1 diabetes.

Molecular mimicry between epitopes common to microbial antigens and host proteins has often been implicated in induction of autoimmune diseases. Epitopes similar enough to crossreact but different enough to break immunologic tolerance are believed to induce a spreading of the immune response from foreign to self-antigens. Although the microbial agent might be cleared, crossreactive antibodies or T cells, once mounted, would continue to assault the host (1). Sequence homologies between a whole panel of potential autoantigens and viral antigens as well as heat shock proteins (hsp) were identified in sequence alignment studies and a role for molecular mimicry was suggested in the pathogenesis of the corresponding autoimmune diseases (2, 3). The finding of sequence homology is, however, no direct evidence for biologically active molecular mimicry. Crossreactive antibodies and/or T lymphocytes must be detected in an autoimmune disease before molecular mimicry might be established as etiological mechanism. Animal models like experimental allergic encephalomyelitis demonstrated induction of autoimmune disease by molecular mimicry effects (4).

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In type 1 (insulin-dependent) diabetes mellitus (IDDM) where pancreatic β cells are destroyed by an autoimmune process, many unrelated viruses have been implicated in the induction of the disease (5). Only in children with congenital rubella syndrome has a clear correlation between virus infection and the high incidence of IDDM emerged (6). Recent findings related the high frequency of IDDM in individuals with congenital rubella syndrome to molecular mimicry effects, since a mouse monoclonal antibody revealed cross-reactivity between a rubella virus capsid protein and a 52-kD islet antigen (7). A major, well-characterized autoantigen in type 1 diabetes is the enzyme glutamate decarboxylase (GAD) (8) which exists in two forms, GAD_{65} and GAD_{67}, only one of which, GAD_{65}, is expressed in human islets (9). GAD is recognized by autoantibodies as well as autoreactive T cells (10, 11). Recently, Kaufman et al. (12) reported a sequence homology between GAD_{65} and the 2C protein of coxsackievirus B4 (CB4) and Jones et al. (3) described a sequence homology of GAD_{65} to the mycobacterial and the human hsp60 (Fig. 1 *A*). This raised the question whether molecular mimicry between GAD_{65} and these proteins is actively involved in induction of IDDM.

We recently derived human monoclonal autoantibodies to GAD_{65} (MICA 1–6) from a patient with newly diagnosed IDDM, which allowed a detailed characterization of diabetes associated epitopes in GAD_{65} (13, 14). In studies with deletion mutants of GAD_{65}, the regions of GAD covering the homology sequences to CD4 were absolutely required for binding of MICA 1, 3, 4, and 6. Although the NH_2-terminal amino acids 1–244 could be removed from the GAD molecule without affecting binding of the MICA, the removal of an additional 50 amino acids, which included the homology region to CB4, prevented binding of MICA 1, 3, 4, and 6, and of all GAD-positive IDDM sera tested with this GAD deletion mutant (Fig. 1 *B*). The homology region of hsp60 was required for a binding of MICA 1, 2, and 3 to the GAD_{65} molecule (14). Although MICA 1, 3, 4, and 6 recognized conformational epitopes, the linear sequence homology region may provide a crucial part of the epitope. Since the MICA epitopes represent a set of diabetes associated epitopes, these antibodies provide a unique tool to investigate the existence of molecular mimicry between GAD_{65} and proteins of CD4 or hsp60. In addition, for the first time in humans, the MICA allow complex viral antigens to be probed for conformational molecular mimicry effects with GAD_{65}, that may escape detection in sequence alignment studies. We here analyzed a panel of viruses previously implicated in the development of IDDM for molecular mimicry with diabetes-associated GAD_{65} epitopes and studied in greater detail the possible crossreactivity of CB4-2C and GAD_{65} epitopes using GAD-positive sera from patients with IDDM and CB4-positive sera from normal individuals. Our data give no evidence for a crossreactivity between GAD_{65} and the CB4-2C protein on the antibody level. Neither hsp60 nor viral antigens of rubellavirus, cytomegalovirus, and coxsackievirus B1-B6 displayed molecular mimicry to the diabetes-specific GAD_{65} epitopes defined by the MICA.

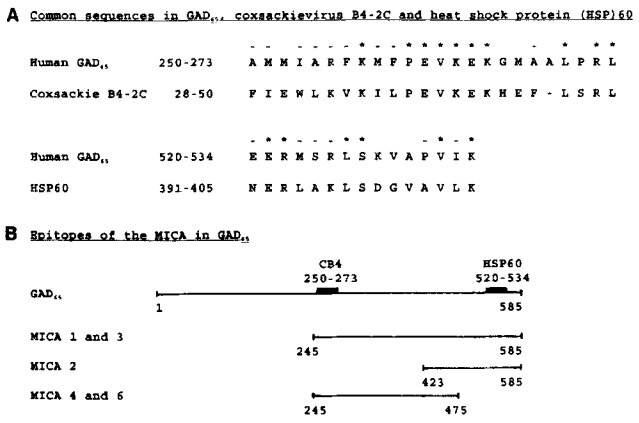

Figure 1. Homology of human GAD_{65} with the 2C protein of coxsackie B4 and with human hsp60. (*A*) Sequence homology regions of GAD_{65} and coxsackievirus B4-2C and GAD_{65} and hsp60 are aligned. Identical positions are marked by an asterisk and conservative amino acid changes by a minus sign. (*B*) The homology regions are indicated in a linearized plot of the full-length GAD_{65} molecule. Core sequences of GAD that are required for recognition of GAD_{65} by the individual MICA are indicated.

Materials and Methods

Viruses and Cell Lines. The vero, HeLa, and HEL cell lines were obtained from the American Type Culture Collection (Rockville, MD) and Sf9 cells from Invitrogen (San Diego, CA). The GMK cell line and the picornaviruses used in this study have been described before (15). Coxsackieviruses and respective titers were: B1 (P. O. Dalldorf) $10^{8.8}$ $TCID_{50}$/ml, B2 (Ohio 1) $10^{5.8}$ $TCID_{50}$/ml, B3 (Nancy) $10^{7.1}$ $TCID_{50}$/ml, B4 (Nα-1-[sp]) $10^{8.5}$ $TCID_{50}$/ml., B5 (Faulkner) $10^{8.7}$ $TCID_{50}$/ml, B6 (1-51-21) $10^{6.9}$ $TCID_{50}$/ml. The viruses were propagated on GMK cells (16) and harvested for antigen preparation after 24 h by repeated freezing and thawing followed by ultrasonification on ice 3 × for 15 s. Cell debris was removed by low speed centrifugation and infectivity titers were assayed by endpoint titration on GMK cells and/or by plaque assay. Uninfected GMK cells processed in parallel served as control antigen. Cytomegalovirus was an isolate from a patient who received bone marrow transplantation. Virus was grown in HEL cells and harvested by Dounce homogenization of the cells after cytopathic effects became visible. Cell debris was removed by centrifugation at 3000 *g* for 10 min. Infectivity titer was 3×10^4 $TCID_{50}$/ml. Uninfected cells processed in parallel served as control antigens. Rubellavirus antigen was purchased from Behringwerke AG (Marburg, Germany).

Antibodies. The MICA were derived from a patient with IDDM as described (13). They were of the IgG class and recognized at least three distinct epitopes specific for GAD_{65} (13). MICA 2 recognized a linear epitope in GAD that was rare in the sera of patients with IDDM, whereas MICA 1, 3, 4, and 6 recognized conformational epitopes similar to those detected in GAD-positive sera from patients with IDDM (14). Mouse anti-hsp60 antibody II C 8 ascites was obtained from T. Gillis (Marshall University School of Medicine, Huntington, WV), the CB4-specific mouse monoclonal antibody M1 was produced by one of us (17), and rabbit anti-2C antibody by C. Hohenadl (Max-Planck-Institute of Biochemistry, Martinsried, Germany) (18).

Patients Sera and Test for CB4 Antibodies. Sera from 42 normal individuals, 22 healthy schoolchildren, and 15 newly diagnosed patients with IDDM were obtained with informed consent. CB4-specific IgM responses were detected by an μ-antibody capture ELISA technique employing CB4 antigens and monospecific mouse

antisera as previously described (19). Neutralizing antibodies to CB4 were tested by a microtechnique in GMK cells as described (20).

Preparation of Mycobacterial hsp60. hsp60 of *Mycobacterium bovis* BCG was expressed in *Escherichia coli* M1546 from plasmid pRIB1300 as described (21). The protein was purified by anion-exchange chromatography (21).

Preparation of GAD_{65}. The human recombinant GAD_{65} sequence was cloned into a baculovirus expression vector and recombinant virus isolated as described (22). A detergent solubilized membrane fraction of GAD_{65} expressing Sf9 cells and mock infected Sf9 cells was prepared according to (10).

Immunoprecipitation Assay. 2×10^6 Sf9 cells were infected by recombinant baculovirus expressing human GAD_{65}. 40 h after infection cells were labeled for 4 h with 100 μCi [^{35}S]methionine. CB4-proteins were labeled 4 h after infection of 6×10^7 HeLa cells with 3.6×10^9 plaque forming units of CB4 by incubation of the cells with 150 μCi [^{35}S]methionine in methionine-free modified Eagle's medium. After washing cells were lysed and extracts prepared as described above for unlabeled GAD_{65}. Samples of cytoplasmic extracts of infected HeLa cells or solubilized membrane fractions of Sf9 cells expressing GAD were precleared by incubation with 25 μl CB4-negative pool serum. To include CB4 antibodies of the IgM class in our analysis, 25 μl test serum was incubated over night with the precleared extract and the immune complexes were precipitated using protein A–Sepharose in a mixture with protein A–Sepharose coated with a goat anti-human IgM-specific antibody (1:1, 2 h, 4°C). The immune complexes were washed in 100 mM Tris, 500 mM LiCl, 1% NP-40, 0.5% mercaptoethanol, and bound antigen was eluted by addition of 62 mM Tris/HCl, pH 6.8, 2% SDS 5% mercaptoethanol, 0.01% bromphenolblue and subjected to polyacrylamid gel electrophoresis. Bound protein was detected after fluorography.

Immunoblotting and Western Blotting. Virus antigens, GAD_{65} (0.63 mg/ml) and the appropriate controls were tested pure and in dilutions of 1:20 and 1:50. 50 μl of each extract were spotted on polyvinyldiene difluoride (PVDF) membranes (Immobilon; Millipore Corp., Bedford, MA) using a slot blot apparatus (Schleicher & Schuell Inc., Keene, NH). Proteins separated on polyacrylamide gels were transfered to PVDF by semidry blotting. Blotting membranes were stained by for 2 h with MICA culture supernatants (2 μg/ml), human monoclonal IgG control antibody (2 μg/ml), rabbit anti-2C antibody (1:100), mouse anti-hsp60 II C 8 ascites (1:400) or supernatant of the CB4-specific mouse monoclonal antibody M1 (17) (undiluted).

Results

Reactivity of MICA 1–6 with Viral Antigens. Viral antigens from coxsackie B1–B6, from cytomegalovirus, herpes simplex virus and rubellavirus were tested for their reactivity with the individual MICA in an immunoblotting assay using the native antigens. Extracts of uninfected host cells, GAD_{65} expressed in baculovirus permissive Sf9 cells and wild-type baculovirus infected Sf9 cells expressing no GAD were included as controls (Fig. 2). Whereas spotted GAD_{65} was detected by all six MICA, neither the viral antigens nor the controls were recognized by MICA 1–6. CB4 antigens were specifically stained by the CB4-specific mouse monoclonal antibody M1 used as a positive control.

Reactivity of MICA 1–6 with hsp60. Different dilutions of a GAD_{65} extract and of purified hsp60 from *M. bovis* BCG were analyzed in a slot blot assay (Fig. 3). The MICA did not crossreact with the hsp60 suggesting that the

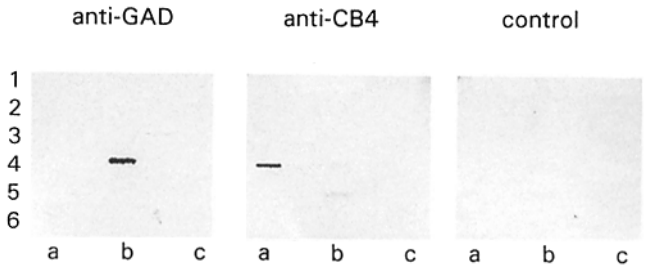

Figure 2. Reactivity of MICA 1–6 with viral antigens. Viral antigens of coxsackie B1–B6, cytomegalovirus, rubellavirus, and herpes simplex virus were spotted on membrane sheets and tested for their reactivity with the MICA. MICA 4 was selected as example for the typical reactivity of all MICA that stained solely GAD. The anti-coxsackievirus B4 (CB4)-specific control antibody M1 reacted with the CB4 antigen only and an unrelated human monoclonal IgG antibody revealed no reactivity with the tested virus antigens. Positions of the antigens: 1a, CB1; 2a, CB2; 3a, CB3; 4a, CB4; 5a, CB5; 6a, CB6; 1b, 2b, 3b, mock infected GMK; 4b, GAD_{65}; 5b, Sf9 control extract; 1c, Cytomegalovirus; 2c, mock infected HEL; 3c, mock infected Vero; 4c herpes simplex virus, 5c rubellavirus.

GAD_{65} region of amino acids 520–534, which is homologous to hsp60, is not directly involved in the epitope recognition of MICA 1, 2, and 3 which required this region of GAD for binding. Alternatively, the homology between the two proteins is too weak to induce crossreactivity by an autoantibody isolated from a patient with type 1 diabetes.

Reactivity of CB4-positive Sera with GAD_{65}. We further analyzed whether CB4 infection in humans induced antibodies crossreactive with GAD_{65}. 15 sera positive for CB4 IgM antibodies in the ELISA and 15 CB4 IgM-negative sera from normal individuals, 22 sera from normal individuals with neutralizing antibodies to CB4, and 20 normal sera negative for neutralizing CB4 antibodies were tested for their reactivity with GAD in an immunoprecipitation assay using ^{35}S-labeled GAD_{65} expressed in a baculovirus system. A combined analysis of IgM and IgG antibodies was performed for the CB4 IgM-positive sera. The results are summarized in Table 1. No reactivity with GAD_{65} was observed with all sera. When seven CB4–IgM-positive and eight CB4–IgM-negative sera from patients with IDDM were tested in the same assay, no correlation was found between the CB4-positivity and the GAD-reactivity of these sera. CB4 infection, therefore, did not induce antibodies crossreactive with GAD_{65} and GAD antibodies appeared independent of positivity for CB4 in patients with IDDM.

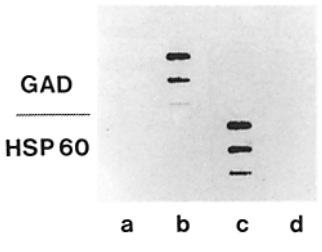

Figure 3. Reactivity of MICA 1–6 with hsp60 of *M. bovis* BCG. GAD_{65} was expressed in baculovirus Sf9 cells and a detergent solubilized membrane fraction of the cells (5 μg total protein/ml), was spotted on membranes together with purified hsp60 (2 μg/ml). For each antigen three dilutions were applied (from top to bottom: pure, 1:5, and 1:25). Lane *a* was stained by an unrelated human monoclonal antibody, lane *b* by MICA 2, lane *c* by the anti-hsp60-specific mouse monoclonal antibody IIC8, and lane *d* by an unrelated mouse monoclonal antibody. All six MICA showed identical results.

Table 1. *Correlation of Anti-coxsackie B4 (CB4)-reactive Antibodies with Anti-GAD-reactive Antibodies in Normal Individuals and Patients with IDDM*

Individuals	No. of sera tested	GAD_{65}positive
Normal		
CB4 IgM positive*	15	0
CB4 IgM negative	15	0
CB4 NA positive‡	22	0
CB4 NA negative	20	0
IDDM		
CB4 IgM positive	8	3
CB4 IgM negative	7	4

* Anti-CB4 IgM antibodies were determined by ELISA.
‡ NA, neutralizing antibodies were determined by a microtechnique in GMK cells.

Reactivity of GAD-positive Sera with CB4-2C. MICA 1–6 and 10 GAD-positive sera from 10 newly diagnosed patients with IDDM as well as 10 sera from normal individuals positive for neutralizing CB4 antibodies and 2 individuals negative for CB4 were analyzed for their reactivity with CB4-2C. HeLa cells were infected with CB4 and labeled by [^{35}S]methionine incorporation. Extracts of CB4 infected HeLa cells were immunoprecipitated with the MICA or the individual sera after preclearing with a CB4-negative serum. Precipitation of viral antigens was detected after separation of the samples by PAGE. No reactivity of the MICA with CB4 antigens was detected (Fig. 4 *A*) concordant with the results presented in Fig. 2. In contrast, all sera recognized a whole panel of bands irrespective of their positivity in the CB4-specific ELISA or the neutralizing test. To determine whether 2C was among the proteins precipitated by the sera, the immunoprecipitates were subjected to Western blotting and stained with an anti-2C rabbit serum raised against a 20-amino acid consensus epitope of the 2C protein of coxsackie B viruses (18). The results are shown in Fig. 4. 3 of the 10 GAD-positive sera from patients with IDDM revealed a reactivity with the 2C protein as well as 3 of 10 GAD-negative sera tested from normal individuals. The frequency of anti-2C antibodies was, therefore, identical in GAD-positive individuals with IDDM and GAD-negative normal individuals. This suggests no correlation of an anti-2C response with autoreactivity to GAD in patients developing type 1 diabetes.

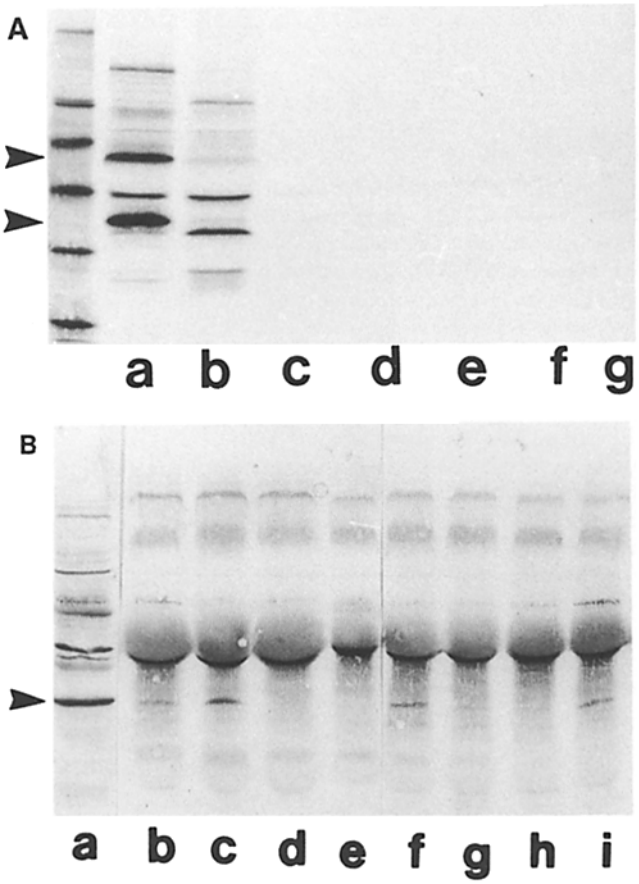

Figure 4. Reactivity with coxsackievirus B4-2C. (*A*) [^{35}S]methionine-labeled extracts of HeLa cells infected with coxsackievirus B4 were immunoprecipitated by (*a*) anti-2C-specific rabbit serum, (*b*) anti-CB4–specific mouse monoclonal antibody M1, (*c*) rabbit control serum, (*d*) MICA 1, (*e*) MICA 2, (*f*) MICA 4, and (*g*) MICA 6, and samples were analyzed by PAGE and fluorography. The arrows indicate the 2ABC precursor protein of 2C (65 kD) and the protein 2C (37 kD), which were both precipitated by the 2C-specific rabbit serum. M1 recognized the viral structural protein VP1 and precursors of VP1 (*b*). The MICA did not recognize any of the proteins. (*B*) HeLa cells infected with coxsackievirus B4 were immunoprecipitated by 10 sera from GAD-negative normal individuals and 10 sera from GAD-positive patients at onset of type 1 diabetes. The samples were subjected to Western blotting and stained for detection of coxsackie B4 2C by a rabbit serum raised against a linear epitope in 2C. The 2C protein in the extract used for immunoprecipitation (*a*) is indicated by an arrow. Results of four GAD-negative normal sera (*b–e*) and four GAD-positive diabetic sera (*f–i*) are shown.

Discussion

Since the isolation of CB4 particles from islets of a patient who died with acute onset of IDDM and the induction of diabetes in mice infected with these particles, CB4 infections were frequently claimed to be correlated with IDDM. In non-human primates and some mouse strains CB4 infections produced transient diabetes (23, 24), but in humans the large number of serological and epidemiological studies revealed no clear picture of an implication of CB4 infection in IDDM (reviewed in 5). After identification of the 64-kD islet antigen as GAD (8) a sequence homology between this major autoantigen in diabetes and the 2C protein of CB4 was identified (12). Molecular mimicry effects between GAD and CB4, therefore, were suggested to play a role in islet cell destruction and development of IDDM. We isolated six diabetes-associated GAD-specific human monoclonal autoantibodies, four of which required the CB4 homology region in GAD_{65} for a binding to the GAD_{65} molecule (14). Now we demonstrate that these antibodies do not crossreact with native

CB4 antigens and particularly not with the 2C protein. The linear sequence homology region shared between GAD_{65} and CB4-C2, although being an important part of the conformational epitopes of MICA 1, 3, 4, and 6 did not mediate a crossreactivity between both proteins.

The coxsackievirus 2C protein is a nonstructural virus protein that is produced by the infected cell did not incorporated in the infectious particle itself. 2C may have a function in the replication of viral RNA (25) and is highly conserved among coxsackieviruses B1–B6 (26). Sera from normal individuals irrespective of positivity for neutralizing anti-CB4 antibodies or anti-CB4-IgM-ELISA antibodies all recognized CB4 virus proteins. The fact that the two sera negative for antibodies to CB1-CB6 still recognized a pattern of CB4 antigens may be due to the presence of antibodies to other picornaviruses that are known to be highly crossreactive to coxsackie antigens (16, 27). Reactivity to the viral protein 2C was detected in 30% of CB4-positive normal individuals by Western blotting. GAD-positive sera of patients with type 1 diabetes revealed the same frequency of an anti-2C response like GAD-negative normal individuals. This argues against a crossreactivity between CB4-2C and GAD_{65} progressing to a development of type 1 diabetes in susceptible individuals. Neither the CB4-specific antibodies in sera of CB4 positive individuals nor GAD antibodies in IDDM, therefore, provided any evidence that tolerance to the linear epitope of GAD which is homologous to CB4-2C was broken. This suggests no molecular mimicry effects between GAD_{65} and the CB4-2C protein in the humoral immune response of type 1 diabetes.

Controversial data exist about the implication of hsps in the development of IDDM (28, 29). One group reported that hsp60 was among the antigens recognized by the diabetes associated 64-kD antibodies (28). Other groups, however, clearly demonstrated that 64-kD antibodies recognize GAD (8) and detected no anti-hsp60 antibodies in sera from patients with IDDM (29). In spite of the reported sequence similarity between GAD and hsp60 (Fig. 1) (3) we demonstrated herein that the observed sequence homology did not induce crossreactivity by a set of human monoclonal autoantibodies. In an animal model of IDDM, the NOD mouse, T cell clones reactive to mycobacterial hsp60 have been shown to induce insulitis and hyperglycemia (30). The T cell–reactive peptides in hsp60 were confined to amino acids 437–474, and were, therefore, different from the homology region observed between hsp60 and GAD_{65}. Neither the T cell epitopes in hsp60 characterized in the NOD mouse system nor the typical set of diabetes associated autoantibodies in humans tested here, therefore, suggest any relevance for molecular mimicry effects between hsp60 and GAD_{65} in type 1 diabetes.

The humoral antibody response in humans tested here suggests no relevance for molecular mimicry effects between GAD_{65}, the CB4-2C protein, hsp60, and other virus antigens which have been suggested to be involved in induction of type 1 diabetes. No data are available about possible T cell–mediated molecular mimicry to GAD in humans as T cell reactive epitopes in GAD remain to be determined. In NOD mice the late activation of T cells to the CB4 homologous region in GAD provides evidence against the hypothesis that molecular mimicry between CB4 and GAD_{65} triggers the autoimmune response on the T cell level in this animal model of IDDM (31). We agree with others that direct destruction of islet cells by viruses (32), upregulation of MHC class I molecules (33, 34), induction of an inappropriate MHC class II expression on islets (35), enhancement of cytokine production in the periphery of islets after virus infections (33, 34), or a combination of these mechanisms may be the relevant factors leading to IDDM in a critical immunogenetic background rather than molecular mimicry effects between viral antigens and GAD_{65}.

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