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Dynamic changes of GAD65 autoantibody epitope specificities in individuals at risk of developing type 1 diabetes

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Abstract *Aims/hypothesis:* Progression to type 1 diabetes is associated with intramolecular epitope spreading to disease-specific antibody epitopes located in the middle region of glutamic acid decarboxylase 65 (GAD65). *Methods:* The relationship between intramolecular epitope spreading of autoantibodies specific to GAD65 in relation to the risk of developing type 1 diabetes was tested in 22 high-risk individuals and 38 low-risk individuals. We determined the conformational epitopes in this longitudinal study by means of competition experiments using recombinant Fab of four GAD65-specific monoclonal antibodies. *Results:* Sera from high-risk children in the preclinical stage recognise a specific combination of GAD65 antibody epitopes located in the middle and the C-terminus of GAD65. High risk of progressing to disease is associated with the emergence of antibodies specific for conformational epitopes at the N-terminus and the middle region. Binding to already established antibody epitopes located in the middle and at the N-terminus increases and shows a significant relation (p= 0.005) with HLA, which confers risk of developing diabetes. *Conclusions/interpretation:* In type 1 diabetes, GAD65 antibodies are initially generated against the middle and C-terminal regions of GAD65. In genetically predisposed subjects the autoimmune response may then undergo intramolecular epitope spreading towards epitopes on the N-terminus and further epitopes located in the middle. These findings clearly demonstrate that the GAD65 autoantibody response in the preclinical stage of type 1 diabetes is dynamic and related to the HLA genotypes that confer risk of diabetes. GAD65-specific Fab should prove useful in predicting progression from islet autoimmunity to clinical onset of type 1 diabetes.

Keywords Autoantibody · Epitope · Type 1 diabetes

Abbreviations GAD65: 65-kDa isoform of glutamate decarboxylase · GAD65Ab: GAD65 autoantibody · IA-2: islet antigen-2 · PGS: Protein G sepharose · rFab: recombinant Fab

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Introduction

Autoantibodies to islet cell antigens are present in the majority of patients at the clinical diagnosis of type 1 diabetes. These autoantibodies are primarily directed against three defined antigens: the 65-kDa isoform of glutamate decarboxylase (GAD65), insulin and the protein tyrosine-like islet antigen-2 (IA-2). All three autoantibodies can also be found in the serum of pre-diabetic individuals, sometimes years before clinical onset of the disease [1–4]. Their combined presence makes it possible to identify individuals at high risk of developing type 1 diabetes [5–9]. While auto-

antibodies to GAD65, IA-2 and insulin are clearly markers for type 1 diabetes, up until recently they were viewed as the product of a bystander response to the autoimmune destruction of the pancreatic beta cells. Recent findings suggest that autoantibodies to GAD65 (GAD65Ab) may play a pathogenic role by modulating the GAD65 presentation to the immune system [10–12].

Epitopes recognised by GAD65Ab at the clinical onset of diabetes have been the focus of many studies [13–16]. We and others have established significant differences in the GAD65Ab epitope patterns present in patients with type 1 diabetes, compared with other GAD65Ab-positive phenotypes [13, 17–19]. Recently we described two conformational epitopes that are located in the middle region of GAD65 and were specifically recognised by GAD65Ab from type 1 diabetic patients [17]. However, very little is known about the development of GAD65Abs and their epitope specificity in the pre-diabetic period. The characterisation of monoclonal GAD65Ab from patients with type 1 diabetes demonstrated that these antibodies had undergone somatic mutations, indicating affinity maturation, during the progression to type 1 diabetes [20, 21]. This gives rise to the question of whether disease-associated GAD65Ab specificities are the product of antibody maturation, or an inherent characteristic of GAD65Ab present in pre-diabetic individuals. In this longitudinal study, we compared GAD65Ab epitopes in children who are at high risk of developing diabetes with those present in children who are at low risk of developing the disease.

Subjects and methods

Human sera Serum samples were obtained from participants in the Karlsburg Type 1 Diabetes Risk Study. In this study, 9,419 randomly selected, healthy schoolchildren (6–17 years old) underwent screening for autoantibodies related to type 1 diabetes, and autoantibody-positive children were followed longitudinally as previously described [22, 23]. A total of 387 serum samples from 86 GAD65Ab-positive children identified in this study were analysed for binding to human GAD65 and rat GAD67. Twenty-six individuals were excluded because of antibody reactivity with GAD67 (n=13), low GAD65Ab titre of the longitudinal samples (n=10), or limited follow-up data, with the number of samples below 2 (n=3) (Fig. 1). The removal of GAD65/67 cross-reactive sera was necessary as the recombinant Fabs (rFab) used in this study are GAD65-specific and do not bind to GAD67 [21, 24]. The GAD65Ab epitopes of the remaining 301 serum samples from 60 GAD65Ab-positive children were analysed (Table 1). A median of four samples (n=2–9) per individual was analysed. Children whose sera tested positive for more than one diabetes-related autoantibody in the initial screen (GAD65Ab, IA-2Ab, or insulin autoantibodies) were classified as high-risk individuals (n=22) (Fig. 1). Of these, nine developed type 1 diabetes during the follow-up period. The last of these children developed diabetes after a follow-up of 55 months. The remaining children (n=38) were positive for GAD65Ab alone and therefore classified as low-risk individuals. None of these children developed type 1 diabetes in the follow-up or developed reactivity to additional autoantibodies. The study protocol was authorised by the Ministry of Culture and Education of Mecklenburg-Vorpommern and approved by the ethics committee of the Ernst-Moritz-Arndt-Univer-

Table 1 Study-related parameters of the analysed individuals

	High risk (n=22)	Low risk (n=38)
GAD65Ab titre (index)		
Initial sample (range)	0.7 (0.1–1.9)	0.3 (0.1–1.5)
Final sample (range)	0.8 (0.22–1.8)	0.4 (0.1–2.0)
Median no. samples per individual (min–max)	5 (2–9)	3 (2–7)
Follow-up period in months (range)	48 (2–55)	42 (11–55)
Age in years (range)	12 (7–16)	11 (6–15)
Sex (female/male)	11/11	21/17
HLA-DQ risk genotype frequency (n)		
02/02	2	3
02/0501	2	2
02/0302	4	5
0302/0304	1	0
0302/0402	1	0
0302/0501	2	0
0302/0302	2	1
0302/0502	0	1

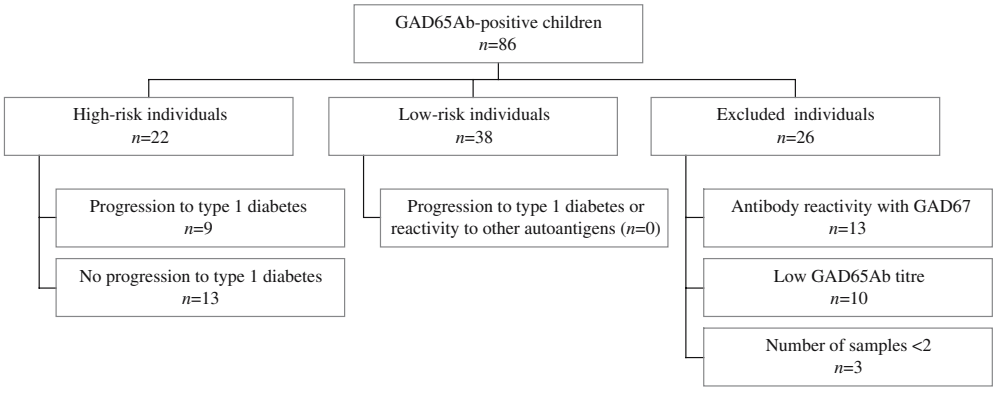

Fig. 1 Flow-chart illustrating the different study groups used in this study

sity (Greifswald, Germany). Informed consent was obtained from the parents or legal guardians, and the investigations were carried out in accordance with the principles of the Declaration of Helsinki, as revised in 2000.

Expression and purification of the antibody rFabs The four rFabs used in this study have been described in detail [17]. Briefly, rFab DPA, DPD and DPC were isolated from a patient with type 1 diabetes [21]. These rFabs recognise epitopes at amino acid residues 483–585 (C-terminus), 96–173 (N-terminus), and 195–412 (middle) respectively [25]. b96.11 was isolated from a patient with autoimmune polyendocrine syndrome type 1 and recognises an epitope at amino acid residues 308–365 (middle) [24, 25]. rFabs were expressed in bacteria as previously described [17]. Briefly, *E. coli* 25F2 cells containing the recombinant plasmids were grown for 16 h at 30°C in complete MOPS medium [26]. Cells were then subcultured and grown in the absence of phosphate at 30°C for 4 h. The rFabs were isolated from the bacteria as described previously [27] and purified by two subsequent affinity chromatography steps on Ni-NTA Agarose (Qiagen, Valencia, CA, USA) and protein G sepharose (PGS) (Zymed Laboratories, Carlton Court, CA, USA). Fractions were examined by immunoblot for the presence of rFabs and by radioligand binding test for GAD65 binding. Active fractions were pooled and the protein concentration was determined. The yield of functional purified rFab was about 0.5–1 mg/l bacterial culture.

Competition studies of rFab with monoclonal antibodies and serum Recombinant [^{35}S]-labelled human GAD65 and rat GAD67 were produced in an in vitro coupled transcription/translation system with SP6 RNA polymerase and nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI, USA), as described previously [28, 29]. The in vitro translated [^{35}S]-antigen was stored at −70°C and used within 2 weeks. Binding of rFab to radiolabelled antigen was determined as described previously, using PGS as the precipitating agent [17].

The capacity of the rFab to inhibit GAD65 binding by human serum GAD65Ab was tested in a competitive radioligand binding test using protein A sepharose (Zymed Laboratories) to separate antibody-bound from free antigen. The rFabs were added at the optimal concentration (0.7–1 μg/ml) as determined in competition assays using the intact monoclonal antibody as a competitor. The background competition for each rFab was established in competition experiments with normal control sera. The background was subtracted prior to calculation of percentage inhibition. The cutoff for specific competition was set at greater than 15% by using as a negative control rFab NQ22/61.1 (a kind gift from Dr J. Foote, Fred Hutchinson Research Center, Seattle), specific to an irrelevant target, phenyl oxazolone, at 5 μg/ml.

HLA-DQB1 typing HLA-DQB1 genotyping was performed by non-radioactive oligonucleotide hybridisation of enzymatically amplified DNA, as previously described [22]. Because DQB1 0201 and 0202 differ by a dimorphism at codon 135, located in the third exon, and are thus not differentiated by regular DQB1 genotyping, DQ2-positive individuals are given as DQB1 02. HLA-DQB1 risk genotypes were classified as follows (number of subjects with a particular genotype shown within parenthesis): 02/02 (5), 02/0302 (9), 02/0501 (4), 0302/0304 (1), 0302/0402 (1), 0302/0501 (2), 0302/0302 (3), 0302/0502 (1). HLA DQB1 genotypes with no or with a negative association with type 1 diabetes were: 0301/0301 (3), 0301/0402 (1), 0301/0302 (4), 0301/0602 (1), 0301/0501 (1), 02/0301 (4), 02/0602 (3), 02/0402 (1), 02/0603 (1), 0501/0604 (1), 0304/02 (1), 0602/0602 (1), 0602/0503 (1), 0602/0303 (1), 0302/0603 (3), 0502/0201 (1), 0402/0501 (1), 0402/0604 (1), 0601/0301 (1), 02/0604 (1), 0502/0602 (1), 0604/0503 (1).

Statistical analyses Binding of GAD65Ab to GAD65 in the presence of rFab was expressed as follows:

$$\left(cpm\ of\ \left[^{35}S\right]-GAD65\ bound\ in\ the\ presence\ of\ rFab\right) / \left(cpm\ of\ \left[^{35}S\right]-GAD65\ bound\ in\ the\ absence\ of\ rFab\right) \times 100$$

The GAD65Ab index was defined as follows: (cpm of tested sample−average cpm of 2 negative standards)/(cpm of positive standard−average cpm of 2 negative standards). The Juvenile Diabetes Foundation islet cell antibody standard [30], which is also GAD65Ab-positive, was used as the GAD65Ab-positive standard. A randomly selected control serum from a healthy volunteer was used as the negative standard.

All samples were analysed in triplicate determinations and the average intra-assay coefficient of variation was 4% (range 0.6–9%). Positive and negative controls were included on each plate to correct for interassay variations. The upper level of normal (0.047) was established by analysing sera obtained from non-diabetic subjects (n=50). In the third DASP Workshop our assay showed 84% sensitivity and 91% specificity for GAD65Ab. Differences in competition between different serum groups were tested for significance with the non-parametric Mann–Whitney U-test. Differences in competition within serum groups were tested using the Wilcoxon matched pair test. Linear regression curves were compared using the Prism statistical analysis tool (GraphPad Software, San Diego, CA, USA). A p value of less than 0.05 was considered significant.

Results

GAD65Ab titre and epitopes recognised in healthy schoolchildren We first established the GAD65Ab titre (Table 1) and epitope specificity of all individuals at the initial sample. When comparing the level of competition achieved with individual rFabs in the first sample taken from children in both groups, no significant differences were observed (Fig. 2). No correlation was observed between the GAD65Ab titre and the level of competition achieved with any of the rFabs (data not shown). This is in accordance with our

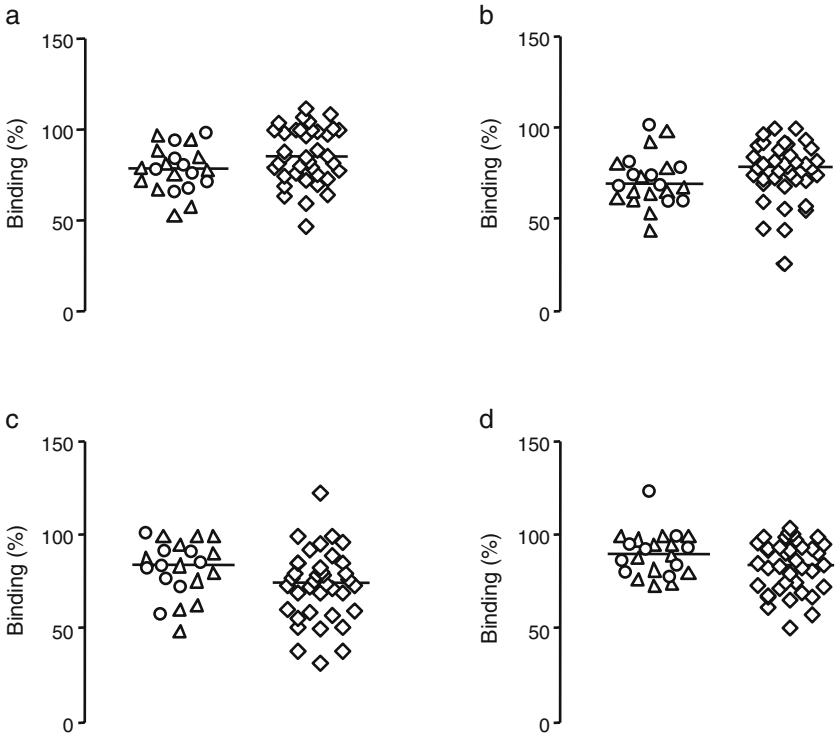

Fig. 2 Binding to GAD65 in the presence of rFab DPA (**a**), b96.11 (**b**), DPC (**c**), and DPD (**d**). Binding of initial samples from high-risk individuals (*triangles*), individuals that later progressed to type 1 diabetes (*circles*) and low-risk individuals (*diamonds*) is given in percent. Median binding is indicated by a *horizontal line*

previous findings [17]. Finally, we evaluated whether there were any changes in the GAD65Ab titre over time. Because the last individual developed diabetes at 55 months of follow-up, we limited the period of analysis for all individuals accordingly. During this time period, no significant changes of GAD65Ab titre were observed for any of the groups (Table 1).

GAD65Ab epitope pattern in the initial sample While the GAD65Ab in both groups recognised similar epitopes, we found significant differences in particular combinations of epitope specificities present in individual patients when comparing the two groups. At the initial sampling, most of the sera in both groups had GAD65Abs that were competed with by a median of two rFabs. Nearly all samples (93–100%) recognised epitopes located in the middle of the molecule, defined as competition by DPC and/or b96.11 (Fig. 3a, b). While the most frequent epitope combination for high-risk individuals in the initial sample included the C-terminus (competed with by rFab DPA) and the middle region (competed with by rFab b96.11; 70% of the samples, shaded regions in Fig. 2a), only 37% of the low-risk individuals had GAD65Ab that were reactive with this epitope combination ($p<0.0001$).

GAD65Ab epitope pattern in the last sample The differences in epitope combinations observed in the initial sample were even more pronounced upon analysis of the last sample. Both the number of epitopes and the epitope pattern recognised had changed for the high-risk individuals (Fig. 3c). While a median of two rFabs still competed with GAD65Ab present in the sera of the low-risk individuals, a median of four rFabs competed with GAD65Ab present in the sera of high-risk individuals. Among the high-risk individuals, the percentage of sera in which all four rFabs competed with GAD65Abs increased from 13 to 60% ($p<0.0001$) between the first and last sample. The remainder

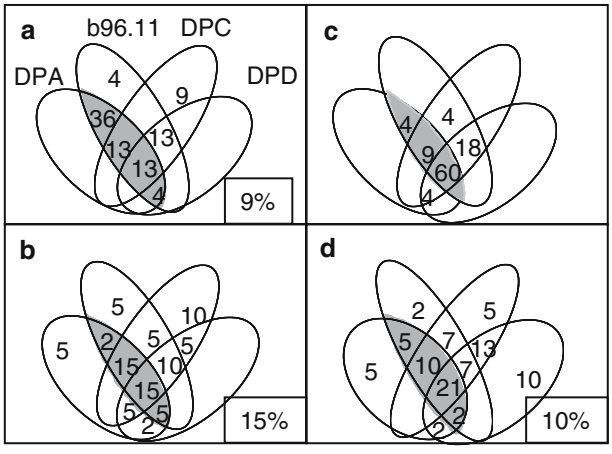

Fig. 3 Venn diagrams for sera from high-risk (**a**) and low-risk individuals (**b**) at the initial sample time and for sera from high-risk (**c**) and low-risk (**d**) individuals at the last sample time. Sera were analysed for competition with the indicated rFab to determine their combination of GAD65Ab specificities. The numbers in the diagrams depict the percentage of serum samples in each group that competed with the respective rFabs. Absolute numbers represented by the above percentages are: (**a**, **c**): 60% (n=13), 36% (n=8), 18% (n=4), 13% (n=3), 9% (n=2), and 4% (n=1); (**b**, **d**): 21% (n=8), 15% (n=6), 13% (n=5), 10% (n=4), 7% (n=3), 5% (n=2), 2% (n=1). The shaded areas highlight the percentage of serum samples that competed with a combination of rFab b96.11 (middle-) and DPA (C-terminus-specific). The percentage of serum samples that were not competed with by any of the four rFabs are shown in a box at the lower right corner of each panel

competed with different combinations of two or three rFabs in the last sample. The epitope pattern in the low-risk individuals remained unchanged between the initial and the last serum samples, with only 21% of the last samples having GAD65Abs competed with by all four rFabs, and 32% of the samples still competed with by one rFab or fewer.

High-risk individuals show significant epitope changes over time To compare the change of epitope recognition over time between different subject groups, we investigated the degree of competition conferred by each rFab by linear regression analysis (data not shown). For the low-risk individuals, we did not observe any significant change in degree of epitope binding using any of the four rFabs. On the other hand, significant increases in degree of epitope binding over time for epitopes represented by rFab b96.11, DPC and DPD were noted in the high-risk individuals (p values: <0.0001, 0.0035 and <0.0001 respectively).

GAD65Ab specificities in the last sample from high-risk individuals differ from those found in low-risk individuals The change in epitope binding over time resulted in significant differences in competition levels. In the high-risk group, GAD65Abs with epitope specificities competed with by rFab b96.11, DPC and DPD were found more frequently in the last sample than in the initial sample, as assessed by degree of competition for each rFab (p value: 0.002, 0.002 and 0.001 respectively) (data not shown). However, no significant difference in epitope specificities between the first and the last sample of the low-risk individuals was observed for any of the rFabs.

Finally, when comparing the degree of epitope binding in the last sample, we observed a significant difference between both groups (Fig. 4). GAD65Abs in the sera of high-risk individuals were significantly better competed with by rFab b96.11 than those in sera of low-risk individuals (p=0.001). No differences in the degree of epitope binding between groups were observed for the other three rFabs, and no differences were observed when comparing individuals in the high-risk group who progressed to diabetes with high-risk individuals who did not progress to diabetes.

Relationship between GAD65Ab specificities and risk HLA markers Possible associations between the HLA-DQB1 genotype and GAD65Ab specificity were analysed (Table 2). No association between individuals with the risk-conferring HLA genotype and GAD65Ab specific to the DPA-defined epitope was found. However, significantly more individuals with the risk-conferring HLA genotype showed an increase in binding to the b96.11-defined epitope over time as compared with individuals with HLA genotypes that were not or were or negatively associated with risk of type 1 diabetes (p=0.0004). The same association was found between individuals with the risk-conferring HLA genotype and the combination of GAD65Ab specific to the DPA- and b96.11-defined epitopes (p=0.0018).

To better understand how the dynamic changes in epitope binding compare with conventional measures of risk, we analysed the sensitivity and specificity for risk defined by multiple islet autoantibodies. We found that in 72% (16/22) of the high-risk individuals GAD65Ab showed an increase in binding to the b96.11-defined epitope, while this was observed only for 10% (4/38) of the low-risk individuals. This results in a positive predictive value of 80%. In our study population the HLA-genotype analysis comes to a positive predictive value of 54% (Table 1).

Epitope specificities change gradually with no correlation with time before onset When correlating changes in epitope specificities with time before clinical onset of the disease in individual patients, no clear relationship was observed (Fig. 5). One individual had a dramatic increase in binding to all four epitopes within the last 2 months before diag-

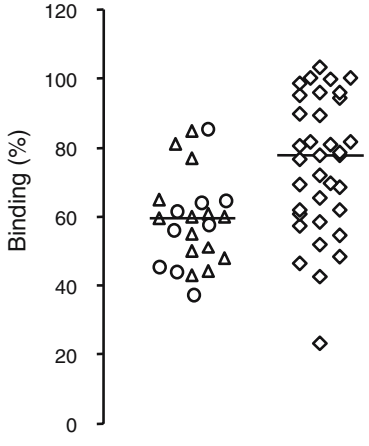

Fig. 4 Binding, in final serum samples, to GAD65 in the presence of rFab b96.11. Sera from high-risk individuals (*left, triangles and circles*) and low-risk individuals (*right, diamonds*) were analysed. Individuals that progressed to type 1 diabetes during follow-up are represented as white circles. Median binding is indicated by a horizontal line

Table 2 Association between HLA genotype and GAD65Ab specificity

	Risk HLA genotype (n=26)	Not associated or negatively associated with type 1 diabetes (n=34)
Sera specific to DPA-defined epitope (first sample)	18	17
Sera with increasing GAD65Ab specificity to b96.11-defined epitope (over time)	14	6
Sera specific to b96.11- and DPA-defined epitopes (first sample)	17	12
Median GAD65Ab titre (range)	0.37 (0.14–1.8)	0.27 (0.1–1.6)

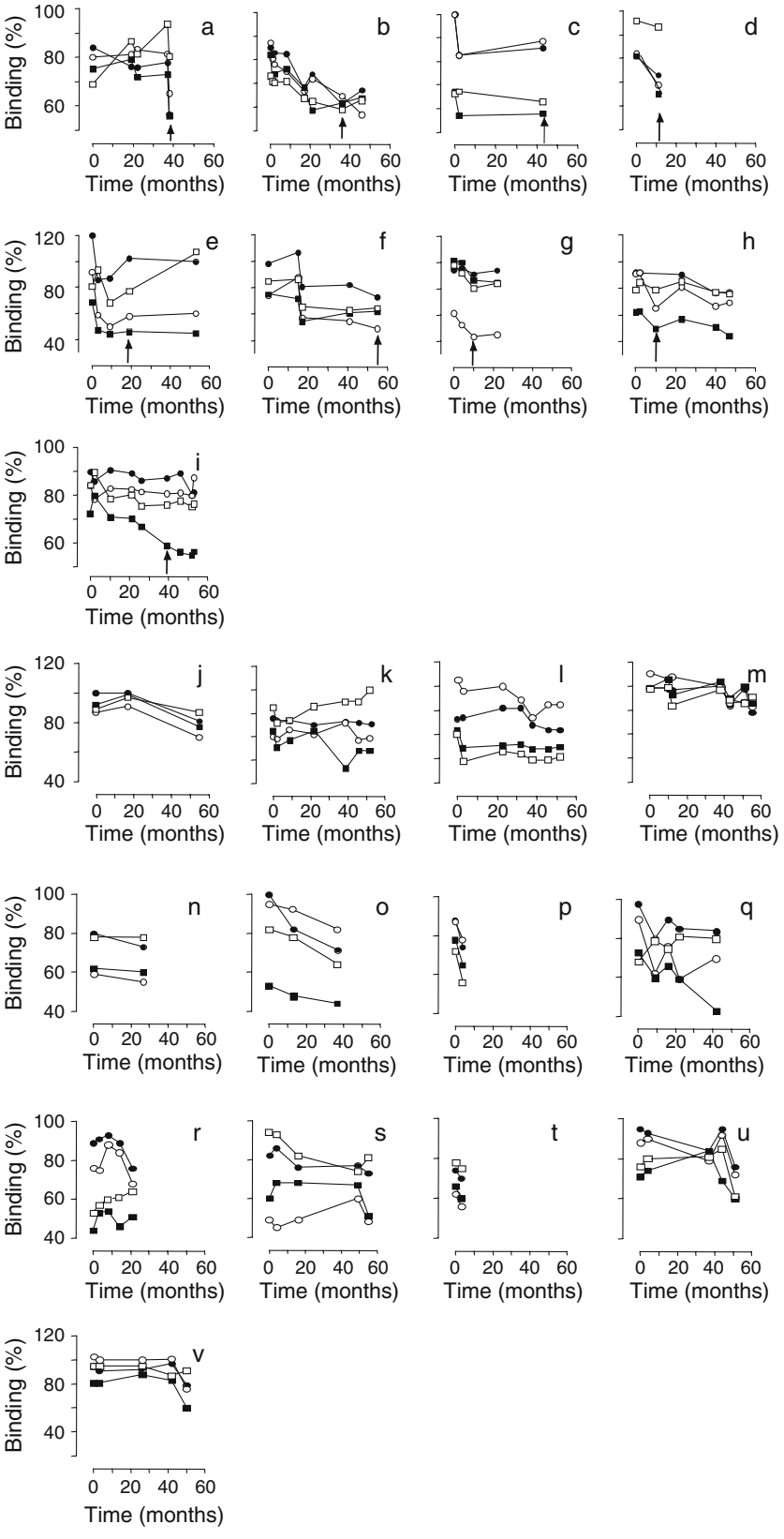

Fig. 5 **a**–**v** Epitope specificities of the high-risk individuals, shown as a function of time. Panels show binding to GAD65 in the presence of rFab DPA (*white squares*), b96.11 (*black squares*), DPC (*white circles*), and DPD (*black circles*). Nine of the individuals progressed to type 1 diabetes (**a**–**i**), the time of clinical diagnosis being indicated by the *arrow*

nosis (Fig. 5a). Most of the other individuals exhibited a more gradual change in their epitope specificities over time. For five individuals, samples were available after onset of the disease (Fig. 5b, e, g–i). No further significant change in epitope specificities was observed after onset of disease, with the exception of one child, in whom a decrease in binding to the DPA-recognised C-terminal epitope was observed (Fig. 5e).

Discussion

Our previous analysis of disease-associated GAD65Ab epitopes in type 1 diabetes revealed two conformational epitopes that are located in the middle of the molecule and were specifically recognised by sera of type 1 diabetes patients [17]. In the present study, we addressed the question of whether this epitope pattern develops as an individual progresses to type 1 diabetes, or whether it is an inherent feature of the pre-diabetic GAD65Ab response. We investigated GAD65Ab epitope profiles in sera obtained from a longitudinal study of GAD65Ab-positive school children, some of whom were at higher risk of progressing to type 1 diabetes, as defined by multiple islet autoantibody positivity.

In high-risk individuals, of whom more than 40% developed diabetes during the follow-up period, we observed that: (1) sera recognise a specific combination of GAD65Ab epitopes directed towards a middle epitope and a C-terminal epitope (recognised by rFab b96.11 and DPA respectively); (2) high risk of progression to disease is associated with the emergence of antibodies specific for conformational epitopes at the N-terminus and the middle region; and (3) the degree of binding to already established Ab epitopes located in the middle and the N-terminus increases in high-risk individuals, but not in low-risk individuals. These Ab binding characteristics may reflect epitope spreading and antibody maturation. No significant differences in the epitope binding pattern or in the longitudinal changes in epitope binding were observed between progressors and non-progressors in the high-risk group. The remarkably stable epitope pattern in the low-risk individuals is in agreement with a previous report showing that GAD65Ab-positive individuals who did not progress to type 1 diabetes demonstrated a similarly stable epitope reactivity [31].

Intramolecular Ab epitope spreading is a phenomenon in which new epitopes within the same molecule are recognised over time (reviewed in [32, 33]). While this has been well demonstrated in various experimentally induced or spontaneous animal models of autoimmunity, the evidence for epitope spreading in human autoimmune diseases is limited (reviewed in [34]). Possible intramolecular Ab epitope spreading has been reported in patients with systemic lupus erythematosus [35]. Intramolecular Ab epitope spreading associated with progression to disease was recently reported for endemic pemphigus foliaceus (fogo selvagem) [36]. Because of the difficulty in identifying sufficient numbers of individuals that have been longitudinally followed until onset of type 1 diabetes, GAD65Ab reactivity during the pre-diabetic period has remained poorly defined. In the few studies addressing this issue, no specific changes in GAD65Ab reactivities were observed as the individuals progressed to disease onset [31, 37, 38]. However, in these previous reports, GAD65/67 fusion proteins were used to analyse GAD65Ab epitope binding. Comparing GAD65Ab epitope analysis using GAD65/67 fusion proteins and GAD65-specific rFabs, we observed that the conformational epitopes located in the middle of GAD65 are very sensitive to conformational changes and may not be well preserved in fusion proteins [39]. The disease-specific GAD65Abs that recognise these conformational epitopes may therefore not bind to the fusion protein; thus, changes in GAD65Ab specificity to these middle epitopes would not be detected.

While binding to the C-terminal epitope recognised by DPA appears to be unaffected, the recognition of both middle (DPC and b96.11) and N-terminal (DPD) autoantibody epitopes progressively increases during progression to diabetes. The finding of epitope spreading towards N-terminal epitopes confirms results from an earlier study of pre-diabetic children of parents with type 1 diabetes [31]. Early reactivity to the middle and C-terminal epitopes of GAD65 has previously been described in offspring of parents with type 1 diabetes [14, 18, 37, 40]. Since the inclusion criterion for our study was GAD65Ab positivity, we do not have data on the transition from GAD65Ab negativity to GAD65Ab positivity. Therefore, the question of whether GAD65Ab reactivity to the C-terminal and middle region of the molecule arise together or successively cannot be addressed in this study.

To our knowledge, this is the first time that GAD65Ab epitope changes specifically associated with the risk of developing diabetes have been described. These changes occurred at different times before the onset of disease in those individuals who developed the disease. While in most cases, the epitope specificities changed gradually, in some cases rapid changes in epitope recognition occurred months or years before onset. It is notable that the epitope specificities remained relatively stable after the clinical onset of disease, which is in agreement with previous findings [41, 42].

We conclude that the GAD65Ab response in high-risk individuals is dynamic. The cause of these dynamic changes in GAD65Ab binding remains unclear. Two possible contributors to this phenomenon are antigen presentation specific to genetically predisposed individuals and environmental factors. The association between risk-conferring HLA types and GAD65Ab epitope specificity may explain previous studies demonstrating an earlier age at onset in children with high-risk HLA [43–45]. The age-dependent risk between HLA and type 1 diabetes is not understood but may be marked by an HLA-dependent development of epitope-specific GAD65Ab. The dynamic changes in binding to the b96.11-defined epitope may contribute to our ability to predict risk, apart from the standard risk assessment based on HLA genotype.

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