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Early Development and Spreading of Autoantibodies to Epitopes of IA-2 and Their Association with Progression to Type 1 Diabetes

Heike E. Naserke,* Anette-G. Ziegler,† Vito Lampasona,‡ and Ezio Bonifacio‡‡

Autoimmunity precedes clinical type 1 diabetes, and indicators of maturing autoimmune responses may be useful markers for disease prediction. To study this, epitope maturation of autoantibodies to the related protein tyrosine phosphatase (PTP)-like autoantigens IA-2 and IA-2β was examined in sequential samples from birth in a cohort of 21 offspring developing multiple islet autoantibodies and a similar cohort of 48 relatives of patients with type 1 diabetes recruited at an older age. Initial reactivity in offspring was heterogeneous against the IA-2 juxtamembrane region (10/21) and PTP domains (13/21), and both specificity and extent of initial IA-2/IA-2β autoantibodies were associated with HLA class II genotype. Intra-IA-2 epitope spreading and/or intermolecular spreading to IA-2β epitopes were observed in seven offspring. In contrast, in older relatives, IA-2/IA-2β Ab reactivity was stable and spreading rare. Development of diabetes in children was associated with the presence of Abs to the IA-2 juxtamembrane region. Other indicators of maturing immune response to multiple determinants (7–9). A higher titer of islet cell Abs (ICA) and, moreso, with the number of different islet autoantibodies present (2–6). Other indicators of fetal and neonatal age are structurally related proteins that are around 70% similar in their antigenic intracellular regions (11, 12). Autoantibodies to each have been mapped and recognize epitopes that are specific to each protein and epitopes that are shared by both IA-2 and IA-2β (7, 13, 14). Epitope reactivity to these Abs is therefore a useful model for the study of humoral intramolecular determinant spreading. In this study we have examined which are the early epitopes recognized, whether epitope specificities spread and change during the prediabetic period, and whether specific epitopes, or changes were indicators of diabetes development.

Materials and Methods

Subjects

Offspring of parents with diabetes (BABY-DIAB). The German BABY-DIAB study includes 1429 offspring of parents with type 1 diabetes and 547 offspring of mothers with gestational diabetes prospectively studied from birth (10). Of those recruited at birth, 682 offspring of type 1 diabetes parents and 238 offspring of gestational diabetes mothers have reached the age of 2 yrs and participated in the 2-yr follow-up. All offspring have been tested for autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA), the protein tyrosine phosphatase IA-2 (IA-2A), and islet cells (ICA). Twenty offspring of parents with type 1 diabetes and one offspring from a mother with gestational diabetes were identified to have at least two of these autoantibodies by the age of 2 yrs. These include all those in whom IA-2A were found, since no offspring had IA-2A in the absence of other islet autoantibodies. From these 21 offspring, samples were collected in yearly intervals from birth up to the age of 8 yrs and were included in this study. The median follow-up time (time to diabetes or last contact) of the offspring from birth was 4.4 yrs (range 1.3 to 8.3 yrs). A total of 92 samples were available for this study. Eleven of the children were male, 13 had a mother with diabetes (12 with type 1 diabetes, one with gestational diabetes), 6 had a father with type 1 diabetes, and in 2 children both parents had type 1 diabetes. Nine of the offspring developed overt type 1 diabetes during their participation in the study (median age of onset 3.2 yrs, range 1.3 to 7.1 yrs, Table 1). Diabetes onset was defined as a 2-h blood glucose value exceeding 11.4 mmol/L (200 mg/dl) in the oral glucose tolerance test. Insulin treatment commenced on the day of diagnosis.

First degree relatives of patients with type 1 diabetes (Munich family study). Samples were obtained from 48 first degree relatives of patients with type 1 diabetes who have been tested for IAA, GADA, IA-2A, and ICA in yearly intervals in the context of the Munich family study and who were found to have at least two of these autoantibodies (5). Twenty were
siblings, 25 were offspring, and 3 were parents of patients with type 1 diabetes; 18 were male. The median age at first screening sample was 12.6 yrs (range 1–58 yrs) and the median follow-up time was 3.3 yrs (range 0.1–91 yrs). A total of 132 samples were tested. Twenty of the relatives developed overt type 1 diabetes during follow-up (median age at onset 15.5 yrs, range 1.9–29.4 yrs).

Characterization of Abs to IA-2 and IA-2β epitopes

Abs to IA-2 and IA-2β epitopes were measured by radio binding assay (7). All samples were tested against the IA-2ic, IA-2βic, IA-2α, IA-2β, IA-2PTP, and IA-2βPTP constructs as previously described (7). Bacterially expressed recombinant IA-2PTP, IA-2βPTP, and IA-2α and IA-2β (juxtamembrane region) proteins were used for competition since they have been repeatedly shown to be capable of inhibiting Ab binding to recombinant and native IA-2 or IA-2β Ags (7, 11, 13–16). Based upon direct binding and competition, Abs were characterized into four reactivities: 1) reactivity to epitopes in the PTP domain that are shared between IA-2 and IA-2β-specific PTP; 3) reactivity to epitopes in the PTP domain that are unique to IA-2 (IA-2-specific PTP); 2) reactivity to epitopes in the juxtamembrane region of IA-2; and 4) Reactivity to epitopes in the PTP domain that are unique to IA-2β (IA-2β-specific PTP).

HLA typing

HLA-DR and -DQ alleles were determined using PCR-amplified DNA and nonradioactive sequence-specific oligonucleotide probes (17).

Statistical analysis

Ab prevalences and their association were compared using the χ² or Fisher exact test. High risk HLA genotypes were defined as HLA DRB1 03,04 and HLA DRB1 04,04 since these have been shown to confer the highest risk for type 1 diabetes (18). Kaplan-Meier life table analysis was used to determine the cumulative risk for relatives to develop type 1 diabetes. For the subjects of the BABY-DIAB study, follow-up started at birth and ended with diabetes onset or with the day of last contact with the family; for subjects of the family study, it started with the date at birth and ended with diabetes onset or with the day of last contact. For life table analysis of data combined from both cohorts, follow-up started on the day of first detection of IA-2/IA-2β Abs. Differences in survival were compared by the log-rank test. Confidence intervals (95% CI) of the cumulative risk were calculated from the SE. For all statistical methods the Statistical Package for Social Sciences (SPSS, Chicago, II) was used.

Results

Appearance of IA-2/IA-2β reactivity

Of the 21 offspring from the BABY-DIAB cohort, 17 developed autoantibodies to IA-2/IA-2β or portions of IA-2/IA-2β (Table I). In six of these, Abs to a single region or specificity were identified in the first sample with IA-2/IA-2β Abs. This was the IA-2 juxtamembrane region in four subjects, IA-2-specific PTP domain epitopes in one, and cross-reactive PTP epitopes in one. The majority (10/17) of subjects with IA-2/IA-2β reactivity had Abs to the IA-2 juxtamembrane region in the earliest sample (Tables I and II). However, these Abs were not consistently the first IA-2/IA-2β reactivity to be detected, and IA-2/IA-2β PTP domain Abs were also found in addition to (six subjects), or in the absence of (seven subjects), IA-2JM Abs in the first sample. The appearance of IA-2JM Abs occurred independently from that of PTP domain reactivity.

Of the 48 first degree relatives with multiple islet autoantibodies, 31 (65%) had reactivity to IA-2/IA-2β (Table II). Twenty-seven had IA-2/IA-2β Abs already in the first available sample, while 4 relatives converted and developed IA-2/IA-2β Abs during follow-up. IA-2JM Abs were found in 18 (58%) relatives, IA-2-specific PTP in 20 (65%), cross-reactive PTP in 20 (65%), and
IA-2β specific PTP in 5 (16%) (Table II). A single reactivity was found in 13 (42%) cases (6 with IA-2JM, 3 with IA-2-specific PTP, and 4 with cross-reactive PTP), and 18 (58%) had more than one Ab reactivity. Of the family study relatives who converted during follow-up, one developed strong cross-reactive PTP Abs 4 yrs after the first sample, which was taken at the age of 1 yr. The second had IA-2JM Abs at 8 yrs of age (1.6 yrs after the first available sample), the third was 59 yrs of age when she developed IA-2JM and IA-2-specific PTP Abs 7.3 yrs after the first available sample, and the fourth developed cross-reactive PTP Abs at 3 yrs of age, 0.5 yrs after the first sample.

Spreading of IA-2/IA-2β reactivity

The presence of additional IA-2/IA-2β Ab reactivities in follow-up samples was seen in seven (40%) offspring from the BABY-DIAB cohort and in only one (3%) of the family study relatives, where reactivity was relatively stable over time (Table III). Offspring in the BABY-DIAB cohort who did not show spreading had either very little follow-up or multiple reactivity already in their first sample. While there was no consistent spreading of the reactivity, some patterns could be distinguished. Spreading to IA-2-specific PTP Abs was most common and seen in six subjects (Nos. 1032.4, 3929, 4262, 2223, 1649, and the family study relative No. 1032.3). In subjects 1032.4, 3929, and 4262, IA-2-specific PTP Abs appear to spread from the IA-2 juxtamembrane region, while in subjects 1649 and 1032.3 spreading is from shared IA-2/IA-2β PTP epitopes, and in subject 2223 IA-2-specific PTP reactivity spread from either the IA-2 juxtamembrane region or shared IA-2/IA-2β PTP epitopes. Only two subjects developed IA-2JM reactivity in later samples (#1628, 4005). Loss or decrease of IA-2JM Abs during follow-up, as shown for subjects 1032.4, 4262, and 2223, was also observed in two other offspring (Nos. 4161 and 1085).

IA-2/IA-2β Abs and HLA

HLA DR and/or DQ genotypes were obtained in 19 of the BABY-DIAB cohort (Table I). Epitope specificity and the extent of epitope reactivity recognized were associated with HLA genotype. The high susceptibility HLA DRB1 03,04 or DRB1 04,04 genotypes were present in 10 of 13 offspring with PTP domain Abs in

<table>
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<th>Subject</th>
<th>Age at Sample (yrs)</th>
<th>IA-2Aa (units)</th>
<th>IA-2JMb</th>
<th>IA-2-specific PTP</th>
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a Threshold for positivity for IA-2A was 5 units; * Indicates onset of type 1 diabetes.

b Amount of reactivity from negative (–) to strong positivity (+++).

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the first sample and 4 of 10 with IA-2JM Abs. All four with IA-
2JM Abs and the high susceptibility genotypes also had strong and
multiple PTP domain reactivity, and none of those with only IA-
2JM Abs in their first sample had these genotypes (p = 0.03,
IA-2JM only, vs PTP domain Abs, Fisher exact test). Moreover, all
six offspring with three or four reactivities had the high suscepti-
bility genotypes compared with only one of five with a single
reactivity (p = 0.02 vs three or four reactivities, Fisher exact test),
and none of those without IA-2 reactivity. Therefore, the high
susceptibility genotypes were associated with PTP domain Abs
and early multiple IA-2/IA-2β reactivity, while the initial IA-2/
IA-2β autoantibodies in those without these genotypes were lim-
ited to the juxtamembrane region of IA-2 and few epitopes (p <
0.005, Fisher exact test).

IA-2/IA-2β Abs and progression to disease
Nine offspring developed type 1 diabetes, and eight of these had
IA-2/IA-2β reactivity (Table I). All eight had IA-2JM Abs. Two
had IA-2JM Abs as a single reactivity; one started with IA-2JM
Abs in the first sample and developed all four IA-2/IA-2β reac-
tivities during follow-up; another offspring had JM and βPTP-
specific Abs first and also developed all reactivities later; one had
IA-2JM, IA-2-specific PTP and cross-reactive PTP Abs; and two
others had all reactivities already in the first sample. Only one of
the nine subjects started without IA-2JM Abs but developed these
in a later sample. The cumulative life table risk by 5 yrs of age to
develop type 1 diabetes was 52% (95% CI, 23–81%) in offspring
with all IA-2/IA-2β reactivities developed diabetes, there was no
significant relationship between the number of IA-2/IA-2β reac-
tivities and progression to disease.

Of 48 family study relatives, 20 (42%) developed type 1 diabe-
etes during follow-up and 18 (90%) of these had IA-2/IA-2β Abs.
Diabetes risk in those with IA-2JM Abs was similar to that ob-
served in the BABY-DIAB children, but, unlike the BABY-DIAB
cohort, several family study relatives without IA-2JM Abs devel-
oped type 1 diabetes and no significant relationship was found
between the presence or absence of IA-2JM Abs and diabetes de-
velopment in this cohort (Table IV). Family study relatives without
cross-reactive PTP domain Abs had the lowest 5-yr risk.

Three of the BABY-DIAB children and 13 relatives of the Mu-
nich family study had only a single IA-2/IA-2β reactivity in all
sequential follow-up samples. Eight had Abs only to the JM re-
gion, four cross-reactive, and four IA-2-specific PTP Abs (Fig. 2).
Five of those with only IA-2JM Abs have developed diabetes com-
pared with only two of those with a single PTP domain reactivity.
In subjects with a single epitope reactivity, the 5-yr risk for pro-
gression to type 1 diabetes was 67% (95% CI, 32–100%) in those
with IA-2JM Abs compared with 34% (95% CI, 0–75%) in sub-
jects with single PTP domain reactivities (p = 0.10).

Discussion
Autoantibodies to islet Ags are important markers of preclinical
type 1 diabetes. The temporal development of autoantibody re-
sponses and epitope spreading in the prediabetic phase is poorly
defined. Such information will be important to understand the
maturation of the immune response in human type 1 diabetes, to
improve risk assessment of progression to disease, and to define
Autoimmunity to IA-2/IA-2β often developed within the first years of life with heterogeneity and interindividual differences in the regions of IA-2/IA-2β that were recognized by Abs. In all cases, initial reactivity was against epitopes found in IA-2, consistent with the suggestion that IA-2 and not IA-2β is the primary PTP-like autoantigen in type 1 diabetes (7). Abs to the juxtamembrane region of IA-2 were the first of these reactivities in the majority, but not all of those developing Abs. The initial humoral autoimmunity to these autoantigens was linked to the HLA class II genotype of subjects. Those with high diabetes susceptibility genotypes immediately developed a broad reactivity to multiple epitopes expressed in both IA-2 and IA-2β, or, if reactivity was not broad, it was against only the PTP domain region. Those with other HLA class II genotypes almost always had IA-2JM Abs and a limited initial reactivity. While associations of islet autoantibodies with specific HLA class II alleles has been shown (19–21), this is the first demonstration in man that the epitopes of related autoantigens recognized by an autoimmune response may be HLA associated. Such an association may result from Ab-specific modification of the epitopes available for presentation on HLA class II molecules and indirectly gives support to the hypothesis that IDDM1 gene diabetes susceptibility is due to presentation of distinct Ag peptides by different HLA class II molecules.

We have previously demonstrated intermolecular spreading of humoral autoimmunity between diabetes-associated autoantigens in the BABY-DIAB cohort (10). In the present study, we also demonstrate that reactivity to single or related autoantigens often expands in the course of the disease in children before diabetes onset. Intra- and intermolecular spreading of the humoral response to IA-2/IA-2β occurred frequently in the BABY-DIAB children, and the majority had developed reactivity to multiple epitopes within the first 5 yrs of life. Despite no consistent pattern of epitope spreading, additional development of IA-2-specific PTP Abs was most frequent, while the additional development of IA-2JM Abs was rare. In contrast, in relatives of the Munich family study, IA-2/IA-2β epitope reaction was much more stable, and spreading was seen in only one subject, who was in fact young (3 yrs of age), and an offspring of a mother with type 1 diabetes.

Family study relatives were older than those of the BABY-DIAB cohort, suggesting that spreading and maturation is more likely to occur early in life. This is also supported by our data from the Barts-Oxford family study cohort, where a de novo detection of IA-2 Abs was relatively infrequent and was usually restricted in its epitope reactivity (7). Unlike what was recently reported in a US cohort (8), we did not find that spreading of Ab reactivity was associated with progression to type 1 diabetes both in the offspring or older relatives. Interestingly, IA-2/IA-2β Ab reactivity was less widespread in the family study relatives than in the BABY-DIAB offspring, and only half had Abs reacting with multiple epitopes of IA-2/IA-2β. This may suggest either that some reactivity is lost over time or that those with high reactivity are more likely to develop diabetes early. Indeed, we found a decrease or loss of IA-2JM Abs over time in some children.

Data in the BOX family study suggested that progression was associated not only with the presence of IA-2 Abs but also with the extent of Ab reactivity to different epitopes (7). The same analysis (one or two reactivities vs >two reactivities) in the Munich family study cohort showed a trend for a higher progression to diabetes in those with broad IA-2/IA-2β epitope reactivity (p = 0.07). Combining the data from the BOX and Munich family study cohorts

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Table IV. Cumulative risk of type 1 diabetes in subjects with IA-2/IA-2β Abs

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* p values were determined by comparing survival with the log-rank test.

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Figure 2. Kaplan-Meier life table analysis of progression to diabetes in the 16 subjects from the BABY-DIAB and Munich family study cohorts with a single IA-2/IA-2β Ab reactivity. Diabetes risk was higher in subjects with IA-2JM Abs only (67% (95% CI, 32–100%); solid line) compared with the risk of subjects with a single PTP domain reactivity (34% (95% CI, 0–75%); p = 0.10; broken line).
maintained a significant association between multiple IA-2/IA-2β epitope reactivity and diabetes development ($p = 0.01$, not shown). This relationship between epitope numbers and disease was not confirmed in the BABY-DIAB cohort where, first, those with a limited epitope reactivity were few and, second, offspring with IA-2 Abs restricted to the juxtamembrane region developed disease. Similarly, in both the Munich and BOX family study cohorts, a number of relatives having IA-2 Abs recognizing only the juxtamembrane region developed diabetes. In all three cohorts we have identified 7 such individuals who developed type 1 diabetes from a total of 37 who had IA-2/IA-2β Ab reactivity before diabetes development. This is a significant proportion and demonstrates the importance of using constructs that detect these Abs. It should be noted also that, in one of these individuals (No. 10324), moderate to strong levels of IA-2JM Abs were detected before Abs against the complete intracellular region and, in a second (No. 4161), IA-2JM Abs were detected in the absence of reactivity to the IA-2ic construct, suggesting that the juxtamembrane region may contain cryptic Ab epitopes. This is consistent with the possibility that autoimmunity to IA-2/IA-2β is secondary to β cell damage.

A significant association of the IA-2JM Abs with a relatively rapid development of diabetes was found in BABY-DIAB offspring. This is remarkable because these Abs were often found in offspring without high susceptibility HLA genotypes and they conferred a high risk for progression to disease even when found in the absence of reactivity to other regions of IA-2/IA-2β. This paradox suggests that, while HLA is clearly important for diabetes development, other independent factors, which include certain autoantibody reactivities, may be just as critical for very early diabetes development. Family study relatives with IA-2JM Abs had a similar risk for diabetes as did the BABY-DIAB offspring, but, unlike the BABY-DIAB cohort, several relatives developed diabetes in the absence of these Abs. This may be explained by, and is consistent with, the observation that IA-2JM Abs decreased over time in some children. A development of diabetes that is associated with an autoantibody response to a specific epitope or region of an autoantigen is intriguing. IA-2JM Abs have particular characteristics. First they are specific to IA-2 and never cross-react with IA-2β. Second, their appearance as a first IA-2/IA-2β reactivity is HLA class II genotype associated and appears to be independent of the appearance of IA-2/IA-2β PTP domain Abs. Third, and importantly, when bound to Ag in vitro they render the IA-2 molecule more accessible to destruction by trypsin (13). Others have shown that Abs can modify the processing of Ag, resulting in both enhancement and inhibition of T cell responses (22, 23). It could be hypothesized, therefore, that the presence of IA-2JM Abs on B lymphocytes results in an extensive protease digestion of IA-2 after internalization, a resultant increased presentation of peptides to T lymphocytes, and, consequently, a more aggressive and/or widespread T lymphocyte reactivity. Such a scenario could explain the relatively frequent and rapid development of diabetes in infants who develop these specific Abs. Clearly, however, there remain interindividual differences in disease development despite the presence of IA-2JM or even multiple IA-2/IA-2β reactivities.

In summary, our data show that the IA-2 juxtamembrane region is an early autoantibody target, that these Abs appear independently from Abs to the PTP domains of IA-2 and IA-2β, that there is a distinct HLA association of the appearance of IA-2JM Abs from that of PTP domain Abs or multiple IA-2/IA-2β Ab reactivity, and that early IA-2JM Abs are associated with a high risk for developing type 1 diabetes. Humoral determinant spreading against IA-2/IA-2β is common in infancy and relatively rare in older subjects, indicating that diabetes-associated autoimmunity is a dynamic process in young children, but relatively stable in adults, and suggesting, as in animal models of the disease, that the autoimmune response is relatively quick to mature. This rapid maturation to a relatively stable response, together with the observation that type 1 diabetes occurs regardless of specific changes in the IA-2/IA-2β epitopes recognized by Abs, suggests that the analysis of autoantibody epitopes is unlikely to provide satisfactory indicators of treatment efficacy in clinical intervention trials aimed at preventing diabetes onset. Moreover, in an analogy with animal models where disease prevention is more successful when intervention is applied before the commencement of destructive autoimmunity (24), it might be expected that intervention therapies in man applied after the autoimmune response has reached maturity may also be relatively ineffective in halting disease development and that their benefit may be limited to delaying diabetes onset.

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