



IA-2 autoantibody affinity in children at risk for type 1 diabetes

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Abstract Autoantibodies to insulinoma-associated protein 2 (IA-2A) are associated with increased risk for type 1 diabetes. Here we examined IA-2A affinity and epitope specificity to assess heterogeneity in response intensity in relation to pathogenesis and diabetes risk in 50 children who were prospectively followed from birth. At first IA-2A appearance, affinity ranged from 10^7 to 10^{11} L/mol and was high ($>1.0 \times 10^9$ L/mol) in 41 (82%) children. IA-2A affinity was not associated with epitope specificity or HLA class II haplotype. On follow-up, affinity increased or remained high, and IA-2A were commonly against epitopes within the protein tyrosine phosphatase-like IA-2 domain and the homologue protein IA-2 β . IA-2A were preceded or accompanied by other islet autoantibodies in 49 (98%) children, of which 34 progressed to diabetes. IA-2A affinity did not stratify diabetes risk. In conclusion, the IA-2A response in children is intense with rapid maturation against immunogenic epitopes and a strong association with diabetes development.

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Abbreviations: GADA, autoantibodies to glutamate decarboxylase; IA-2A, autoantibodies to insulinoma-associated protein 2; IAA, autoantibodies to insulin; IQR, interquartile range; JM, juxtamembrane; PTP, protein tyrosine phosphatase; T1D, type 1 diabetes; ZnT8A, autoantibodies to zinc transporter 8.

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1. Introduction

Type 1 diabetes (T1D) is preceded by the development of autoantibodies to multiple islet antigens such as autoantibodies to insulin (IAA), the 65 kDa isoform of glutamate decarboxylase (GADA), zinc transporter 8 (ZnT8A), and insulinoma-associated protein 2 (IA-2A) [1–6]. Diabetes risk is related to the magnitude and maturity of these autoantibody responses [7–10]. IAA and GADA are typically the first islet autoantibodies to appear in genetically at-risk children [11–14], and these early autoantibody responses can be heterogeneous with respect to antibody affinity, epitope specificity, association with HLA DR-DQ, and diabetes risk [9,10]. Based on these findings, we have proposed that both the nature and intensity of the early immunizing event and the host HLA are critical in determining the subsequent disease pathogenesis in autoimmune diabetes.

IA-2A are also prominent features of pre-T1D. They are almost always detected together with IAA, GADA and/or ZnT8A and are therefore very specific for T1D [2–5,15]. Furthermore, high-titer IA-2A have been associated with high risk of progression to disease [7,16], and spreading of the IA-2A response to include the homologue protein IA-2 β identifies a subgroup of individuals at particularly high risk of rapid development of diabetes [5,17,18]. Here, we asked whether IA-2A affinities vary between children at the time of first IA-2A appearance and change over time, and whether IA-2A affinities are associated with IA-2A epitope binding and/or progression of islet autoimmunity to clinical T1D.

2. Material and methods

2.1. Study cohort and samples

Samples analyzed in this study were obtained from children with a T1D family history who were prospectively followed from birth in the German BABYDIAB study [11] for the development of islet autoimmunity and diabetes. Sera were tested for the appearance of IAA, GADA, IA-2A and ZnT8A at birth and at age 9 months, 2, 5, 8, 11, 14, 17 and 20 years, and autoantibody positive children were tested in 6- to 12-month intervals. A child was considered to have a positive autoantibody outcome if at least two consecutive positive samples for the autoantibody were found, excluding positive results that were likely to be due to placental transfer of maternal autoantibodies. The BABYDIAB study was approved by the ethical committees of Bavaria, Germany (Bayerische Landesärztekammer No. 95357). All participants in this study gave informed consent, and the study was carried out in accordance with the Declaration of Helsinki as revised in 2000.

Fifty children were selected for this study on the basis of having developed IA-2A that were confirmed positive in a subsequent antibody test and having sufficient serum available for IA-2A affinity measurement. We used the first IA-2A positive sample from 40 children and, because of serum limitations, the next available IA-2A positive sample from 10 children (median time from first IA-2A positive sample: 0.85 years, range 0.48–4.3 years). Samples were obtained at median age of 3.8 years (interquartile range, IQR, 2.6–7.2 years). Thirty-four (68%) of the 50 children developed T1D after median IA-2A positive

follow-up of 3.2 years (IQR, 0.96–5.31). IA-2A positive follow-up sera with sufficient volume for affinity measurement were available from 30 of the 50 children.

2.2. Islet autoantibody measurements

IAA, GADA, IA-2A, and ZnT8A were determined using radio-binding assays as previously described [2,11]. Briefly, IAA were measured by Protein A/G radio-binding assays using [¹²⁵I]-recombinant human insulin labeled at tyrosine aa14. GADA, IA-2A, and ZnT8A were measured by Protein A radio-binding assays using [³⁵S]-methionine-labeled *in vitro* transcribed/translated recombinant human GAD65 (aa1-585), IA-2 (aa605-979), and the R325 and W325 variants of ZnT8 (aa268-369), respectively. The upper limit of normal for each assay was determined using Q-Q plots and corresponded to the 99th percentile of 836 control children. Children were considered islet autoantibody positive when two consecutive serum samples were positive. Autoantibody assays were evaluated by the Diabetes Antibody Standardization Program (laboratory 121) [19–21].

IA-2A epitope specificity was determined by Protein A radio-binding assays using [³⁵S]-methionine-labeled *in vitro* transcribed/translated fragments of recombinant human IA-2 and IA-2 β as previously described [22,23]. IA-2 aa605-682, IA-2 aa687-979, and IA-2 β aa662-1033 constructs were used to identify antibodies binding to the juxtamembrane (JM) and the protein tyrosine phosphatase (PTP)-like region of IA-2, and the homologous protein IA-2 β , respectively. The upper 99th percentile of control sera was used as the threshold of positivity for each construct.

2.3. IA-2A affinity measurement

IA-2A affinity was measured by competitive binding experiments similar to previously described IAA and GADA affinity assays [9,10]. Serum (2 μ l) was incubated in duplicate for 48 h at 4 °C in TBST buffer (50 mol/L Tris, 150 mmol/L NaCl, 1% Tween 20, pH 7.4) in presence of 1.0×10^{-15} mol of [¹²⁵I]-recombinant human IA-2 aa604–979 [24] (Medipan, Berlin, Germany) and 5 increasing quantities of unlabeled human IA-2 aa604–979 (5.1×10^{-11} , 5.1×10^{-10} , 5.1×10^{-9} , 5.1×10^{-8} and 5.1×10^{-7} mol, respectively; RSR Ltd., Cardiff, UK) or TBST buffer only in a final volume of 47 μ l. Immune complexes were precipitated for 60 min with 1.5 mg Protein A sepharose (GE Healthcare, Chalfont St Giles, UK) pre-swelled in 50 μ l TBST. Protein A sepharose was subsequently washed 5 times with 1.8 ml of ice cold TBST buffer. Bound [¹²⁵I]-IA-2 was measured using a Gamma counter (Packard Instruments Co., Meriden, Connecticut, USA). Results were expressed as counts per minute (cpm). IC₅₀ and K_d values were calculated by non-linear regression analysis using the GraphPad Prism3 program (GraphPad Software Inc., San Diego, USA), and IA-2A affinity was expressed as reciprocal K_d value (L/mol). Displacement curves were computed directly from the cpm for each competition reaction, and background binding or maximal displacement of radio-labeled ligand was not subtracted from bound cpm (Fig. 1). The reproducibility of IA-2A affinity measurements was determined from replicates of an IA-2A positive serum that was included in each experiment. The inter-assay

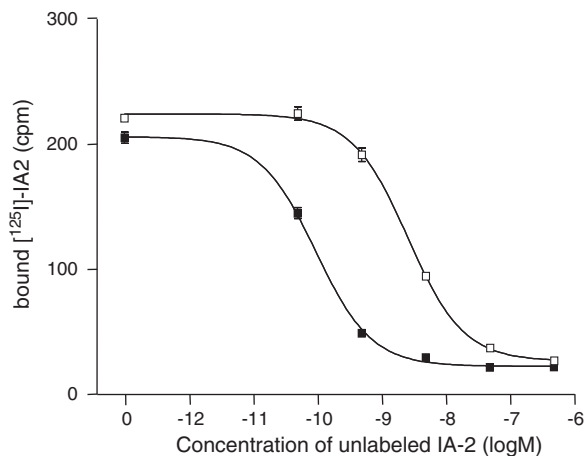


Figure 1 Example competitive binding curves. IA-2A binding to [^{125} I]-labeled human IA-2 (ordinate scale) at increasing concentrations of competitive unlabeled human IA-2 (abscissa) is shown for a serum with high-affinity IA-2A of 1.3×10^{10} L/mol (filled squares) and a serum with low-affinity IA-2A of 3.8×10^8 L/mol (open squares). Both curves are consistent with a one-site binding model. The shift of the second curve to the right indicates lower IAA affinity.

coefficient of variation at a mean affinity of 2.3×10^{10} L/mol was 21.4% ($n = 10$ experiments).

2.4. HLA typing

HLA-DRB1-DQB1 alleles were typed using polymerase chain reaction (PCR)-amplified genomic DNA and non-radioactive sequence-specific oligonucleotide probes [25].

2.5. Statistical analysis

The Mann–Whitney U test and the Kruskal–Wallis test were used to compare IA-2A affinities between groups. Spearman's correlation was used to determine the correlation between variables. For all analyses, a two-tailed P value of <0.05 was considered significant. Statistical analyses were performed using GraphPad Prism3 program (GraphPad Software Inc., San Diego, USA).

3. Results

3.1. Initial IA-2 autoantibody responses

IA-2A developed together with or after IAA, GADA and/or ZnT8A in 49 (98%) of the 50 children, and only one IA-2A positive child was negative for the other three islet autoantibodies. At first IA-2A appearance, affinities ranged from 2.8×10^7 L/mol to 1.0×10^{11} L/mol (median 5.5×10^9 L/mol) and were high ($>1 \times 10^9$ L/mol) in 41 (82%) children. Antibody binding to IA-2-JM epitopes was detected in 38% of samples, 60% bound to IA-2-PTP, 56% to IA-2 β , and 52% to multiple IA-2 epitopes. IA-2A affinity was not significantly associated with age ($r = 0.14$; $p = 0.35$; Fig. 2A), IA-2A titer ($r = 0.25$; $p = 0.08$; Fig. 2B), HLA

DRB1*04 alleles ($p = 0.96$) (Fig. 2C) or IA-2A epitope binding pattern ($p = 0.46$) (Fig. 2D).

3.2. IA-2 autoantibody responses on follow-up

On follow-up, IA-2A affinity remained relatively constant and high (Fig. 3). Among 30 children who had multiple IA-2A positive sera of sufficient volume available for affinity measurement, high-affinity IA-2A ($>1 \times 10^9$ L/mol) were detected in the last follow-up samples of 27 (90%) children. A marked increase from low-affinity ($<1.0 \times 10^8$ L/mol) to high-affinity IA-2A occurred in 3 children. Spreading of IA-2A reactivity to epitopes within the IA-2-PTP region and IA-2 β was common (Table 1; child number 1–27) and frequently associated with progression to T1D (74% of those children became diabetic on follow-up). Three children did not develop IA-2-PTP-reactive antibodies (Table 1; child number 28–30), among them the only child who remained single IA-2A positive (child number 29). Of note, these 3 children had lower IA-2A affinities as compared to children with IA-2-PTP-reactive IA-2A ($p = 0.008$) and they did not develop diabetes. Nevertheless, IA-2A affinity could overall not stratify progression to T1D among the IA-2A positive children.

4. Discussion

Autoantibodies to IA-2 are associated with a high risk for developing type 1 diabetes [5–8]. Here we showed that low affinity IA-2A were rare, and that IA-2A affinity was almost always very high from the first detection of autoantibodies. These data were consistent with the strong association of IA-2A with multiple islet autoantibodies and with previous findings where we and others have demonstrated that also IAA [9,26] and GADA [10] were of high affinity when they occurred together with other islet autoantibodies. These data support the notion that the development of multiple islet autoantibodies shows evidence of strong and prolonged or repeated stimulation by multiple antigens.

Study strengths are the cohort and timing of samples which were at or close to the first appearance of IA-2A in 50 children followed from birth in the BABYDIAB study [11]. Furthermore, we could follow-up IA-2A affinity and epitope reactivity in over half of the children, which allowed us to observe affinity maturation and epitope spreading. Limitations include the restriction of the cohort to children who have an affected family member, and the relatively small number of IA-2A positive children in our study. Thus the findings may not be representative of IA-2 autoimmunity that occurs in older individuals or in children without a first-degree T1D family history, where single IA-2A may be more frequent [3,6].

Although the affinity of IA-2A was found to vary more than 1,000-fold at seroconversion, much of this variation was within the high affinity range, and the few cases of lower affinity IA-2A at seroconversion increased to high affinity on follow-up. All but one of the IA-2A positive children had developed other islet autoantibodies (IAA, GADA and/or ZnT8A) at first IA-2A appearance. IA-2A affinity could not stratify T1D risk in the multiple autoantibody positive children, which is in line with what has previously been shown for IAA and GADA [9,10,26]. GADA and IAA

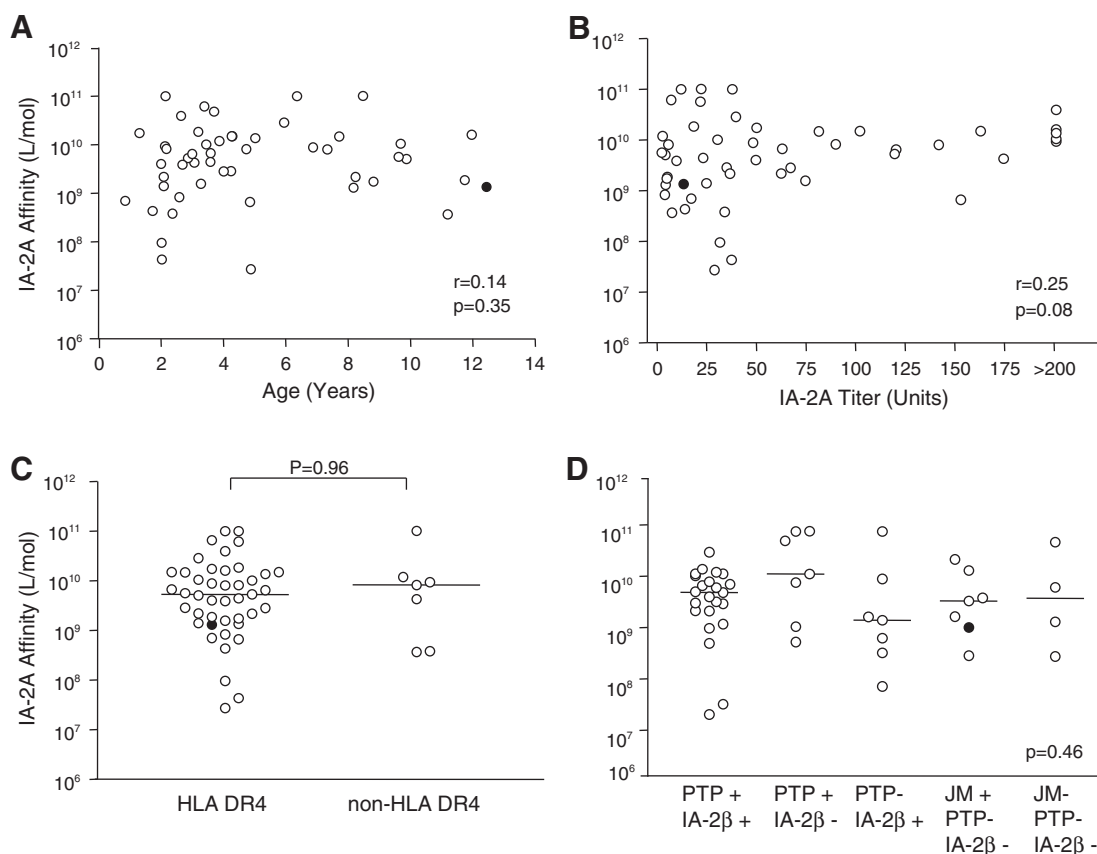


Figure 2 Relationship between IA-2A affinity and the age of IA-2A appearance, IA-2A titer, HLA, or IA-2A epitope binding in the first available IA-2A positive sera from 50 children. (A and B) IA-2A affinity (ordinate scale) plotted against the age of the child at the time of blood sampling (A) or the titer of IA-2A (B) (abscissa). (C) IA-2A affinity in children who had HLA DRB1*04 alleles (HLA DR4) compared with those who did not have these alleles (non-HLA DR4). (D) Affinity of IA-2A grouped according to their binding to epitopes contained within residues 687–979 (PTP) and residues 605–682 (JM) of IA-2, and residues 662–1033 of IA-2 β . The child who had single IA-2A is identified (filled circle). In (C) and (D), the median affinity is indicated for each group.

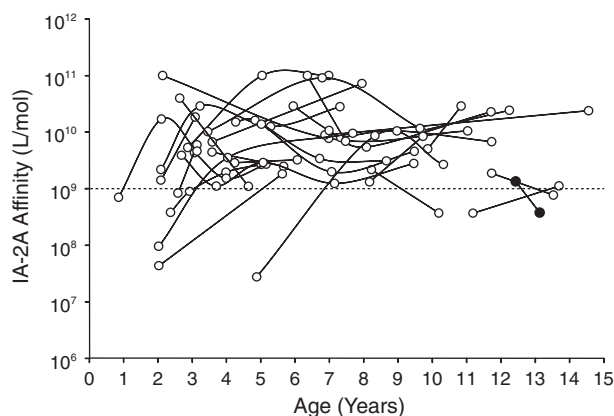


Figure 3 IA-2A affinity over time in 100 follow-up serum samples from 30 children. Consecutive samples from individual children are connected by lines. The child who had single IA-2A is identified (filled circles). Affinity of 1.0×10^9 L/mol is indicated (dotted line).

differed in that their affinity ranged up to 10^6 -fold, with a much larger proportion of these antibodies having low affinity. The lower affinity was reflected by a much more frequent appearance of either GADA or IAA on their own, and in many cases the persistence of single autoantibodies. GADA and IAA affinity associated with antibody binding to specific epitopes and HLA class II haplotypes, and was effective in stratifying risk for diabetes in children with single autoantibodies [9,10].

Along with the relatively homogeneous affinity, IA-2A epitope specificity was commonly against epitopes located in the IA-2-PTP region as observed in previous studies [22,27–30]. Reactivity to JM epitopes was not infrequent, particularly early in the IA-2A response, but rarely remained the dominant antibody response on follow-up. The one child with persistent antibody binding restricted to the JM region had no other islet autoantibodies and has not developed diabetes.

5. Conclusions

Our data show that the IA-2A response is intense with rapid maturation against immunogenic epitopes. The response is

Table 1 IA-2 autoantibody (IA-2A) epitope binding patterns in the first IA-2A positive sera of 30 children, and on follow-up. Antibody binding to the juxtamembrane (JM) and the protein tyrosine phosphatase (PTP)-like region of IA-2, and to IA-2 β is indicated. IA-2A affinity is shown for the last available IA-2A positive follow-up sample of each child. It is indicated whether a child has developed type 1 diabetes (T1D).

Child No.	Initial IA-2A reactivity			IA-2A reactivity on follow-up			IA-2A affinity ($\times 10^9$ L/mol)	T1D
	IA-2-PTP	IA-2 β	IA-2-JM	IA-2-PTP	IA-2 β	IA-2-JM		
1	+	+	+	+	+	+	1.8	Yes
2	+	+	+	+	+	+	72.0	Yes
3	+	+	—	+	+	+	6.8	Yes
4	+	+	—	+	+	+	2.8	No
5	+	+	—	+	+	—	1.1	Yes
6	+	+	—	+	+	—	2.7	Yes
7	+	+	—	+	+	—	3.1	Yes
8	+	+	—	+	+	—	4.6	Yes
9	+	+	—	+	+	—	8.5	Yes
10	+	+	—	+	+	—	25.0	Yes
11	+	+	—	+	+	—	3.2	Yes
12	+	+	—	+	+	—	2.7	No
13	+	—	—	+	+	+	4.6	Yes
14	+	—	—	+	+	—	19.0	Yes
15	+	—	—	+	+	—	24.0	Yes
16	+	—	—	+	+	—	12.0	No
17	+	—	—	+	+	—	6.9	No
18	—	+	+	+	+	+	6.0	Yes
19	—	+	—	+	+	+	11.0	Yes
20	—	+	—	+	+	—	0.8	No
21	—	+	—	+	+	—	9.6	No
22	—	—	+	+	+	+	2.7	Yes
23	—	—	+	+	+	+	2.5	Yes
24	—	—	+	+	+	+	29.0	Yes
25	—	—	+	+	+	+	28.0	No
26	—	—	+	+	+	+	23.0	No
27	—	—	—	+	+	—	100.0	Yes
28	—	+	+	—	+	+	0.4	No
29 ^a	—	—	+	—	—	+	0.4	No
30	—	—	—	—	—	—	1.1	No

^a Child 29 is the only single IA-2A positive child.

commonly preceded or accompanied by other islet autoantibodies suggesting that this immunogenicity is likely to be due to the intensity of the immunization process rather than a propensity of IA-2 to elicit immune responses.

6 . Conflict of interest statement

Michael Powell, Jadwiga Furmaniak and Bernard Rees Smith are employees of RSR Ltd. RSR Ltd is a developer of medical diagnostics including kits for measuring diabetes autoantibodies. Otherwise the authors declare that there are no conflicts of interest.

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