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Karlsburg Type I diabetes risk study of a general population: frequencies and interactions of the four major Type I diabetes-associated autoantibodies studied in 9419 schoolchildren

M. Strebelow[1], **M. Schlosser**[1], **B. Ziegler**[1], **I. Rjasanowski**[2], **M. Ziegler**[1]

[1] Institute of Pathophysiology Karlsburg, University of Greifswald, Germany
[2] Centre of Diabetes and Metabolic Disorders Karlsburg, Germany

Abstract

Aims/hypothesis. The Karlsburg Type I (insulin-dependent) diabetes risk study on schoolchildren aims to evaluate the predictive diagnostic value of diabetes-associated autoantibodies in the general population.
Methods. We took capillary serum from 9419 schoolchildren, aged 6–17 years, for testing of autoantibodies (AAbs) to glutamic acid decarboxylase (GADA), protein tyrosine phosphatase (IA2A) and insulin (IAA) by 125I-antigen binding. We also tested for autoantibodies to cytoplasmic islet cell antigens (ICA) immunohistochemically.
Results. By testing of 9419 sera for the four AAbs at cut-off at or greater than the 98th centile for the radioassayed AAbs and at or greater than 10 Juvenile Diabetes Foundation (JDF) units for ICA, 8.1 % of schoolchildren had at least one AAb. We found that 3.04, 2.97, 2.35, and 0.86 % had IAA, GADA, IA2A or ICA, respectively. 7.3 % had only one AAb and 0.8 % (75) had two or more AAbs, reflecting a risk to develop diabetes. Thus, by primary screening by combined testing of GADA and IA2A, 98.7 % (74/75) would be identified. At high AAb levels, cut-off at or greater than the 99.8th centile and at or greater than 40 JDF units for ICA, 0.23 % (22/9419) of schoolchildren, similar to the disease prevalence of 0.3 %, had two or more AAbs. Ten of 17 children tested had reduced ($p < 0.001$) first-phase insulin secretion by intravenous glucose tolerance test. Six of 22 subjects developed Type I diabetes within a follow-up of 19 ± 10 months.
Conclusion/interpretation. For children older than 5 years the combined anti-GAD/IA2 test with cut-off at or greater than the 98th centile should be used for primary screening followed by testing for IAA and ICA. Subjects at risk for diabetes have two or more AAbs at or greater than the 98th centile. Subjects at risk for rapid progression to Type I diabetes have two or more AAbs at or greater than the 99.8th centile. [Diabetologia (1999) 42: 661–670]

Keywords Type I (insulin-dependent) diabetes, prediction, screening approach, schoolchildren, normal population, autoantibodies, GADA, IA2A, IAA, ICA.

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Corresponding author: Dr. M. Ziegler, Institute of Pathophysiology, University of Greifswald, Greifswalder Strasse 11 a, D-17 495 Karlsburg, Germany
Abbreviations: AAbs, Autoantibodies; ICA, islet cell antigens; GADA, glutamic acid decarboxylase; IA2A, protein tyrosine phosphatase; IAA, insulin autoantibodies; JDFU, Juvenile Diabetes Foundation units; KU, Karlsburg arbitrary units; FDR, first degree relative; FPIR, first-phase insulin response; ROC, receiver-operating characteristic.

Type I (insulin-dependent) diabetes mellitus is a chronic autoimmune disease characterized by selective destruction of the insulin-producing beta cells in the pancreatic islet, accompanied by antibody formation against beta-cell components [1, 2]. Although Type I diabetes appears to be a T-cell mediated disease, the autoantibodies (AAbs) to beta-cell antigens may be useful markers of beta-cell destruction that precedes clinical manifestation [3]. These include the heterogeneous islet cell cytoplasmic antibodies (ICA), AAbs to glutamic acid decarboxylase (GADA) and to the protein tyrosine phosphatase-

like molecule IA2 (IA2A), and insulin autoantibodies (IAA). More recently, a direct correlation between humoral and cellular immune reactivity to the autoantigen IA2 has been proved, tending to confirm its role in the pathogenesis of Type I diabetes [4].

Increasingly accurate prediction of Type I diabetes, based on AAb markers, has become possible in first-degree relatives (FDR) of diabetic patients [5]. ICA were highly predictive in this context, and their predictive diagnostic value could be enhanced by combined analysis with further beta-cell autoantibodies [6–9]. Although a positive family history of Type I diabetes is a risk factor for the disease, some 90% of cases have no affected first-degree relative [10]. The present challenge is to achieve predictive diagnostic power in the general population, although the overall risk of Type I diabetes is considerably lower than in subjects with a family history of diabetes.

Conflicting results have been published for ICA in schoolchildren and several studies found that the ICA prevalence greatly exceeded the overall risk of Type I diabetes in the general population [11–13]. This implies that ICA testing yields a high proportion of false-positives in a general population. Similar results have been reported for IAA [13].

The aim of the Karlsburg Type I diabetes risk study is to evaluate a strategy to assess the risk of progression to clinical manifestation of the disease in a large general population of schoolchildren by a prospective study based on single and combined antibody measurement and on analysis of genetic markers of Type I diabetes susceptibility. The aim of the first part presented here was the cross-sectional study of 9419 schoolchildren to evaluate the frequency and levels of the four major Type I diabetes-associated autoantibodies ICA, GADA, IA2A, and IAA as well as their interactions with one another and with age and sex. A strategy for assessing the risk of progression to diabetes is suggested by comparing these AAb data with those of 86 subjects with newly diagnosed Type I diabetes, including those of six schoolchildren who progressed to insulin-dependent diabetes during the short follow-up period of the Karlsburg schoolchildren study.

Subjects and methods

Normal schoolchild population. We selected randomly 13 953 schoolchildren in the Greifswald region for testing. Of these children 12 558 (mean age 11 ± 3; median 11; ranging from 6–17 years; 6088 boys, 6470 girls) were tested. The study protocol was approved by the Ministry of Culture and Education of Mecklenburg-Vorpommern and the ethics committee of the Ernst Moritz Arndt University, Greifswald. Informed consent was obtained from the parents of the schoolchildren. Capillary blood samples were collected from January 1995 until June 1996 and the sera were stored at –20 °C until assayed. As ascertained by questionnaires none of the children had first-degree relatives with Type I diabetes. From 9419 children (4722 girls, 4697 boys) sufficient blood serum was available to detect all four AAbs, GADA, IA2A, IAA, and ICA. From this cohort a control group of 6877 schoolchildren without a family history of either type of diabetes or of autoimmune thyroid diseases and not positive for two or more diabetes-associated AAbs (IA2A, GADA, IAA, and ICA) were selected to define the cut-off limits for AAb positivity.

Patients with newly diagnosed Type I diabetes. To examine the diagnostic sensitivity of the assays used for detection of AAbs and their combinations, 86 age-matched newly diagnosed Type I diabetic patients were recruited for the study within 3 days after starting insulin therapy (41 girls, 45 boys; mean age 12 ± 3 years; median 12; ranging from 6 to 17 years; 17 children < 10 and 69 ≥ 10 years). From all patients and their parents informed consent was obtained.

Autoantibody detection. Testing for AAbs against the biochemically characterized antigens GAD65, IA2ic and insulin were carried out by the 125I-labelled recombinant antigen binding tests and ICA were determined immunohistochemically.

Detection of autoantibodies to GAD65. For detection of GADA human recombinant GAD65 (Synectics Biotechnology AB, Stockholm, Sweden) was used after iodination as described in detail [14, 15]. 20 µl (12 000 cpm) of 125I-GAD65 were incubated with 20 µl serum in duplicates for 18 h at 4 °C. The immune complexes were precipitated with 100 µl of Protein A suspension (Calbiochem, Bad Soden, Germany). Bound 125I-GAD65 were expressed as arbitrary Karlsburg units (KU/l) derived from a standard curve constructed using a GADA-positive human serum diluted 1/1000 defined to be 100 KU/l. The interassay and intraassay coefficient of variation (CV) were 4 and 1.9% at 6 KU/l, 7.2 and 2.3% at 12.0 KU/l, and 6.8 and 4.2% at 22 KU/l ($n = 20$), respectively. The cut-off was defined as 2.14 KU/l (98th centile of the control group). This assay had a sensitivity and specificity of 74.5 and 98%, respectively, in the Multiple Autoantibody Workshop 1995 and of 100% for both in the Third International GAD Antibody Workshop 1997.

Detection of autoantibodies to IA2. Recombinant IA2ic was a kind gift of N. Morgenthaler (BRAHMS Diagnostica, Berlin, Germany) and produced by coupled in vitro transcription and translation of human IA2ic cDNA [16, 17]. The assay was done according the anti-IA2 test from BRAHMS Diagnostica. 200 µl (20 000 cpm) of 125I-IA2ic were added to 20 µl serum in duplicate tubes. After incubation for 18 h at room temperature the immune complexes were precipitated with 100 µl of Protein A suspension as described for the GADA assay. The levels of IA2A were calculated by a standard curve of an IA2A-positive serum pool defined to be 140 KU/l at dilution 1/4. The interassay and intraassay CV were 7.7 and 5.4% at 3 KU/l, 6.7 and 2.6% at 9 KU/l, and 5.5 and 4.9% at 145 KU/l ($n = 20$), respectively. The cut-off was defined to be 1.4 KU/l (98th centile of the control group).

Detection of insulin autoantibodies (IAA). IAA were detected according Vardi et al. [18, 19] with a slight modification. Fifty µl (25 000 cpm) of 0.1 nmol/l A14 mono-125I-human insulin (specific activity 360 mCi/mg, a kind gift of Hoechst, Frankfurt, Germany) were incubated with 50 µl serum in duplicates for 3 days at 4 °C with and without 333 nmol/l unlabelled human insulin to display the specific 125I-insulin binding. Antibody-bound 125I-insulin was precipitated by polyethylene gly-

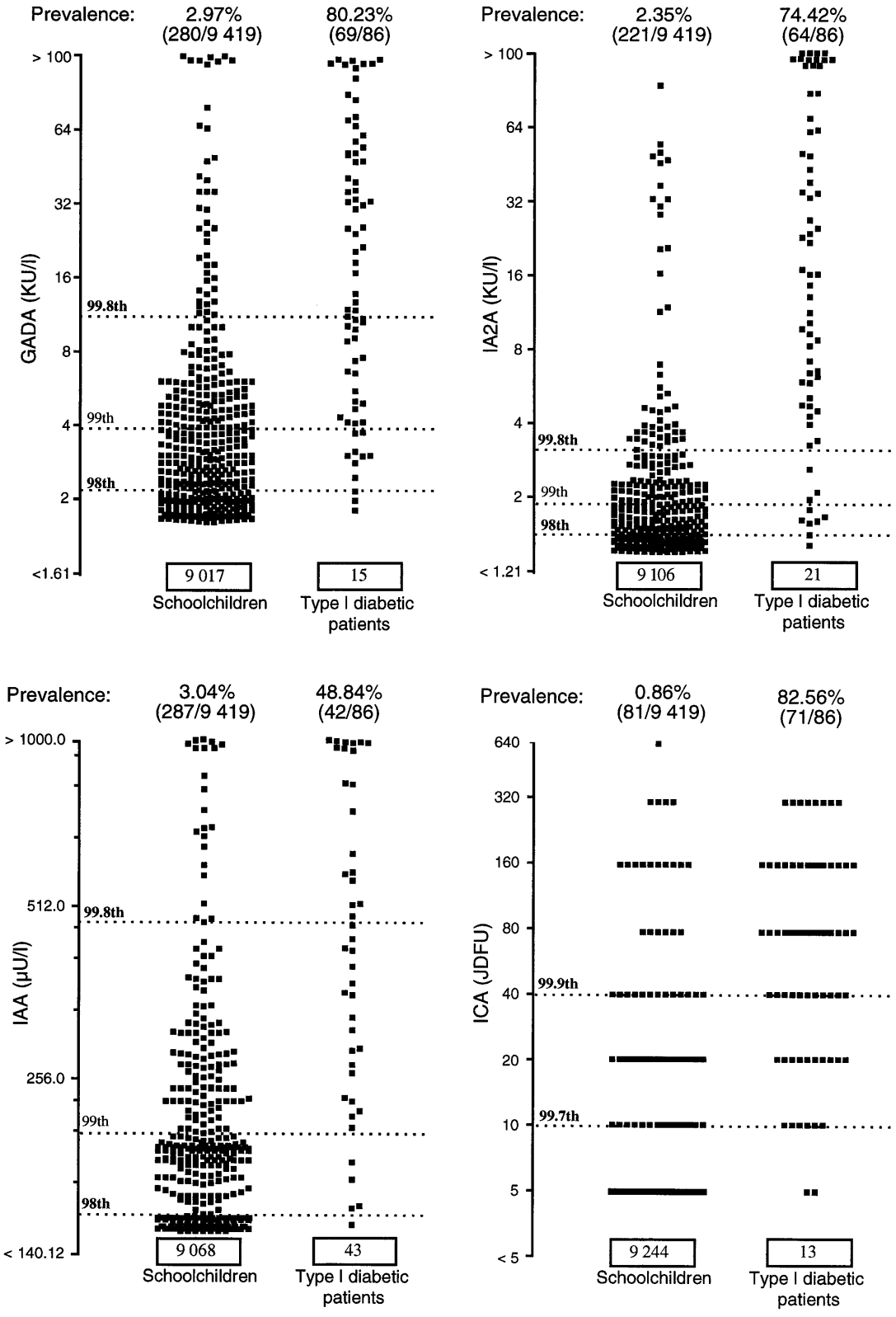


Fig. 1. Frequencies and levels of GADA, IA2A, IAA, and ICA in 9419 schoolchildren tested and in 86 age-matched children with newly diagnosed Type I diabetes. The numbers of children with AAb levels below the threshold 97th centile or ICA less than 5 JDFU are written in the boxes. Dotted lines represent cut-off limits at the 98th centile used for primary screening and 99.8th centiles used to identify subjects at high risk of developing Type I diabetes. The AAb prevalences (%, number of cases in brackets) given at the top represent AAb positivity of primary screening at or above centile 98 (for GADA, IA2A, and IAA) and for ICA equal or greater than 10 JDFU

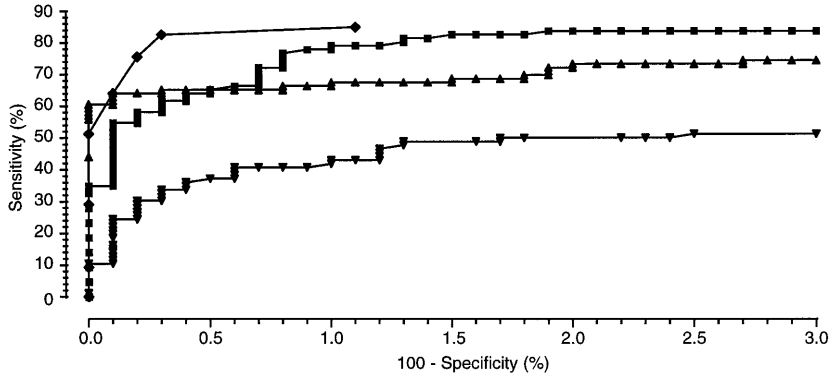


Fig. 2. Receiver-operating characteristic (ROC) plots for GADA (■), IA2A (▲), IAA (▼), and ICA (◆) constructed for each assay by varying the thresholds for AAb positivity. The sensitivities were determined from the results of 86 newly diagnosed Type I diabetic children and the specificity from 6877 control subjects with no family history of diabetes and autoimmune thyroid disease and not positive for two or more Type I diabetes-associated AAbs. At thresholds between the 97th and 99.4th centile GADA provides the highest sensitivity among the three AAbs to biochemically defined islet antigens, while above the 99.5th centile IA2A is the most sensitive marker of the beta-cell autoimmunity. ICA immunohistochemically tested, however, shows the highest sensitivity between the 99th and 99.7th centile. ICA are heterogeneous though and might include AAbs against three or more various antigens

col [19]. Specific bound 125I-insulin was calculated as μU/l for each sample by subtracting the counts of the tubes with excess of unlabelled insulin from those with 125I-insulin alone. IAA levels equal or greater than 147.56 μU/l were defined to be positive (98th centile of the control group). The interassay and intraassay CV were 12.6 and 2.5 % at 744 μU/l, 14.6 and 3.0 % at 1116 μU/l, and 6.8 and 5.9 % at 3986 μU/l ($n = 20$), respectively. The assay achieved 100 % sensitivity and 100 % specificity in the 6th IAA Proficiency Test 1997.

Detection of islet cell antibodies (ICA). ICA were measured by indirect immunofluorescence on cryosections of human pancreas as described before [20, 21]. Serum samples were incubated overnight at 4 °C and endpoint titres of test samples were converted to Juvenile Diabetes Foundation Units (JDFU). The threshold of detection was 5 JDFU, and titres equal or greater than 10 JDFU (99.7th centile of the control group) were considered positive. The assay achieved 100 % sensitivity and 100 % specificity in the 13th ICA Workshop 1998.

Metabolic studies. Intravenous glucose tolerance tests (IVGTTs) were done in schoolchildren at risk of developing diabetes expected from levels and co-occurrence of AAbs detected by primary screening and confirmed by the second AAb tests done with i.v. serum samples 8–12 weeks later. The first-phase insulin release (FPIR) was calculated as the sum of the 1 and 3 min insulin values after glucose injection according to the ICARUS protocol with 0.5 g glucose/kg of body weight as a 40 % solution injected over a 2-min period [22, 23]. The first centile for FPIR is 215 pmol/l, and the 5th centile is 328 pmol/l determined in 120 healthy control subjects (mean age 12 ± 3 years, ranging from 6 to 16; 64 girls, 56 boys).

Statistical analysis. Receiver-operating characteristic (ROC) analysis for GADA, IA2A, IAA, and ICA were carried out using the software package MedCalc, (Version 4.16 h, Mariakerke, Belgium). Sensitivity (from the 86 children with new-onset Type I diabetes) and specificity (from the 6877 healthy schoolchildren) of the immunoassays used were calculated by varying the thresholds of AAb levels over the 97.5th up to 99.98th centile. Distribution of GADA, IA2A, IAA, and ICA levels in children with Type I diabetes ($n = 86$) and in schoolchildren assayed for all four AAb specificities ($n = 9419$) was tested using the Kolmogorov-Smirnow goodness of fit test. Mann Whitney U-test was used to analyse skewed distributions of AAb levels in the different groups investigated. Relations between the different AAb levels and specificities were analysed by the two-sided Spearmen's nonparametric correlation analysis. Differences between individual groups studied were analysed by explorative two-sided testing using Chi square statistics and Fisher's exact test. All statistical analyses and calculation of centiles were done using the Statistical Package for Social Sciences, Version 8.0.0. (SPSS, Chicago, Ill., USA). Values are given as median (interquartile range) or mean ± SD. A two-tailed value $p < 0.05$ was considered to indicate statistical significance.

Results

Frequencies and levels of Type I diabetes-associated autoantibodies. Using a cut-off at or greater than the 98th centile for the AAbs GADA, IA2A, IAA and 10 JDFU for ICA the diagnostic sensitivity of the single AAb assays used amounts to 80.23, 74.42, 48.84, and 82.56 %, respectively, and the AAb frequencies by the same cut-offs were 2.97, 2.35, 3.04, and 0.86 % in schoolchildren of the Greifswald region (Fig. 1).

ROC curves derived from AAb distributions in 6877 schoolchildren used as control subjects (specificity) and in 86 age-matched Type I diabetic patients at diagnosis (sensitivity) for GADA, IA2A, IAA, and ICA are shown in Figure 2. Sensitivities at cut-offs between the 97th and 99.5th centile in diabetic schoolchildren were highest for ICA (85 %), followed by GADA (84–62 %), IA2A (76–65 %), and IAA (50–33 %). At highest specificity (99.98th centile), the highest sensitivity was obtained for IA2A of 60.5 %, whereas the sensitivity of GADA, IAA and ICA was decreased to 34.9, 10.5 and 51 %, respectively. As shown in Figure 2 optimum sensitivity for pri-

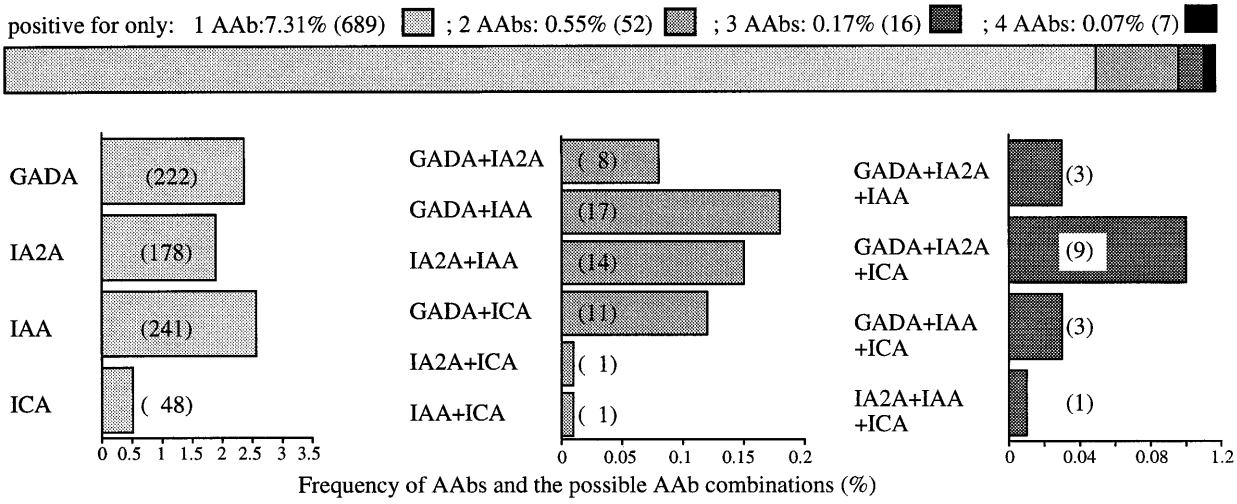
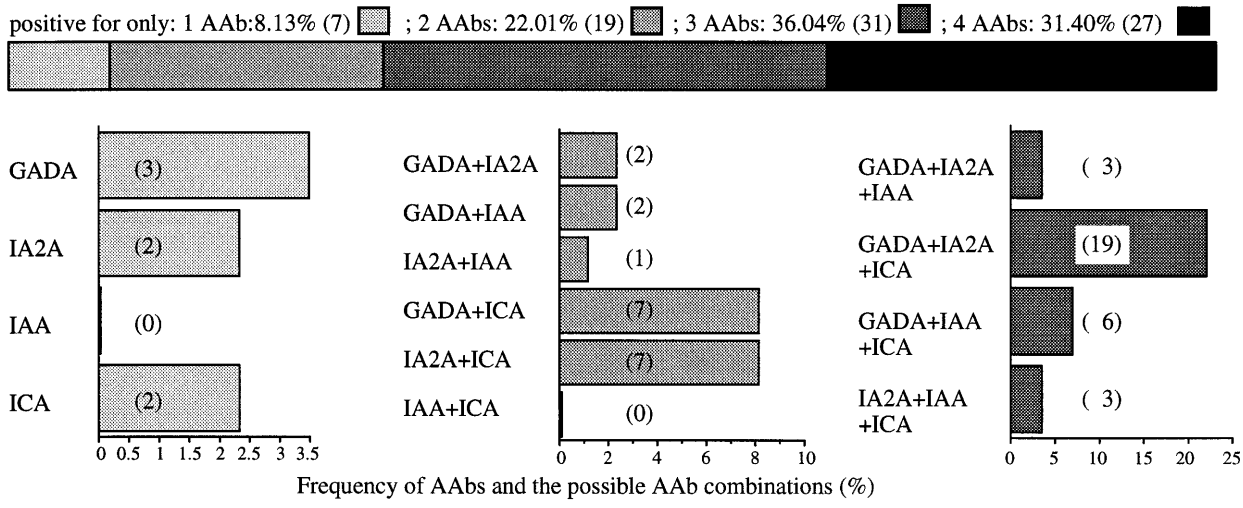


Fig. 3. Frequencies of positivity for GADA, IA2A, IAA, and ICA and their combinations in 9419 schoolchildren at cut-off limits at the 98th centile or greater for GADA, IA2A, and IAA and 10 JDFU or more for ICA. 48 schoolchildren had only ICA at 10 JDFU or more. There were, however, 400 schoolchildren (222 + 178) who had only GADA or IA2A, but all subjects with two or more AAbs, with one exception, would be identified by combined primary testing of GADA and IA2A

mary screening is reached at 2% background positivity (98th centile) for all four AAbs.

Frequency of autoantibodies and their combinations in schoolchildren compared with those of Type I diabetic children. A complete overview about occurrence of GADA, IA2A, IAA, and ICA and their possible combinations at cut-off at or greater than the 98th centile for the radioassayed AAbs and at or greater than the 10 JDFU for ICA measured in 9419 schoolchildren is given in Figure 3 and compared with the AAb distribution in 86 children with newly diagnosed Type I diabetes (Fig. 4). A history of Type I diabetes in a first-degree relative was present in 9.30% (8/86) of subjects. There were, however, no differences in the frequencies and levels of any of the AAbs between diabetic children with and without an affected first-degree relative. As shown in Figure 5, 8.11% (764/9419) of schoolchildren had at least one AAb level at or greater than the 98th centile, the highest AAb frequency was found for IAA (3.04%) and GADA (2.97%) followed by IA2A (2.35%), and ICA (0.86%). Most of the AAb positive schoolchildren, however, (7.31%: 689/9419) had only one AAb, 52 (0.55%) had two, 16 (0.17%) had three and 7 (0.07%) had 4 AAbs. Frequencies of all possible AAb combinations are presented in Figure 3. 84 of 86 (97.67%) diabetic children had in-

Fig. 4. Diagnostic sensitivity of GADA, IA2A, IAA, and ICA and their combinations in 86 children with newly diagnosed Type I diabetes at cut-offs at the 98th centile or greater for GADA, IA2A, IAA, and 10 JDFU or more for ICA. All patients positive for two or more AAbs would be identified by combined testing of GADA and IA2A



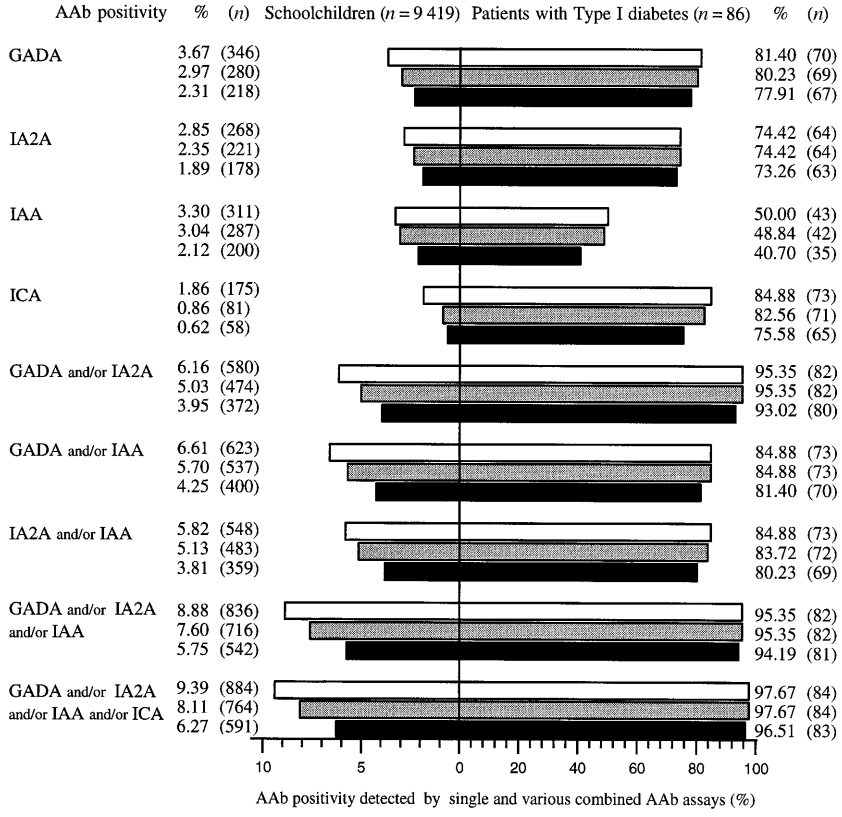


Fig. 5. Frequencies (%) of AAb positivity using single and various combinations of AAb assays at different thresholds (centile: ≥ 97.5 □, ≥ 98 ▒, ≥ 98.5 ■ for GADA, IA2A, IAA, and always ≥ 10 JDFU for ICA) detected in 9419 schoolchildren (left) and in 86 children with newly diagnosed Type I diabetes (right). With the exception of the combination IA2A and/or IAA, the sensitivity in children with Type I diabetes was not diminished by increasing the centile from 97.5 to 98 or greater. Using the two-assay combination GADA and/or IA2A at the 98th centile 95.35% of children with Type I diabetes were AAb positive, i.e. 97.62% (82/84) of all AAb positive patients and 66.20% (474/764) of all AAb positive schoolchildren were identified by this combination

creased levels of at least one AAb with the highest frequency for ICA (82.56%), followed by GADA (80.23%), IA2A (74.42%), and IAA (48.84%). 77 of 84 (91.66%) children had two or more AAbs. The distribution of the AAb combinations is given in Figure 4. It is noteworthy that no child with Type I diabetes, and only one healthy schoolchild, was positive for the AAb combination of IAA and ICA.

Relation between sensitivity and specificity at different numbers and levels of autoantibodies. The frequencies of AAb positivity by the use of single AAb assays and their combinations depending on different thresholds is shown in Figure 5. Among the single AAbs against defined beta-cell antigens GADA had the highest diagnostic sensitivity of 80.23%, which was enhanced most strongly by a combination with IA2A up to 95.35% at both cut-off limits at the 97.5th and 98th centiles and could not be further increased by additional IAA testing. The overall sensitivity of 97.67% by testing all four AAbs in children with Type I diabetes at the centile 97.5 or greater was not diminished by increasing the centile to 98 or greater. This is also valid for the diagnostic sensitivity of GADA or IA2A (95.35%) or both, GADA or IAA (84.88%) or both, and GADA or IA2A or IAA or two or all three (95.35%). By further enhancement to the cut-off limit at the 98.5th centile or greater, however, the sensitivity was decreased. Because of the low AAb levels in the general population, frequency of AAb positivity of each assay combination was decreased with further enhancement of cut-offs (Fig. 5, left). Using the two-assay combinations for the three autoantibodies GADA, IA2A and IAA, the highest diagnostic sensitivity was found for GADA and IA2A at the cut-off limit at the 98th centile or greater detecting 95.35% (82/86) of cases.

Association of autoantibodies with sex and age. Frequencies of AAbs of the 9419 schoolchildren at cut-off at the 98th centile or greater differed between 4722 girls and 4697 boys for IAA: 3.4 (161 girls) vs 2.7% (126 boys), $p = 0.03$; for GADA or IAA or both: 6.2 (294 girls) vs 5.2% (243 boys), $p = 0.02$; for children with at least one AAb: 8.7 (413 girls) vs 7.5% (351 boys), $p = 0.02$; and with three or four AAbs: 0.12 (6 girls) vs 0.36% (17 boys), $p = 0.018$. In

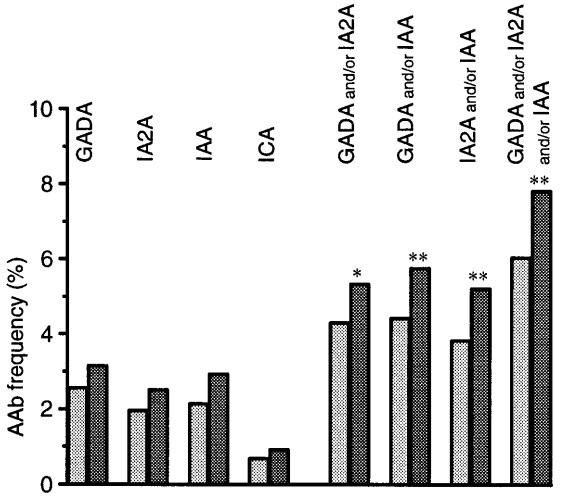


Fig. 6. AAb frequencies (%) of single autoantibodies (GADA, IA2A, IAA, and ICA) and autoantibody combinations in relation to the age of 2763 schoolchildren less than 10 years (▨) and 6655 schoolchildren 10 years and older (■). All combined AAb tests provided significantly increased AAb frequencies in the group 10 years and older (*$p < 0.05$, **$p < 0.01$), while frequencies of single AAbs were only slightly increased in the older schoolchildren. The enhanced AAb frequency in schoolchildren aged 10 years or more is consistent with the raised incidence of Type I diabetes during puberty

86 (41 girls, 45 boys) Type I diabetic children the frequency of GADA or IA2A or both was increased in boys: 90.3 (37 girls) vs 100 % (45 boys), $p < 0.01$.

29.3 % (2763/9419) of schoolchildren tested for all AAbs were less than 10 years of age. There was no difference of single AAb frequencies between these two age groups (Fig. 6). Combined AAb testing, however, results in significantly increased AAb frequencies in the older groups: for GADA or IA2A or both, $p < 0.05$; GADA or IAA or both, $p < 0.01$; IA2A or IAA or both, $p < 0.01$; and for all three AAbs against the molecularly characterized beta-cell antigens, $p < 0.01$. There were no differences of AAb levels between either boys and girls or the two age groups in both AAb-positive schoolchildren and diabetic children.

Relation of the autoantibodies to one another in schoolchildren and Type I diabetic children. Depending on the level of ICA (10 or 20 vs ≥ 40 JDFU) the frequency of GADA, IA2A as well as the AAb positivity by testing GADA or IA2A or both, and GADA or IA2A or IAA or two or all three in schoolchildren was significantly increased ($p < 0.01$–0.001) (Fig. 7). In contrast, in Type I diabetic children the frequencies of AAbs to the biochemically characterized beta-cell antigens were already high in co-occurrence with low ICA level (10–20 JDFU) and were not further increased in the presence of ICA equal or greater than 40 JDFU.

In schoolchildren as well as in diabetic children, the levels of each of the four AAbs were significantly enhanced ($p < 0.05$) in subjects with two or more AAbs compared with those with only one AAb at the 98th centile or greater (Table 1).

Furthermore, analysing all AAb positive schoolchildren and diabetic children, only GADA vs ICA ($r = 0.218$ and $r = 0.307$) and IA2A vs ICA ($r = 0.543$ and $r = 0.403$) were significantly associated ($p < 0.01$) but GADA vs IA2A was not in both cohorts. There was no association between IAA and any of the other three AAbs in either schoolchildren or diabetic children.

Schoolchildren at highest risk of developing diabetes. IVGTTs have been carried out up to now in 17 of the 22 schoolchildren positive for at least two autoantibodies by the high centile 99.8 or greater for GADA, IA2A and IAA, and for ICA at 40 or more

Fig. 7. Frequencies (≥ 98th centile) of GADA (▨), IAA (▨), IA2A (▨), and of AAb positivity by combined testing of GADA and/or IA2A (■), and of GADA and/or IA2A and/or IAA (■) depending on the ICA levels measured in 81 ICA-positive schoolchildren (left) and in 71 ICA-positive children with Type I diabetes (right). As expected, the frequencies of GADA, IA2A, GADA and/or IA2A as well as GADA and/or IA2A and/or IAA were only significantly increased in schoolchildren at ICA of 40 JDFU or more (**$p < 0.01$, ***$p < 0.001$) because of the high frequency of AAb co-occurrence at clinical onset independent of ICA levels

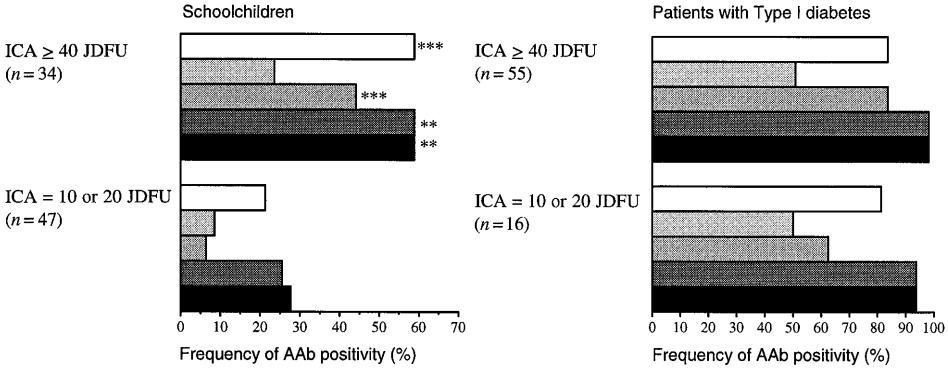


Table 1. Comparison of antigen binding levels between sera with one and two or more autoantibodies from AAb-positive schoolchildren and children with Type I diabetes

AAb specificity	Schoolchildren (n = 764)		Children with Type I diabetes (n = 84)	
	positive for 1 AAb (n = 689)	positive for ≥ 2 AAb (n = 75)	positive for 1 AAb (n = 7)	positive for ≥ 2 AAb (n = 77)
GADA (KU/l)	0.46 (0.10–2.63)	4.40[a] (2.20–15.80)	0.97 (0.67–4.10)	13.63[a] (3.98–50.12)
IA2A (KU/l)	0.41 (0.12–1.41)	1.68[a] (0.42–3.71)	0.97 (0.63–1.97)	9.28[a] (1.67–49.19)
IAA (μU/l)	50.84 (1.24–182.28)	164.92[a] (39.68–208.32)	28.52 (21.08–81.84)	153.76[a] (53.01–482.05)
ICA (JDFU)	0 (0–0)	3[a] (0–40)	0 (0–10)	80[a] (20–160)

Data are median (interquartile range); [a] $p < 0.05$ (Mann Whitney U-test)

Table 2. Biochemical and clinical data in prediabetic schoolchildren

Subject	Age (years)	Sex	Months before onset	GADA (KU/l)	IA2A (KU/l)	IAA (μU/l)	ICA (JDFU)	IVGTT FPIR[a] (pmol/l)
1	12	female	12	250.0	0.84	42.1	160	250
2	12	male	37	13.6	93.8	0	320	285
3	7	male	24	15.8	32.6	124.0	320	209
4	11	female	28	23.9	30.5	345.9	640	310
5	12	male	6	184.4	46.9	148.8	160	n.d.
6	15	female	11	501.0	3.7	0	320	n.d.

Values indicate AAb positivity ≥ 98.th percentile and significantly diminished first-phase insulin release (FPIR; [a] 10 ± 6 months after initial screening)

JDFU, assumed to be at highest risk for rapid developing diabetes. 58.8 % (10/17) had a significantly decreased ($p = 0.0012$) first-phase insulin response of 243.6 ± 38 pmol/l (mean ± SD, $n = 10$).

Six of these subjects (27.3 %), five older than 10 years, progressed to diabetes during the short follow-up period of 38 ± 8 months. All six subjects were positive for GADA greater than the 99.8th centile and had ICA equal or greater than 160 JDFU during the primary screening (Table 2). Four of 5 additionally IA2A-positive subjects had levels equal or greater than the 99.98th centile. Only two had additional IAA greater than the 98th centile. All prediabetic children had at least two AAbs. The first-phase insulin response of prediabetic children studied, measured 10 ± 6 months after initial screening, was decreased below the 5th centile (328 pmol/l) including one below the first centile (215 pmol/l) calculated from 120 healthy control subjects.

Discussion

A major goal in diabetes research is the prediction and prevention of Type I diabetes. Because approximately 90 % of subjects with newly diagnosed Type I diabetes have no affected first-degree relatives [10], predictive testing and risk assessment has to be extended to the general population. The screening procedure aims for high sensitivity to avoid missing future cases, combined with sufficient specificity to minimise false-positive results to avoid unnecessary treatment and potential anxieties related to predicted risk. These aims inevitably conflict because the specificity of a screening method is reciprocally related to its sensitivity. The aim of this study was to develop a strategy for assessing risk of progression to Type I diabetes in a general population involving 9419 schoolchildren with no family history of Type I diabetes of the Greifswald region. In our study 8.11 % (764/9419) schoolchildren had at least one AAb in the primary screening using the cut-off limit at the 98th centile or greater for the radioassayed GADA, IA2A, IAA and equal or greater than 10 JDFU for ICA and the frequency of each single AAb was 2.97, 2.35, 3.04, and 0.86 %, respectively. That means the AAb frequency was much higher than the overall prevalence (0.3 %) of Type I diabetes in this region. Only 0.8 % (75/9419), however, had two or more AAbs at this cut-off. Using the thresholds at the 99.8th centile or greater and ICA equal or greater than 40 JDFU, the frequency of two and more Type I diabetes-associated AAbs was reduced to 0.23 % (22/9419) similar to the prevalence of Type I diabetes. These 22 schoolchildren were considered at high risk for rapid progression to diabetes. In 10 of 17 (58.8 %) subjects of this group the first-phase insulin secretion tested was already significantly ($p < 0.01$) reduced. It is notewor-

thy that in spite of the short follow-up period (19 ± 10 months) six of these 22 schoolchildren (27.3 %) have already progressed to diabetes. All six children had high levels of ICA equal or greater than 160 JDFU and were positive for GADA but only one had a GADA level greater than the 99.98th centile. Note, however, that in four of the five IA2A positive subjects AAb levels were greater than the 99.98th centile, indicating the highest disease sensitivity of IA2A. 83.3 % (5/6) of those who have progressed to diabetes were older than 10 years. This is in accordance with an enhanced incidence of disease at puberty age [10] and with the increased ($p < 0.05$–0.01) AAb frequency shown in schoolchildren 10 years and older vs that of younger probands.

Schoolchildren of our study with no or with only one AAb have not so far progressed to diabetes, i. e. single AAb frequency in the general population is without predictive diagnostic value as argued previously for ICA as individual risk marker [11]. It has been repeatedly shown by follow-up studies in first-degree relatives that the risk of developing Type I diabetes increases as the number of autoantibodies increases [24]. Although current experience with autoantibody screening in the general population is limited, one prospective study in 2805 schoolchildren detected AAbs to 64 k antigen and more recently to GAD in eight ICA-positive children. Only six had both autoantibodies and four of these subsequently developed diabetes [26]. Another study compared the frequency of ICA, GADA and IAA in 491 Swedish children with newly diagnosed Type I diabetes and matched control subjects, and the positivity for all three AAbs gave a 23 % positive predictive value [13]. A further study [25] measured all four AAbs in 2855 schoolchildren and in 256 children with Type I diabetes and found an association of estimated risk of developing diabetes with increasing levels of AAbs and multiple AAb positivity.

Our results suggest that the co-occurrence of Type I diabetes-associated AAbs detected in persons of a general population has a similar predictive value for developing diabetes as described for first-degree relatives [9, 17, 25]. In both cohorts, the diabetic subjects' and AAb-positive schoolchildren's levels of each AAb were significantly ($p < 0.05$) increased by AAb co-occurrence, which is consistent with data from first-degree relatives of Type I diabetic patients [7, 8] (Table 1). With increasing levels of ICA, the levels of GADA and IA2A are also enhanced in both cohorts subjects and schoolchildren. This is in accordance with the contribution of both GADA and IA2A to ICA detected by immunofluorescence [27, 28]. There was, however, no association between IAA and any of the three other AAbs. IAA as AAb against the only known beta-cell-specific antigen possibly reflects a particular autoimmune reaction [29]. But the additional presence of IAA in ICA positive children increased the predictability of the disease [30]. We found only 2 of 6 schoolchildren who progressed to diabetes IAA positive.

As our study shows, the most effective screening strategy for children aged 6 to 17 years could be derived from the frequencies of all possible two-AAb combinations shown at the threshold of the 98th centile or greater because a higher centile reduced the sensitivity. At this assay specificity the combination of GADA and IA2A has the highest diagnostic sensitivity compared with GADA and IAA or IA2A and IAA or ICA. Surprisingly, in subjects with diabetes the two-AAb combination IAA plus ICA did not occur. That means, as shown in Figure 4, all diabetic children would be identified as AAb-positive by testing only for GADA and IA2A, and only one of the 75 schoolchildren with two and more AAbs, i. e. at high risk, would not be identified by this AAb combination [31, 32].

It is concluded that with the AAb markers currently available the primary screening in a general population of children older than 5 years should be done by combined testing of GADA and IA2A at the cut-off limit of the 98th centile or greater, if possible in a single combined assay [33], followed by second-line testing for ICA and IAA. Subjects at high risk progressing to diabetes would be identified by having at least two antibody specificities greater than the 98th centile. Subjects with two ore more AAb levels equal or greater than the 99.8 centile seem to be at high risk for rapid progression to diabetes. Additional differentiation of an individual risk of developing diabetes could be provided by the determination of first-phase insulin response.

There is, however, one exception where the prognostic information is uncertain because it is unclear how many subjects, bearing immunogenetic markers known to be protective for the disease but also having autoantibodies, will eventually progress to overt diabetes with long-term follow-up. Thus, studies are currently in progress to examine whether the occurrence of multiple autoantibodies in schoolchildren is associated with Type I diabetes-associated alleles [34]. This ongoing follow-up study will enable us to evaluate the risk of developing Type I diabetes depending on immunological and genetic markers in a general population of schoolchildren.

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