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Antibodies to the M_r 64,000 (64 K) protein in islet cell antibody positive non-diabetic individuals indicate high risk for impaired Beta-cell function

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Summary. A prospective study of a normal childhood population identified 44 islet cell antibody positive individuals. These subjects were typed for HLA DR and DQ alleles and investigated for the presence of antibodies to the M_r 64,000 (64K) islet cell antigen, complement-fixing islet cell antibodies and radiobinding insulin autoantibodies to determine their potency in detecting subjects with impaired Beta-cell function. At initial testing 64K antibodies were found in six of 44 islet cell antibody positive subjects (13.6%). The same sera were also positive for complement-fixing islet cell antibodies and five of them had insulin autoantibodies. During the follow-up at 18 months, islet cell antibodies remained detectable in 50% of the subjects studied. In all six cases who were originally positive, 64K antibodies were persistently detectable, whereas complement-fixing islet cell antibodies became negative in two of six and insulin autoantibodies in one of five individuals. HLA DR4 ($p < 0.005$) and absence of asparic acid (Asp) at position 57 of the HLA DQ β chain ($p < 0.05$) were significantly increased in subjects with 64K antibodies compared with control subjects. Of 40 individuals tested in the intravenous glucose tolerance test, three had a first phase insulin response below the first percentile of normal control subjects. Two children developed Type 1 (insulin-dependent) diabetes mellitus after 18 and 26 months, respectively. Each of these subjects was non-Asp homozygous and had persistent islet cell and 64K antibodies. We conclude that 64K antibodies, complement-fixing islet cell antibodies and insulin autoantibodies represent sensitive serological markers in assessing high risk for a progression to Type 1 diabetes in islet cell antibody positive non-diabetic individuals.

Key words: Population study, 64K antibodies, islet cell antibodies, complement-fixing islet cell antibodies, insulin autoantibodies, HLA.

Type 1 (insulin-dependent) diabetes mellitus is caused by a chronic autoimmune process where pancreatic Beta cells are specifically destroyed. In Caucasian diabetic patients 95% possess the HLA DR3 and/or DR4 alleles [1]. Susceptibility to Type 1 diabetes has also been demonstrated in patients with the absence of asparic acid (Asp) at amino acid residue 57 of the HLA DQ β chain [2]. Several studies on first degree relatives have revealed a long pre-diabetic period in which the appearance of Type 1 diabetes-specific autoantibodies and the loss of the early phase insulin response to intravenous glucose indicate a high risk for the precipitation of the disease [3–5]. In family studies, persistent cytoplasmic islet cell antibodies (ICA) at high levels as well as the presence of complement-fixing ICA (CF-ICA) and radiobinding insulin autoantibodies (CIAA) have been correlated with progression to Type 1 diabetes in non-diabetic individuals [6, 7].

The M_r 64,000 (64K) islet cell protein, now recognized as glutamate decarboxylase (GAD) [8] is a major target antigen in Type 1 diabetes. Immunoprecipitating antibodies to this protein have been found in 80% of newly-diagnosed cases, and they have also been detected in the pre-diabetic period in first degree relatives of Type 1 diabetic patients who later developed diabetes [9–11].

The present prospective study, started in 1988 and was designed to investigate the course of the pre-diabetic period in a random childhood population. Separate aspects of this study have been published recently [12, 13]. Here we addressed the question of whether the detection of antibodies to the 64K protein or other Type 1 diabetes specific autoantibodies can be correlated with genetic high risk markers and impaired Beta-cell function.

Subjects and methods

A total of 4208 school children and adolescents (2292 females, 1916 males, mean age 13.9 years, range 7–21 years) from the Ulm/Alb-Donau County in southern Germany were screened for ICA be-

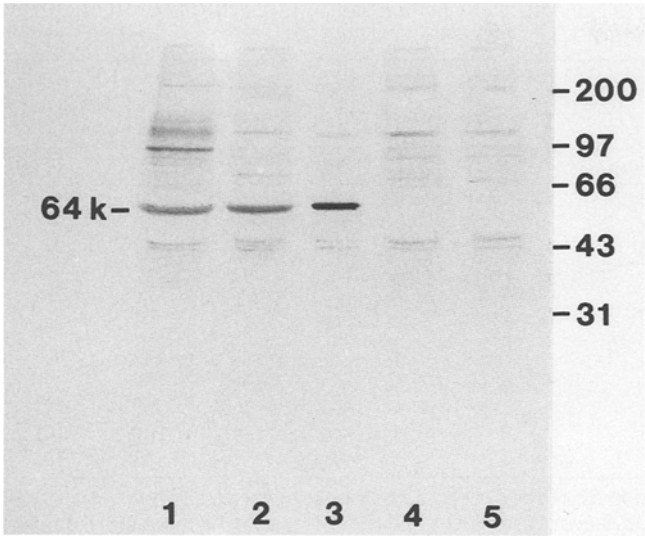

Fig. 1. Fluorogram showing immunoprecipitation of ^{35}S-methionine-labelled porcine islet cell proteins by two islet cell antibody positive subjects (ID numbers 2, 3; lanes 1 and 2), the corresponding sex- and age-matched control subjects (lanes 4 and 5) and by a monoclonal anti-glutamate decarboxylase antibody (lane 3)

tween July 1988 and July 1989. Informed consent was given by the parents of the children studied. The study was approved by the local ethical committee and the Data Protection Council and was performed according to the principles of the Declaration of Helsinki.

ICA were determined by the indirect immunofluorescence test on unfixed cryostat sections of blood group 0 human pancreas [14]. The tests were performed without knowledge of the origin of the sample by two investigators (G. G-F and G. T.). All determinations were carried out on the same tissue and the results were expressed in Juvenile Diabetes Foundation (JDF) Units according to the workshop protocol for the standardisation of ICA. The detection threshold of the assay in our laboratory was 5 JDF Units with values of 100% for validity, consistency, sensitivity and specificity (Second Proficiency Program, LAB ID number: 116). CF-ICA were measured by an indirect immunofluorescence assay [14], performed on the same substrate as for ICA determination. Positive sera were titrated to end-point. ICA positive individuals were retested for ICA and CF-ICA 3, 6, 9, 12 and 18 months after the initial screening.

Antibodies to the 64K antigen (64KA) were determined in parallel with porcine and human islets as antigen using a modification of the immunoprecipitation method described by Christie et al. [15]. Due to the shortage of human islets, porcine preparations were used for follow-up investigations. Islets were isolated by the method originally recorded by Ricordi et al. [16]. The mean purity of the islets was 80–90%.

Islets were labelled with ^{35}S-methionine, homogenised in 20 mmol/l Tris pH 7.4 supplemented with 2 mmol/l phenylmethylsulphonyl fluoride, 2 mmol/l EDTA and 1% Trasylol (Tris-buffer) followed by centrifugation at 36,000 × *g* for 30 min. The pellet was resuspended in Tris-buffer containing 150 mmol/l NaCl followed by the addition of 1% Triton × 100 for 2 h to extract the membrane proteins. After centrifugation at 16,000 × *g* for 30 min the supernatant was pre-cleared with a pool of normal human serum (25 µl/100 µl lysate, equivalent to 300 islets) and then precipitated with the test serum (25 µl/100 µl lysate) for 12 h. The immune complexes were bound to protein A Sepharose (Pharmacia, Freiburg, FRG), washed five times and eluted with 62 mmol/l Tris HCl pH 6.8, 2% SDS, 5% mercaptoethanol. The proteins were separated by SDS-PAGE using a 7.5–15% linear gradient gel according to the method of Laemmli [17] and the precipitates were analysed by fluorography. Each sample was coded and results were interpreted independently by two observers.

CIAA were detected by the competitive radiobinding assay described by Vardi et al. [18]. A total binding insulin level above 45 nU/ml (mean + 3 SD of 100 control subjects) was regarded as positive. In the First Insulin Autoantibody Proficiency Program our laboratory obtained values of 100% of sensitivity and consistency, 92% for validity and 87% for specificity (Lab ID number 116).

The intravenous glucose tolerance test (IVGTT) was performed after an overnight fasting period. Glucose (0.5 g/kg body weight) was infused within 2 min. Plasma insulin concentrations were measured before, and at 1, 3, 5, 10, 20 and 30 min after infusion. Insulin was analysed by double antibody radioimmuno-technique (International CIS, Gif-sur-Yvette Cedex, France). The sum of the 1 + 3 min insulin values after glucose infusion was used as a parameter of the first phase insulin response. A suppressed peak insulin response was defined as a value below the first percentile (48 µU/ml) of 216 normal control subjects (92 females, 128 males, mean age 29.5 years). In ICA positive individuals the IVGTT was repeated at 3–6 month intervals.

HLA analysis of our ICA positive individuals and a control population has been published recently [12]. HLA DR typing was performed by the two colour fluorescence technique. The DQβ chain polymorphism at codon 57 was determined by DNA amplification and analysis with specific oligonucleotide probes.

Statistical analysis

Results were expressed as mean ± SD. Statistical differences between groups were assessed using the Wilcoxon test, the chi-square test with Yates' correction or Fisher's exact test, where appropriate.

Results

Of the 4208 probands 44 (1.05%) were ICA positive at initial screening (19 females, 25 males, mean age 13.6, range 7–19 years). The mean positive ICA level was 33.4 (range 5–160) JDF Units. None had a family history of Type 1 diabetes. 64KA were detected in six of the 44 (13.6%) ICA positive individuals but in none of the 60 ICA negative sex- and age-matched control subjects (Fig. 1). Identical results were obtained whether porcine or human islets were used in our assay (data not shown). The data of the 64KA and their correlation to CF-ICA and CIAA are given in Table 1. The mean ICA levels of the sera containing 64KA and CF-ICA (55 ± 27 JDF Units) or of sera containing CIAA (64 ± 17 JDF Units) were increased as compared to ICA levels in sera that were only ICA positive (30 ± 33 JDF Units) ($p < 0.05$, CIAA vs ICA positive subjects).

In contrast to 808 control subjects the frequency of HLA DR3 and DR4 was significantly increased in ICA positive individuals ($p < 0.0005$) (Table 2). Twenty-nine of 31 (93.5%) ICA positive subjects had a non-Asp haplotype ($p < 0.02$) and 20 (64.5%) were non-Asp homozygous or non-Asp/blank at position 57 of the HLA DQ β chain ($p < 0.005$) compared to 71.5% and 33.4% in 123 control subjects, respectively. Five of six (83.3%) 64KA and CF-ICA positive subjects possessed a DR4 haplotype ($p < 0.005$), two (16.7%) had HLA DR3 and five of these individuals were non-Asp/non-Asp or non-Asp/blank ($p < 0.05$) (Table 1).

Table 1. Islet specific autoantibodies, HLA typing and Beta-cell function in individuals with persistently positive cytoplasmic islet cell antibodies

ID no.	age sex	HLA		ICA (JDF Units)		CF-ICA (titre)		64KA		CIAA (nU/ml)		IVGTT 1 + 3 min insulin (μU/ml)	
		DR	DQ	0 mo	12 mo	0 mo	12 mo	0 mo	12 mo	0 mo	12 mo	0 mo	12 mo
1	14 F	3,4	NA/NA	80	60	1:4	1:4	+	+	178	–	77	139
2	15 M	1,2	NA/NA	60	20	1:2	1:1	+	+	101	104	42	< 7
3	12 M	4,8	NA/NA	80	80	1:2	1:4	+	+	49	66	85	44
4	13 M	3,4	NA/^{blank}	10	10	1:2	–	+	+	–	–	58	70
5	11 M	4,11	NA/AA	60	160	1:2	1:2	+	+	241	301	205	152
6	8 F	4,6	NA/NA	40	10	1:1	–	+	+	115	55	25	36
7	8 F	1,3	NA/NA	40	5	–	–	–	–	–	–	121	n.t.
8	13 M	n.t.	n.t.	20	40	–	–	–	–	–	–	127	258
9	14 M	6,11	A/A	10	40	–	–	–	–	–	–	207	491
10	10 F	4,12	NA/NA	160	40	–	–	–	–	–	–	222	107
11	14 M	4,^{blank}	NA/NA	10	5	–	–	–	–	–	–	472	286

ICA, islet cell antibodies; CF-ICA, complement fixing ICA; 64KA, antibodies to the 64K protein; CIAA, insulin autoantibodies, values above 45 nU/ml are taken as positive; IVGTT, intravenous glucose tolerance test; A, asparic acid; NA, absence of aspartic acid at position 57 of the DQ β chain; Blank, possibility of homozygosity or presence of an unidentified allele; F, female; M, male; mo, months of follow-up; –, negative; n.t., not tested

Table 2. Correlation of the islet cell antibody (ICA) status at the follow-up with the HLA DR and DQ gene frequencies in ICA positive non-diabetic individuals compared to ICA negative control subjects from the same population

ICA status	HLA phenotype			HLA DQ β genotype at position 57			
	n	DR3	DR4	*n*	A/A or A/blank	A/NA	NA/NA or NA/blank
control subjects	808	0.189	0.238	123	0.285	0.382	0.333
ICA positive	36	0.444[a]	0.528[b]	31	0.065	0.290	0.645[c]
persistent ICA	10	0.300	0.700[c]	10	0.100	0.100	0.800[df]
fluctuating ICA	12	0.667[b]	0.333	10	0.000	0.300	0.700[e]
transient ICA	8	0.500	0.500	8	0.125	0.625	0.250

Persistent ICA, ICA positivity at each time point; fluctuating ICA, change between positive and negative ICA results during follow-up; transient ICA, initially positive ICA but persistent ICA negativity at the follow-up; A, aspartic acid; NA, absence of aspartic acid at position 57 of the DQ β chain; blank, possibility of homozygosity or presence of an unidentified allele; n, number of subjects.
[a] $p < 0.0005$, [b] $p < 0.0002$, [c] $p < 0.005$, [d] $p < 0.01$, [e] $p < 0.05$, vs control subjects; [f] $p < 0.05$, vs transient ICA

Table 3. Consistency of cytoplasmic islet cell antibodies (ICA), complement-fixing islet cell antibodies (CF-ICA), antibodies to the 64K antigen (64KA) and insulin autoantibodies (CIAA) at the 18-month follow-up

	Months of follow up				
	3	6	9	12	18
Number of subjects	40	35	34	34	28
ICA positive	22 (21 ± 21)	19 (25 ± 31)	14 (23 ± 18)	17 (29 ± 40)	14 (30 ± 44)
CF-ICA positive	5	5	4	5	5
64KA positive	6	n.t.	n.t.	6	6
CIAA positive	5	4	4	4	n.t.

The ICA levels (mean ± SD) are given in parentheses. n.t., not tested

The data on the course of ICA and CF-ICA during an 18-months follow-up are given in Table 3. After 18 months of initial testing 50% of the originally ICA positive individuals had become ICA negative. As shown in Table 4 the presence of 64KA, CF-ICA and CIAA was positively correlated with persistent ICA. ICA persistency was also associated with HLA DR4 and non-Asp homozygosity (Table 2). Individuals with persistently detectable ICA had higher mean ICA levels (52 ± 45 JDF Units) as compared to those subjects with transient ICA, where ICA were negative at all follow-up examinations (16 ± 10 JDF Units) ($p < 0.02$). 64KA were persistently detectable in all six subjects who were initially positive whereas CF-ICA were only positive in four of the six and CIAA in four of the five initially positive cases. The frequencies of non-Asp/non-Asp was significantly increased in subjects with 64KA, CF-ICA or persistent ICA compared to the group with transient ICA ($p < 0.05$).

Of 44 ICA positive school children 40 were available for IVGTT's. All of them were clinically healthy, had normal fasting blood glucose and a normal body mass index. Two of the 40 had a first phase insulin response less than 48 μU/ml. Both subjects with persistently ICA levels greater than 40 JDF Units, were also persistently positive

Table 4. Correlation of cytoplasmic islet cell antibody (ICA) persistency with ICA levels, complement fixing ICA (CF-ICA), antibodies to the 64K antigen (64KA), insulin autoantibodies (CIAA) and Beta-cell function in the intravenous glucose tolerance test (IVGTT)

ICA status	ICA (JDF Units)				CF-ICA	64KA	CIAA	IVGTT
	< 20	20–40	> 40	Mean level				1 + 3 min < 48 μU/ml
Persistent	3	3	5	52 ± 45[a]	6	6	5	3
Fluctuating	5	3	4	35 ± 32	–	–	–	–
Transient	6	5	–	16 ± 10	–	–	–	–

Persistent ICA, ICA positivity at each timepoint; fluctuating ICA, change between positive and negative ICA results during follow-up; transient ICA, initially positive ICA but persistent ICA negativity at the follow-up.
[a] $p < 0.02$ vs transient ICA

for 64KA and CIAA and showed a blunted insulin response in the IVGTT over a period of 15–18 months. In another subject that was positive for 64KA and CF-ICA the IVGTT decreased below the first percentile after 6 months. During the follow-up one of these children (ID no. 2) with persistent ICA (20–60 JDF Units), 64KA, CF-ICA (titre 1/1 to 1/2) and CIAA (83–195 nU/ml) and another child (ID no. 1) with persistent ICA (40–80 JDF Units), 64KA and CF-ICA (titre 1/2 to 1/4) developed Type 1 diabetes after 16 and 26 months of observation, respectively. In the latter case, CIAA had become negative 17 months before the onset of Type 1 diabetes.

Discussion

The determination of antibodies to the 64K protein, radiobinding competitive insulin autoantibodies (CIAA) and immunogenetic markers have not been included in published population studies so far [19–21]. Based on the study of a random population aged 7–21 years we show that in ICA positive non-diabetic individuals antibodies to the 64K antigen, CF-ICA and CIAA are closely correlated with impaired Beta-cell function, the HLA DR4 phenotype and the absence of Asp at position 57 of the HLA DQ β chain.

With our porcine 64K assay 64KA were detected in six out of 44 (13.6 %) ICA positive individuals. In accordance with the results in a family study reported by Atkinson et al. [10] we also found an association between 64KA and high ICA levels as well as with the presence of insulin autoantibodies. We were able here to show a strong correlation between 64KA and CF-ICA, which are considered as reliable serological markers for active Beta-cell destruction and the progression to diabetes in non-diabetic first degree relatives of Type 1 diabetic patients [4, 6]. The high risk for Type 1 diabetes in the subjects with 64KA and CF-ICA in their serum is stressed by the fact that three of the six cases had a blunted first phase insulin response in the IVGTT and two children developed Type 1 diabetes during the follow-up.

In the report by Atkinson et al. [10] 64KA were positive in 23 out of 28 (82 %) first degree relatives who later developed diabetes. This finding seems to be in sharp contrast to the frequency of 64KA in our study. However, the discrepancy in the prevalences of the 64KA can be explained by the different composition of the populations studied. We have investigated subjects at low risk for Type 1 diabetes and we further selected individuals according to their ICA status. In our study, 64KA were detected in more than half of the cases with persistently positive ICA and in virtually all individuals who showed CF-ICA, CIAA or a blunted first phase insulin response to intravenous glucose. These data are in agreement with a recently published study of Bärmeier et al. [11], who showed that 64KA are excellent markers for a progressive loss of Beta-cell function in first degree relatives of Type 1 diabetic patients.

About half of the initially ICA positive subjects became ICA negative after a period of 18 months. These individuals may bear no increased risk for Type 1 diabetes [4]. Although our laboratory performed very well in the ICA workshops, a variation of 1 to 2 titres must be considered due to intra- assay variation. The majority of our cases with seroconversion had ICA levels of 5 to 10 JDF Units, but some individuals became ICA negative even after ICA levels of 80 JDF Units had been detected. This observation has also been made by other investigators and indicates the limited predictive value of a single ICA determination. In one study of 321 healthy children Landin-Olsson et al. [22] showed that seven out of nine ICA positive non-diabetic individuals became ICA negative in a follow-up study after more than 2 years without developing Type 1 diabetes. Transient ICA have also been reported in the Barts' Windsor family study. Here, susceptibility to Type 1 diabetes was only assumed when ICA positivity had been confirmed on two further occasions after an interval of four months [23]. In contrast to the high fluctuation rate of ICA, CF-ICA and CIAA remained positive in most of our cases, whereas 64KA positive subjects seem to remain persistently positive in all non-diabetic subjects during the follow-up study.

The importance of 64KA, CF-ICA and CIAA as serological high risk markers for Type 1 diabetes is confirmed by the genetic analysis of our ICA positive population. HLA susceptibility for Type 1 diabetes in Caucasian individuals has been described for HLA DR3, DR4 and a non-Asp genotype at position 57 of the HLA DQ β chain [1, 2]. Recently we reported an association of ICA positivity in non-diabetic subjects with HLA DR3 and DR4 and a non-Asp haplotype [12]. In this study we showed for the first time that in a population of ICA positive individuals there is a significant correlation between the appearance of 64KA, CF-ICA and CIAA with HLA DR4 and the non Asp DQ β allele. Five of six 64KA and CF-ICA positive individuals were non-Asp homozygous and possessed a

DR4 phenotype. These frequencies are in agreement with data from Type 1 diabetic patients from the same ethnic origin [12], indicating a high risk for the development of Type 1 diabetes in these subjects.

Although the present study, based on the investigation of 4208 children and adolescents, is too small to make final conclusions, our preliminary results indicate that a single ICA determination is not sufficient to assess the risk for Type 1 diabetes. By additionally detecting 64KA, CF-ICA or CIAA in an ICA positive population it is possible to identify subjects at high risk. As 64KA were shown to lack fluctuation these antibodies could be valuable serological markers for indicating high risk for the development of Type 1 diabetes in a random population, when blood is available only on one occasion. 64KA, CF-ICA and CIAA seem to be closely correlated with an irreversible active autoimmune process culminating in the manifestation of diabetes. Our results suggest that in prediabetic individuals without a family history of Type 1 diabetes the onset of the disease is preceded by immunological mechanisms that are identical with those in subjects with a family history of Type 1 diabetes.

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Since this manuscript has been submitted, another child (ID no. 4) with persistent ICA (5–10 JDF Units) and 64KA developed Type 1 diabetes.

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