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Combined analysis of islet cell antibodies which cross-react with mouse pancreas, antibodies to the M_r 64,000 islet protein, and antibodies to glutamate decarboxylase in subjects at risk for IDDM

L. Chaillous[1], M. Delamaire[2], A. Elmansour[1], D. Maugendre[2], V. Rohmer[3], M. G. Joseph[3], P. Lecomte[4], J. M. Limal[3], B. Charbonnel[1], H. Allannic[2], P. Saï[1]

[1] Laboratoire Universitaire d'Immuno-Endocrinologie associé INRA/ENVN et service d'Endocrinologie, Nantes, France
[2] Centre de transfusion sanguine et Service d'Endocrinologie, Rennes, France
[3] Service d'Endocrinologie et Service de Pédiatrie, Angers, France
[4] Service d'Endocrinologie, Tours, France

Summary With regard to progression to diabetes, ICA cross-reactive with mouse pancreas, antibodies to the M_r 64,000 islet antigen (64K), antibodies immunotrapping brain GAD activity, and IAA were analysed in 53 ICA-positive first-degree relatives of IDDM patients and 18 ICA-positive schoolchildren without a family history of diabetes. Sera from 29 (55 %) relatives did not bind to mouse pancreas, whereas 24 (45 %) displayed cross-species reaction. ICA titres on human and mouse pancreas were weakly correlated in the overall population ($p < 0.05$) but more strongly ($p < 0.01$) in only those subjects who displayed antibodies on tissues from both species. GAD and 64K antibodies were detected in 31 % and 35 % of relatives. In schoolchildren, the frequencies of cross-species reactive ICA (22 %), GAD antibodies (6 %), 64K antibodies (22 %), and IAA (6 %), were lower ($p < 0.05$) than in relatives. A strong correlation ($p < 0.0001$) was observed between GAD and 64K antibodies. GAD or 64K antibodies were strongly correlated with ICA on human pancreas ($p < 0.0001$) but poorly with ICA on mouse pancreas ($p = 0.05$). After pre-incubation of sera with brain homogenate, ICA titres were unaffected on mouse pancreas but reduced on human pancreas. ICA-positive subjects who displayed neither cross-species reactive ICA nor GAD or 64K antibodies were more frequent ($p < 0.05$) among schoolchildren than relatives, whereas subjects who displayed all antibody specificities were more numerous ($p < 0.04$) in relatives. All relatives with ICA binding only to human pancreas, as well as all schoolchildren, permanently displayed an AIRG higher than the first control percentile and remained non-diabetic. Five of ten relatives with cross-species reactive ICA, GAD and 64K antibodies at the same time displayed acute insulin response to glucose which fell below the first control percentile and developed the disease. The cross-species heterogeneity of ICA was thus confirmed in a large series of relatives and revealed in the general population. Detection of cross-species reactive ICA, GAD antibodies, or 64K antibodies enhances the prognostic significance after conventional ICA screening. The combination of these antibodies is more indicative of diabetes development than any antibody alone. Correlations between tests and absorption experiments indicate that GAD 64 is an ICA antigen on human but not on mouse pancreas, and that ICA which recognize GAD 64K coexist with others which react with mouse pancreas but not with GAD. A third ICA subset might have been revealed by high-titred ICA without either cross-species reactivity or GAD or 64K antibodies. This latter state was more frequent in the general population than in relatives and might typify an early immune response which may or may not progress. [Diabetologia (1994) 37: 491–499]

Key words IDDM, risk, islet cell antibodies, mouse pancreas, 64K antibodies, glutamate decarboxylase

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Corresponding author: Dr. P. Saï, Laboratoire d'Immuno-Endocrinologie cellulaire et moléculaire, Faculté de Médecine (3è étage), 1 rue Gaston Veil, F-44035 Nantes, France

Abbreviations: IDDM, insulin-dependent diabetes mellitus; ICA, islet cell antibodies; GAD, glutamic acid decarboxylase; AIRG, acute insulin response to glucose; JDF, Juvenile Diabetes Foundation; IAA, insulin autoantibodies; 64K, M_r 64,000 islet antigen; IRI, immuno-reactive insulin.

In family studies, ICA remain the best risk marker for IDDM [1–3]. However, their predictive value is limited because many ICA-positive subjects do not progress to diabetes [4, 5], whereas others become diabetic even though ICA have not been detected. Furthermore, ICA alone are weakly predictive in the general population [5–10] accounting for 90% of IDDM subjects [11, 12]. Combining the conventional ICA assay with other tests could enhance predictive efficiency in family members and the general population.

The heterogeneity of ICA can be revealed by the staining pattern on human islet cell [13, 14] and by cross-reaction with mouse pancreas [15, 16]. We and others have suggested that ICA which cross-react with mouse pancreas may confer in family members a higher risk for progression to diabetes than ICA without cross-species reaction [15, 16]. However, the frequency of these ICA subtypes was not unambiguously assessed since sample sizes were small. Furthermore, the cross-species heterogeneity of ICA was not studied in the general population.

Antibodies to the M_r 64,000 (64K) islet protein [17], recognized as the small form of glutamate decarboxylase (GAD 65) [18], are sensitive markers of risk [19–22]. Although GAD 65 is the main immunogenic isoform [23], both forms of GAD (GAD 65 and GAD 67) may be recognized by diabetes-associated antibodies [24], and heterogeneous antibody response to the 64K antigens exists [24–28]. Prospective studies on antibodies which immunoprecipitate GAD enzyme activity from tissue extracts [18, 25–27] are still few, even though these antibodies are promising markers [26, 27]. Both GAD isoforms are present in beta cells [29] but GAD 65 is mainly detected in human islets, whereas mouse islets mainly express GAD 67 [30–32]. Since a subset of ICA recognizes GAD [13, 33], the different patterns of cross-species ICA reactivity could be partly related to the differential expression of GAD isoforms between species. Another ICA antigen is identified as a monosialoganglioside [34] which is present in mouse pancreas [35].

More knowledge is needed about the relationship between these heterogeneous antibodies concerning risk of progression to the disease. We thus carried out a combined analysis of ICA cross-reactive with mouse pancreas, 64K antibodies, GAD antibodies, and IAA in ICA-positive subjects of two distinct populations: relatives of IDDM patients and children of the general population who are at lower risk of developing diabetes. Beyond the descriptive view of antibody frequencies, correlations between these various antibodies, which may or may not coexist, were examined.

Subjects and methods

Subjects

Fifty-three subjects (26 female, 27 male) classified as ICA-positive on human pancreas were recruited from a prospective evaluation of 940 first-degree relatives of IDDM patients (prevalence rate of ICA: 6.5%). Their mean age ± SEM, when the first sample was taken, was 20 ± 4 years. None of these relatives displayed diabetes or impaired glucose tolerance when the initial serum was drawn.

Eighteen ICA-positive healthy schoolchildren from the French background population (eight boys, ten girls, mean age 12 ± 3 years, range 6–16 years) without a family history of diabetes were obtained from a cohort of 1000 children (ICA prevalence rate: 1.8%) initially screened in 1989 at the Institut Régional de Santé (Tours, France). The ICA status of this cohort has been previously published by another group [7]. Personal or parental informed consent was obtained, and the study was approved by the ethical committee. Control sera were obtained from blood donors without personal or familial history of diabetes or of other autoimmune diseases.

ICA determination on human pancreas

ICA were detected without knowledge of sample identity by indirect immunofluorescence [36] on sections of a human frozen pancreas. Antibody titres were determined by serial dilutions to end-point, using a fluoresceinated anti-human IgG serum (Wellcome, Datford, Kent, UK). Results were expressed in JDF units. To apply the same unit for comparison with the assay on mouse pancreas, results obtained on human pancreas were sometimes only expressed by end-point titres. Subjects were classified as ICA-positive if they had at least two positive samples ($\geq$ 5 JDF units). One ICA-positive internal standard and the international reference sera from the international workshops were included [37]. In the fourth international ICA workshop, our laboratory had 100% sensitivity and 86% specificity in blinded analysis of test serum samples, with a limit of detection of 2.5 JDF units.

ICA determination on mouse pancreas

Mouse pancreases were obtained from 10-week-old Balb/c mice (Charles River, St Aubin les Elbeuf, France), snap frozen in liquid N_2, and stored at −70 °C until sectioning (5-μm thick). ICA were detected by indirect immunofluorescence, as previously described [16]. All human sera were absorbed overnight at 4 °C with acetone and methanol-extracted mouse liver powder (Sigma, St Louis, Mo., USA). The sections were incubated overnight at room temperature with the human sera serially diluted (1:2 to 1:256) in 10 mmol/l phosphate buffered saline pH 7.2, and developed for 30 min at room temperature with fluoresceinated rabbit F(ab')2 fragments anti-human IgG (H + L) (Bioatlantic, Nantes, France).

In order to follow-up the fluorescence obtained with sequential samples from each subject, the dilutions of each serum were tested on serial sections of the same batch of mouse pancreases. They were then tested on sections from another batch of pancreases on a different day. Each section was scored according to end-point titre. At least 50 islets were evaluated for each dilution. In each assay, one positive internal standard (80 JDF units on human pancreas) and one negative sample serially diluted were included as quality controls. The negative control was read

Table 1. Autoantibody analyses of conventionally ICA positive sera from first-degree relatives of IDDM patients and from schoolchildren without a family history of diabetes

		Frequencies			Concordances		Discordances	
	IAA	ICA on mouse pancreas [1]	GAD antibodies [2]	64K antibodies [3]	Positive for [1] and [2] or [3]	Negative for [1], [2] and [3]	Positive for [1] but negative for [2] and [3]	Negative for [1] but positive for [2] or [3]
Relatives ($n = 53$)	14 (27%)	24 (45%)	16 (31%)	18 (35%)	10 (19%)	19 (36%)	13 (25%)	10 (19%)
Schoolchildren ($n = 18$)	1 (6%)	4 (22%)	1 (6%)	4 (22%)	0 (0%)	10 (57%)	4 (22%)	4 (22%)

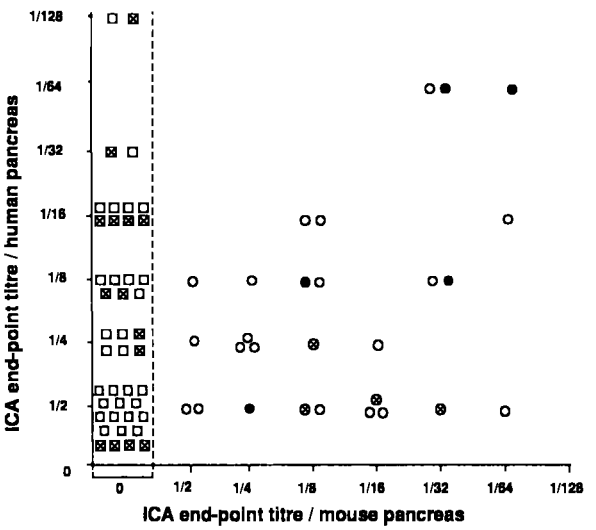

Fig. 1. Detection of ICA on human or on mouse pancreas. To apply the same measuring unit for comparison between human and mouse pancreas, results are expressed by end-point titres. To convert results on human pancreas into JDF units, see Methods. Symbols filled with a cross represent schoolchildren without a family history of diabetes. The other symbols indicate relatives of diabetic patients. In order to allow identification in the following figures, subjects who displayed antibodies on both human and mouse pancreas (○) are differentiated from those who possessed ICA which did not cross-react with mouse pancreas (□). Black symbols (●) indicate subjects who later progressed to diabetes

as negative on 50 occasions. The positive control was always read as positive, with a mean end-point titre of 1/64 and an inter-assay variation of 0.5 titration steps. The effect of GAD in blocking ICA reactivity on human or on mouse pancreas was analysed on four sera using Wistar rat brain homogenate prepared in 1 mmol/l 2-aminoethylisothiouronium bromide, 0.2 mmol/l pyridoxal phosphate, 1 mmol/l EDTA, 1 mmol/l benzamidine, 25 mmol/l potassium phosphate, pH 7.0 (homogenisation buffer) and centrifuged at 100,000 × *g* for 30 min. One volume of serum was incubated with four volumes of homogenate or buffer overnight at 4 °C, and ICA titred on human and on mouse pancreas. All analyses were performed in a blinded fashion by two independent observers who agreed to 96%. Pre-incubation with homogenisation buffer or with rat liver extracts did not affect the ICA titres on pancreases of both species.

Antibodies to the 64K islet antigen

Antibodies to the 64K islet antigen were determined using a modification of the immunoprecipitation method described by Christie et al. [22]. Islets were isolated from pancreases of 7-day-old Wistar rats by collagenase digestion. Batches of 4000 islets were labelled with 1 mCi ^{35}S-menthionine (Amersham Int., Amersham, Bucks., UK), homogenized in 0.25 mmol/l sucrose 10 mmol/l HEPES pH 7.4 supplemented with 1 mmol/l phenylmethylsulphonyl fluoride, 0.1 mm/l p-chloromercuribenzene sulphonic acid (HEPES-buffer) and centrifuged at 10,000 × *g* for 30 min at 4 °C. The pellet was resuspended in HEPES-buffer containing 150 mmol/l NaCl followed by the addition of 2% Triton X-114 for 2 h. After centrifugation at 10,000 × *g* for 15 min, the supernatant was pre-cleared twice with a pool of normal human serum (50 µl/100 µl lysate) and precipitated with the test serum (12.5 µl/50 µl lysate equivalent to 300 islets) for 5 h. The immune complexes were bound to protein A Sepharose (Pharmacia, Uppsala, Sweden), washed five times and eluted with 62 mmol/l Tris HCl pH 6.8, 2% SDS, 5% mercaptoethanol. After separation by SDS-PAGE, all fluorographs were analysed independently by two observers without knowledge of sample identity. Negative and positive control sera were included in each experiment.

Antibodies immunotrapping brain GAD activity

Antibodies to GAD were measured without knowledge of sample identity by determining the enzyme activity immunotrapped by sera from brain extracts [18, 27, 28]. Brains from adult female Wistar rats were homogenized in a 25-fold volume of 1 mmol/l 2-aminoethylisothiouronium bromide, 0.2 mmol/l pyridoxal phosphate, 1 mmol/l EDTA, 1 mmol/l benzamidine, 25 mmol/l potassium phosphate, pH 7 (homogenization buffer), then centrifuged at 100,000 × *g* for 30 min at 4 °C. Aliquots of the extract (60 µl) were incubated with 18 µl test serum for 5 h at 4 °C. Immune complexes were isolated on 20 µl protein A Sepharose and washed four times with 1 ml 10 mmol/l HEPES (pH 7.4) 155 mmol/l NaCl, 10 mmol/l benzamidine, 0.5% Triton X-114, 0.5 mg/ml bovine serum albumin, and once in water. Protein A Sepharose pellets were incubated for 16 h at 37 °C with 25 µl 1 mmol/l glutamic acid and 0.125 µCi [1-^{14}C] glutamic acid in homogenization buffer. $^{14}CO_2$ released was absorbed to filter papers soaked with 50 µl 1 mol/l hyamine hydroxide in methanol and measured by scintillation counting. GAD activity was calculated as percentage of the activity trapped by the same standard antibody-positive control serum used in analyses of 64K antibodies. Sera were regarded as positive if the activity was more than 6%, i.e. greater than 3 SD of the activity in sera from 82 control subjects (mean ± SD: 3 ± 1%). In the first international GAD antibody workshop, our laboratory had 100% sensitivity and 100% specificity in blinded analysis of test serum samples.

Insulin autoantibodies

Sera were tested for the presence of IAA [38, 39], using the ability to bind monoiodinated ^{125}I Tyr-A-14 human insulin (specific activity 250 μCi/μg, Novo, Copenhagen, Denmark). The inter- and intra-assay coefficients of variation were 15 and 10% respectively. In each assay a positive internal standard sample was included. A positive result was defined as a value more than 1.3%, i.e. greater than 3 SD above the mean binding of control sera (0.94 ± 0.12%). These control sera displayed normaly distributed values. The sensitivity of the assay was 0.15%.

Metabolic study

The AIRG were evaluated on the morning after a 12-h overnight fast, as previously described [40]. Glucose (0.5 g/kg body weight of a 30% solution) was injected over 2 min ± 5 s. Blood samples, drawn in less than 15 s, were taken 10 min prior to each bolus and at 1 and 3 min after the end of the injection. IRI concentrations were measured by radioimmunoassay (SB-INSI-5; CIS International, Saclay, France) with ^{125}I-porcine insulin, human insulin as a standard, and guinea pig antiserum to human insulin. Separation was carried out with polyethylene glycol. Sensitivity of the assay was 2.5 μU/ml. The intra- and inter-assay coefficients of variation were less than 10%. AIRG was calculated as the sum of 1 + 3 min insulin values (IRI 1 + 3 min). Inter- and intra-individual variations have been previously reported in control subjects [41]. A polynomial regression ($p < 10^{-5}$) was previously established between age and AIRG with a peak during puberty [42]. With this standard chart for AIRG, a "low" AIRG for each pubertal stage was defined as a value of IRI 1 + 3 min below the first percentile: 29 μU/ml for adults and 40 μU/ml for Tanner's stage 4–5. Oral glucose tolerance tests (75-g glucose load for adult subjects and 1.75 g/kg for children) were performed. Impaired glucose tolerance and diabetes mellitus were defined according to the criteria of the World Health Organization. Blood glucose was measured using a glucose oxidase method.

Statistical analysis

The significance of differences among antibody levels was determined by the Student's *t*-test. The significance of differences among frequencies was determined by chi-square analysis with Yates' correction or by Fisher's exact test. The significance of correlations between antibodies was tested by linear regression analysis or by the Spearman Rho test. Differences were considered significant at *p* less than 0.05.

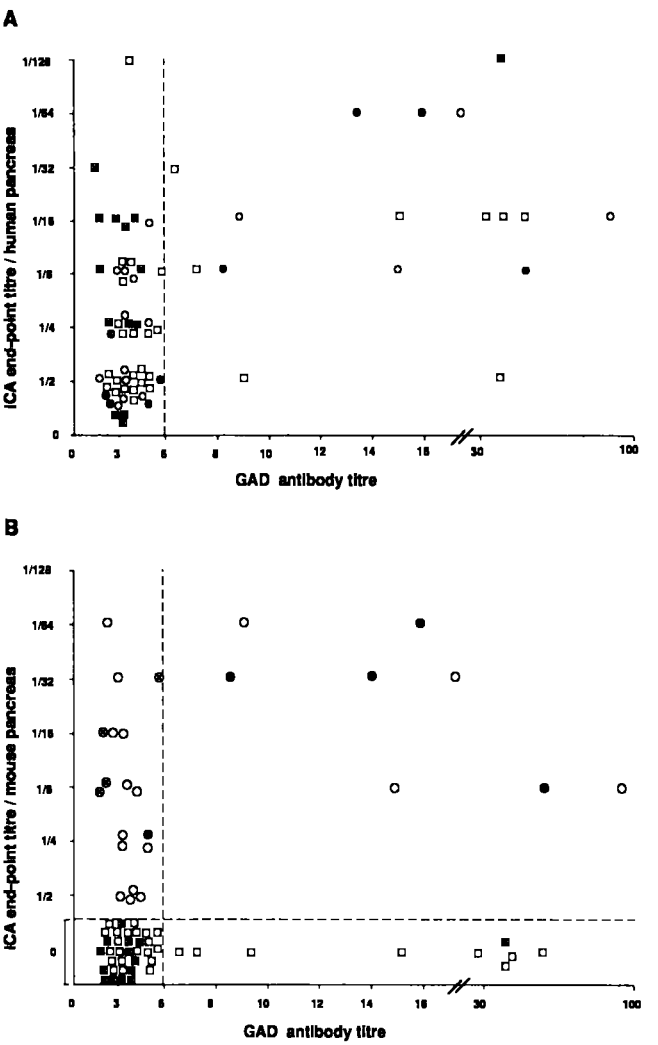

Fig. 2 A, B. Association of ICA detected on human or on mouse pancreas with antibodies to rat brain GAD. ICA titres on human (**A**) or on mouse pancreas (**B**) are compared to GAD antibody titres expressed as the enzyme activity immunoprecipitated from rat brain. The dotted lines indicate the thresholds for positivity. The symbols are identical to those of Figure 1: subjects who displayed antibodies on both human and mouse pancreas (○), subjects who possessed ICA which did not cross-react with mouse pancreas (□). Symbols filled with a cross represent schoolchildren, whereas the other symbols indicate relatives of diabetic patients. Black symbols (●) indicate subjects who progressed to diabetes

Results

Antibody frequencies

Sera from 24 of 53 (45%) first-degree relatives of diabetic patients displayed ICA which cross-reacted with mouse pancreas (Table 1 and Fig. 1), whereas 29 (55%) did not bind at any dilution to mouse pancreas. Mean ICA titres on human pancreas were not different in the subjects with cross-species reactive antibodies (34 ± 10 JDF units) and in those with ICA which only bound to human substrate (29 ± 11 JDF units). Antibodies immunotrapping brain GAD activity and antibodies to the 64K antigen were detected in 16 (31%) and 18 (35%) relatives, respectively (Figs. 2A and 3A). Fourteen (27%) relatives displayed IAA (Fig. 4).

The mean ICA titre on human pancreas was not different in 18 ICA-positive children without a family history of diabetes (35 ± 17 JDF units) and in relatives (30 ± 8 JDF units). The frequencies of ICA which cross-reacted with mouse pancreas (22%), GAD antibodies (6%), 64K antibodies (22%), and IAA (6%) were significantly lower ($p < 0.05$) in these children than in relatives (Table 1, Figs. 1–4).

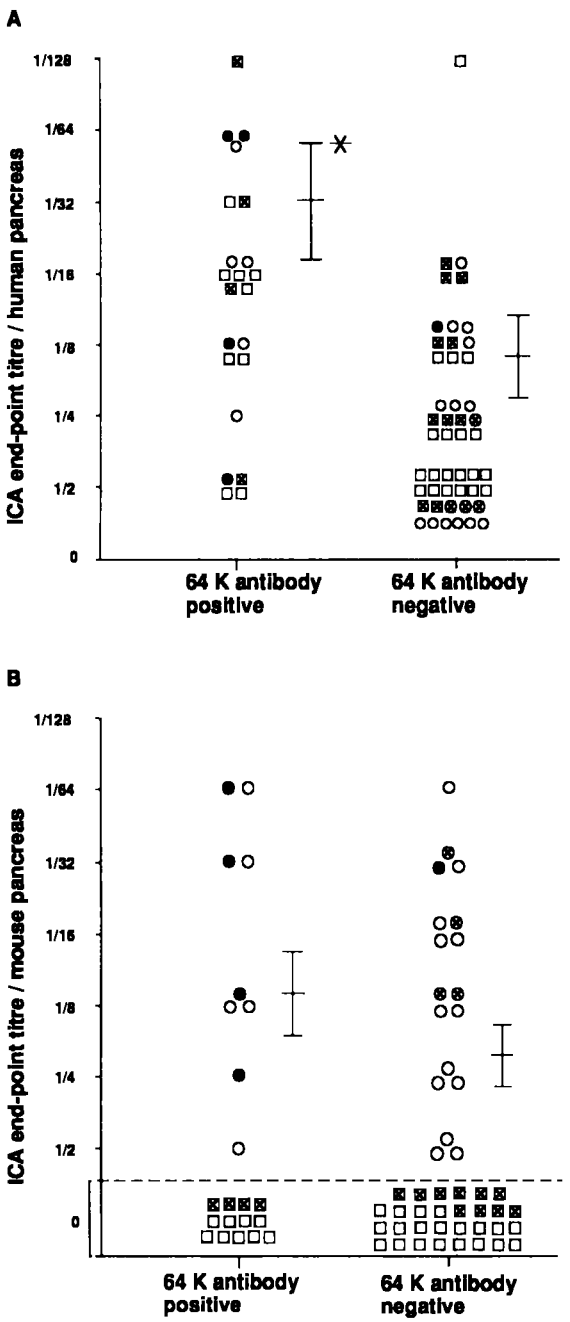

Fig. 3 A, B. Association of islet cell antibodies detected on human (**A**) or on mouse (**B**) pancreas with antibodies to rat islet 64K antigen. The symbols are identical to those of Figure 1: subjects who displayed antibodies on both human and mouse pancreas (○), subjects who possessed ICA which did not cross-react with mouse pancreas (□). Symbols filled with a cross represent schoolchildren, whereas the other symbols indicate relatives of diabetic patients. Black symbols (●) indicate subjects who progressed to diabetes. Asterisks indicate statistical differences in mean ICA titres between 64K antibody positive and 64K antibody negative subjects (* $p < 0.006$)

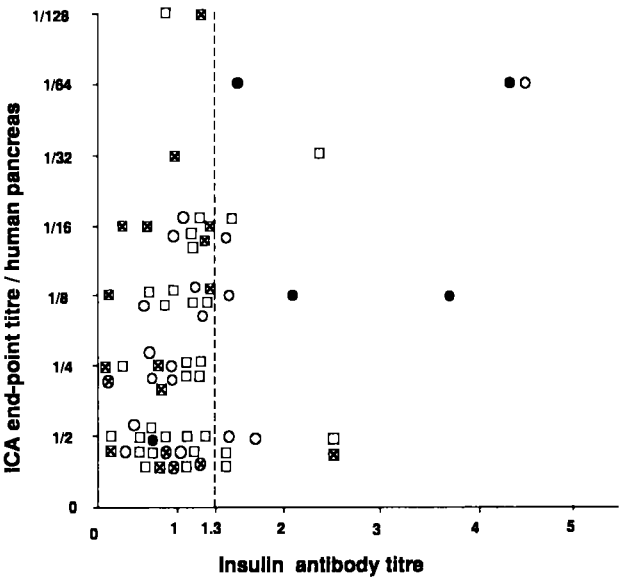

Fig. 4. Association of ICA detected on human or on mouse pancreas with IAA. ICA titres on human pancreas are compared with IAA titres. The dotted line indicates the threshold for IAA positivity. The symbols are identical to those of Figure 1: subjects who displayed antibodies on both human and mouse pancreas (○), subjects who possessed ICA which did not cross-react with mouse pancreas (□). Symbols filled with a cross represent schoolchildren, whereas the other symbols indicate relatives of diabetic patients. Black symbols (●) indicate subjects who progressed to diabetes

Correlations, concordances, and discordances between tests

With respect to all subjects, a correlation ($p < 0.05$) was found between ICA titres on human and mouse pancreas. A stronger correlation ($p < 0.01$) was found between titres on both tissues when only those patients who displayed cross-species reactive ICA were considered.

A strong correlation ($p < 0.0001$) was observed between antibodies immunotrapping brain GAD activity and 64K antibodies. Only two of the 71 individuals possessed GAD antibodies but no detectable 64K antibodies, and seven displayed 64K antibodies but no GAD antibodies.

ICA titres on human pancreas were correlated with GAD antibody titres (Fig. 2 A) or 64K antibodies (Fig. 3 A) in the overall ICA-positive population ($p < 0.006$) or in those subjects who displayed ICA which cross-reacted with mouse pancreas ($p < 0.0001$), but the correlations were weaker ($p < 0.05$) when those who displayed ICA only on human pancreas were considered.

ICA detected on mouse pancreas were more weakly correlated with GAD (Fig. 2 B) or 64K (Fig. 3 B) antibodies than conventional ICA ($p = 0.05$). Thirteen relatives and four schoolchildren displayed cross-species reactive ICA but no GAD or 64K antibodies, whereas 10 relatives and four schoolchildren possessed GAD or 64K antibodies but not cross-species reactive ICA (Table 1).

The ability of brain extracts to inhibit reactivity of sera from four relatives to human or mouse pancreas was tested. These sera contained conventional ICA (1:64, 1:64, 1:64, and 1:16, respectively), ICA binding to

Table 2. Individual data for relatives of IDDM patients who progressed to the disease

Subject n°	Age at first sample (years) /sex	Period from diabetes (months)	HLA DR	ICA				GAD antibodies %		64K antibodies		IAA %		AIRG 1 + 3 min insulin (μU/ml)	
				human pancreas		mouse pancreas									
				0	1	0	1	0	1	0	1	0	1	0	1
1	14/male	30	3.4	5	20	0	1:8	40	58	+	+	2.06	1.20	32	25
2	14/male	1	4	20	n.t.	1:32	n.t.	8.5	n.t.	–	n.t.	3.70	n.t.	29	n.t.
3	11/male	63	4	160	160	1:32	1:64	14	12	+	+	1.50	4.50	68	28
4	13/female	48	3.4	160	160	1:64	1:128	16	20	+	+	2.62	1.40	102	20
5	32/male	24	3.4	5	5	0	1:4	5.0	6.1	+	+	0.71	0.53	18	17

Two results are given for each parameter: (0) result at entry in the study, (1) result at 6 months from diabetes. The titres of ICA on human and on mouse pancreases are given in JDF units and in last positive dilutions, respectively. For GAD antibodies, binding > 6.0% is considered as positive. IAA values > 1.3% are taken as positive. AIRG values below the first percentile of control subjects of similar age (35 μU/ml) are interpreted as a loss of AIRG. n.t., not tested

mouse pancreas (1:32, 1:32, 1:64, and 1:64, respectively), GAD antibodies and 64K antibodies. A blocking effect of brain extracts (loss of fluorescence activity more than two-fold dilutions from the end-point titre) was observed on human pancreas for the four sera whereas the fluorescence observed on mouse pancreas was unaffected (no loss of fluorescence for three sera, and loss equal to one dilution for the fourth serum).

Finally, it is noteworthy that 29 subjects displayed neither cross-species reactive ICA nor GAD or 64K antibodies (Table 1). These subjects were more frequent ($p < 0.05$) among schoolchildren (57%) than among relatives (36%) in spite of similar ICA levels in the two populations (35 ± 17 JDF units vs 30 ± 8 JDF units). Conversely, the presence at the same time of cross-species reactive ICA, GAD and 64K antibodies was more frequent ($p < 0.04$) in relatives (19%) than in schoolchildren (0%).

ICA and IAA correlated together (Fig. 4). in the overall population ($p < 0.004$) or in subjects who displayed cross-species reactive ICA ($p < 0.004$), but not in those who displayed ICA on human pancreas only. Six of the 14 IAA-positive subjects also displayed cross-species reactive ICA, GAD and 64K antibodies at the same time.

Progression to IDDM

In the 29 relatives with ICA binding only to human pancreas during testing of at least four samples corresponding to a 7-year follow-up, the AIRG, quantified every 6 months, remained above the first percentile obtained from control subjects of similar age (data not shown, [16, 42]). None of these 29 relatives progressed to diabetes. Similarly, the 18 ICA-positive children without a family history of diabetes permanently displayed AIRG higher than the first control percentile, and remained non-diabetic after 4 years of follow-up. By contrast, the AIRG of five out of the 24 relatives with cross-species reactive ICA fell below the first control percentile (Table 2). These five relatives developed IDDM. GAD antibodies or 64K were also detected in these five individuals, so that among 10 subjects displaying cross-species reactive ICA and GAD or 64K antibodies at the same time, five progressed to diabetes. Four of these pre-diabetic relatives also possessed IAA, so that four out of six subjects displaying cross-species reactive ICA, IAA, and GAD or 64K antibodies at the same time, progressed to the disease.

Discussion

According to our [16] and other [15] previous studies, we confirm in a much larger series of first-degree relatives of IDDM patients that a subset of ICA cross-reacts with mouse pancreas, whereas another subset only binds to human pancreas. We also reveal similar heterogeneity in children of the general population. All the ICA-positive relatives who progressed to diabetes possessed cross-species reactive ICA, whereas subjects with ICA which only bound to human pancreas did not develop the disease. Furthermore, the lower frequency of cross-species reactive antibodies in children without a family history of diabetes and at low risk of developing diabetes, combined with their presence in all true pre-diabetic relatives, suggests that these antibodies represent markers with prognostic significance for diabetes whereas ICA which do not cross-react may typify a beta-cell lesion which may or may not progress to the disease. This is also consistent with our previous report of a progressive increase in titres of cross-species reactive ICA in relatives who later developed diabetes [16]. In family members and the general population, the limited predictive value of ICA [4, 5, 10] can be overcome by using a several-step procedure. Initial conventional ICA screening, with a low detection threshold to maximize sensitivity, identifies individuals for whom detection of cross-species reactive ICA can then be applied to identify those subjects who will develop the disease. However, a longer follow-up will be required before any speculation can be made about the general population. Populations to be analysed must be even larger than that of the present study

which was based on the screening of 1000 schoolchildren and 940 relatives, and on 4 years and 7 years of follow-up, respectively.

The frequencies of 64K antibodies and of antibodies immunotrapping brain GAD activity were also low in the schoolchildren. Combined with their presence in all true pre-diabetic relatives, this reinforces the notion that these antibodies represent markers for diabetes development [19–22, 28, 29]. Part of the islet 64K antigen is recognized as the GAD 65 isoform, and we confirm the strong correlation between detection of antibodies by immunoprecipitation of the 64K islet antigens and immunotrapping of brain GAD activity [26]. In fact, all five relatives who progressed to the disease had cross-species reactive ICA and GAD or 64K antibodies simultaneously, whereas none of the schoolchildren displayed this association. Thus, combined analysis of these markers may indicate diabetes development better than any given antibody alone.

Discordances between the ICA assays, the immunoprecipitation assay of 64K antibodies with neonatal rat islets, and the immunotrapping assay of GAD from adult rat brain extracts might be attributable to differences in sensitivity between tests, to the existence of antibodies to the enzymatic site of GAD which could hinder detection in the immunotrapping assay [27], or to limitations due to tissue and species specificities of these antibodies even though GAD is highly conserved in mammals. Different patterns of epitope recognition may be also present among individuals. Extraction with Triton X-114 prior to the immunoprecipitation assay may reveal hidden immunoreactive sites. These technical pitfalls and others must be taken into account, but cannot explain all the discordances observed in our study and in another study [23], especially when they concern sera with high-titred antibodies. Thus, information can be drawn from correlations or discordances between tests. Our study revealed correlations between conventional ICA and antibodies detected using the immunoprecipitation or immmunotrapping assays, but several individuals with high ICA titres did not display GAD or 64K antibodies. The qualitative correlation confirms that at least part of conventional ICA positivity is due to a reaction with GAD 64K [33]. However, the discordant cases suggest that antigens other than GAD could also contribute to ICA positivity on human pancreas. On the other hand, ICA detected on mouse pancreas were only poorly correlated with GAD or 64K antibodies, with a substantial number of discordant cases. This poor link is probably indirect since GAD 65 is very weakly expressed by mouse islets and probably not recognized on pancreas of this species [31]. These interpretations appear to be reinforced by our experiments based on pre-absorption of some ICA-positive sera with GAD-containing brain extracts. Although based on only four sera, ICA binding to mouse pancreas is unaffected after pre-absorption whereas, as previously reported [13, 33], ICA positivity on human pancreas is reduced. These experiments should be extended in future studies with more sera tested for absorption of conventional or cross-species reactive ICA, and where recombinant GAD 65 and GAD 67, human and mouse islet extracts are separately applied for blocking.

In a previous study [33], non-diabetic subjects with ICA for more than 5 years had a higher frequency of GAD-reactive ICA than did diabetic patients or true pre-diabetic subjects, and conversions from GAD-reactive to non-GAD reactive ICA were documented near the clinical onset of diabetes. Non-GAD-reactive ICA may thus occur secondarily and provide greater prognostic significance than GAD-reactive ICA which may typify an inductive beta-cell lesion. Even though mainly based on the interpretation of correlations and discordances between tests, our data also reveal distinct ICA types which can coexist: GAD or 64K antibodies on one hand and non-GAD but cross-species reactive ICA on the other. These two ICA subsets were more frequent in relatives, particularly in those who progressed to diabetes, than in schoolchildren. The specificity of cross-species reactive ICA is not characterized but may involve constituents such as monosialogangliosides [34] which, unlike GAD 64K, are expressed by mouse pancreas [35]. Our results might even indicate a third ICA subset (non-GAD-reactive ICA which do not cross-react with mouse pancreas) which might have been revealed in a substantial number of subjects by high-titred ICA without either cross-species reactivity or GAD or 64K antibodies. It was also suggested by the absence of correlation between titres of GAD antibodies and ICA which only bound to human pancreas. This specificity was even more frequent in subjects at the lowest risk (ICA-positive children without a family history of diabetes) than in relatives, and was absent in the subjects who progressed to diabetes. It might thus typify an early immune response that may or may not progress. Hypotheses on sequential worsening with conversions from one ICA subtype to another remain to be proven by absorption experiments during prospective studies. Risk estimation is also improved if IAA levels are taken into account [43, 44]. It appears from our study that IAA has a lower sensitivity as a marker for diabetes development than cross-species reactive ICA, GAD antibodies, or 64K antibodies. However, IAA correlated with ICA in subjects who displayed cross-species reactive antibodies but not in those with antibodies which bound only to human pancreas. In addition, IAA were absent in the children of the general population but present in most of the relatives who progressed to diabetes. This confirms that IAA represent secondary markers of an ongoing beta-cell lesion.

In conclusion, the cross-species heterogeneity of ICA is confirmed in a large series of relatives and revealed in the general population. The detection of ICA

which cross-react with mouse pancreas, GAD antibodies, or 64K antibodies in series after conventional ICA testing identifies subjects at high risk for diabetes. The simultaneous presence of these antibodies may indicate diabetes development better than any antibody alone. GAD 64 is not the antigen recognized by ICA on mouse pancreas but is one of the targets on human pancreas. Non-GAD components expressed by human but not mouse pancreas might be involved in an early response. Secondary responses may involve GAD 64K (almost absent from mouse islets), non-GAD components expressed in mouse islets, and insulin.

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