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Distinct cytoplasmic islet cell antibodies with different risks for Type 1 (insulin-dependent) diabetes mellitus

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Summary. The cytoplasmic islet cell antibody patterns of sera from islet cell antibody positive non-diabetic and diabetic endocrine autoimmune patients, and newly-diagnosed Type 1 (insulin-dependent) diabetic patients were characterised using four layer immunofluorescence with monoclonal anti-proinsulin or anti-glucagon antibodies. Two distinct islet cell antibody types were identified. One gave a diffuse cytoplasmic staining in both Beta and Alpha cells ('whole' islet pattern), and was not affected by pre-incubation with rat brain homogenate. The other had a granular appearance with staining restricted predominantly to Beta cells ('selective' islet pattern) and was completely inhibited by pre-incubation with rat brain homogenate. Some sera appeared to have a 'mixed' islet pattern, in which glucagon-positive cells gave a weaker cytoplasmic staining than proinsulin-positive cells. The granular 'selective' pattern was found in sera from 19 (79%) of 24 non-diabetic endocrine autoimmune patients, in two (22%) endocrine autoimmune patients who developed Type 1 diabetes ($p < 0.0001$ vs non-diabetic endocrine autoimmune patients), and in none of 19 newly-diagnosed diabetic patients. The 'whole' islet pattern was found only in sera from patients who had, or who subsequently progressed to, Type 1 diabetes. This study has identified a novel islet cell antibody specificity and demonstrates that in islet cell antibody positive endocrine autoimmune patients, only islet cell antibodies which stain both Beta and Alpha cells are associated with progression to Type 1 diabetes.

Key words: Islet cell antibodies, Type 1 (insulin-dependent) diabetes mellitus, polyendocrinopathy, indirect immunofluorescence, glutamate decarboxylase.

Type 1 (insulin-dependent) diabetes mellitus is considered to be an autoimmune disease [1]. Islet cell antibodies (ICA) are detected in up to 90% of newly-diagnosed diabetic children [2], and have become the most sensitive serological marker of the disease so far identified. Despite this, and the high predictive value of high titre ICA for developing Type 1 diabetes within families [3], not all individuals with ICA develop diabetes. In particular, in following a cohort of patients with endocrine autoimmunity and detectable ICA ('The Polyendocrine Study'), 62% of patients with ICA greater than 80 Juvenile Diabetes Foundation (JDF) units remained free of diabetes after 10 years [4]. Therefore, in the autoimmune process against the Beta cell, a significant number of individuals produce autoantibodies, but apparently lack elements which then precipitate the progression to disease. A number of factors may be involved. In particular, it has been suggested that ICA include several specificities detectable by immunofluorescence. Indeed, autoantibody specificities binding only to islet Alpha or Delta cells have been described, and these ICA subtypes were not associated with Type 1 diabetes [5].

We, therefore, reassessed the immunofluorescence specificities of cytoplasmic ICA using a four layer immunofluorescent technique in long-standing high titre ICA-positive non-diabetic endocrine autoimmune patients, ICA-positive endocrine autoimmune patients who have developed Type 1 diabetes, and in newly-diagnosed Type 1 diabetic patients. We describe here a novel ICA specificity, and demonstrate that only a sub-group of ICA are associated with progression to Type 1 diabetes.

Subjects and methods

Patients

Sera were analysed from 24 patients with endocrine autoimmunity and ICA greater than 40 Juvenile Diabetes Foundation (JDF) units for a period of more than 7 years without developing diabetes. These patients had a mean age of 47 years (range 20–88); 19 were female. Sera were also obtained from nine ICA-positive (range: 20 – greater

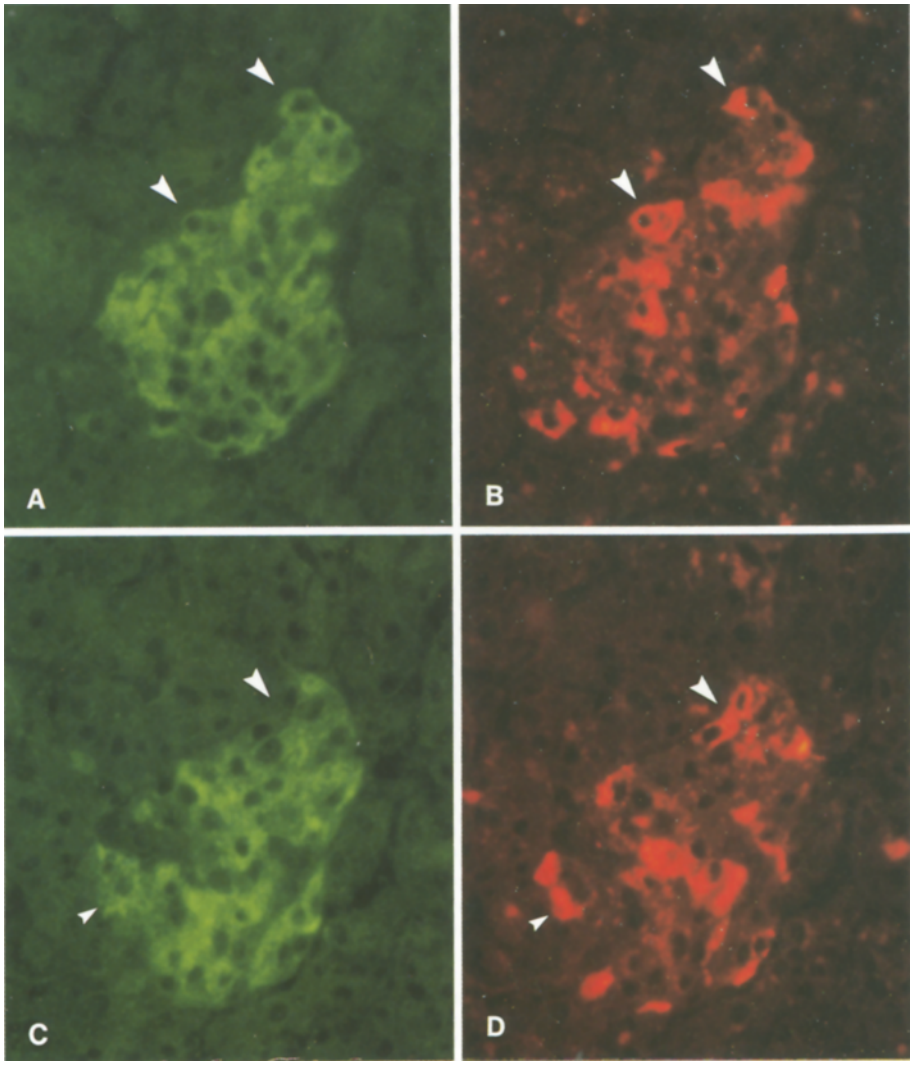

Fig. 1 A–D. Four layer immunofluorescence on a pancreas section stained with a serum from an islet cell antibody (ICA) positive (greater than 40 JDF-units) newly-diagnosed Type 1 (insulin-dependent) diabetic patient and Fluorescein isothiocyarate (FITC) anti-human IgG (**A**), together with anti-glucagon and biotin anti-mouse Ig/Tetramethylrhodamine isothiocyarate (TRITC) avidin (**B**). The glucagon-cells stained in **B** are also stained by the ICA (see arrows), confirming a 'whole' islet pattern. **C** and **D** show the same islet on the following section stained with a serum from an ICA-positive (greater than 40 JDF-units) non-diabetic endocrine autoimmune patient and FITC anti-human IgG (**C**), together with anti-glucagon and biotin anti-mouse Ig/TRITC avidin (**D**). In this section the majority of glucagon-cells are not stained by the ICA (see large arrows) indicating a Beta-cell 'selective' islet pattern. A few glucagon-cells are weakly stained by the same serum (small arrows). The staining in **C** appears more granular than in **A**

than 80 JDF-units) endocrine autoimmune patients who developed Type 1 diabetes during prospective follow-up. These sera were collected from 48 to 0 months prior to insulin dependency. The mean age of these patients at the time the samples were collected was 52 years (range 16–74); seven were female. Sera from 19 newly-diagnosed Type 1 diabetic patients with ICA of more than 40 JDF-units and no endocrine autoimmunity were also tested. These patients had a mean age of 11 years (range 4–16); ten were female.

Methods

ICA were detected in patient sera by indirect immunofluorescence on unfixed cryostat sections of blood group 0 human pancreas, as previously described [3]. Quantification in JDF-units was performed using serial dilutions of serum in 10 mmol/l phosphate buffered saline (PBS) pH 7.2 in parallel with standard samples of 4, 8, 16, 32, and 80 JDF-units ICA, according to the recommendations of the Second International ICA Workshop, as previously described [3].

For the four layer immunofluorescent technique, sections were first incubated with undiluted patient sera for 20 min, washed in PBS, and incubated with Fluorescein isothiocyarate (FITC)-rabbit anti-human IgG (Dako, Glostrup, Denmark) diluted 1/20 in PBS for 20 min. The third layer consisted of a 20-min incubation with either monoclonal antibody to human glucagon (GLU-1-F120; Novo, Copenhagen, Denmark) diluted 1/2, or monoclonal antibody to porcine pro-insulin (PPI-2-F12; Novo) undiluted. The sections were then washed in PBS, and incubated with biotinylated horse anti-mouse Ig (Vector Laboratories Inc, Burlingame, Calif., USA) diluted 1/40. After further washing in PBS, the anti-hormone antibodies were visualised by incubation of sections with Tetramethylrhodamine isothiocyarate (TRITC)-avidin D (Vector Laboratories Inc.) diluted 1/80 in PBS. All samples were tested and read without prior knowledge of their content.

Blocking experiments were carried out on selected sera using rat brain homogenate prepared by homogenisation of Wistar Firth rat brain 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 centrifugation at 100000 *g* for 30 min. In some studies, the rat brain homogenate was pre-cleared with the S3 polyclonal sheep anti-glutamate decarboxylase (GAD) antibody (kindly donated by Dr. M. Tappaz, Lyon, France) or sera with ICA. For pre-clearing, equal volumes of serum and rat brain homogenate were mixed, incubated for 2 h at 4 °C and the immune complexes precipitated by protein A Sepharose (Pharmacia LKB, Uppsala, Sweden). The remaining supernatant was used for inhibition studies. For all inhibitions, one volume of serum was incubated with four volumes of appropriate supernatant, homogenate or buffer overnight at 4 °C, and ICA titred by immunofluorescence.

Results

Two ICA staining patterns were identified by immunofluorescence. One gave a diffuse pattern on both Beta cells (proinsulin-positive) and Alpha cells (glucagon-positive

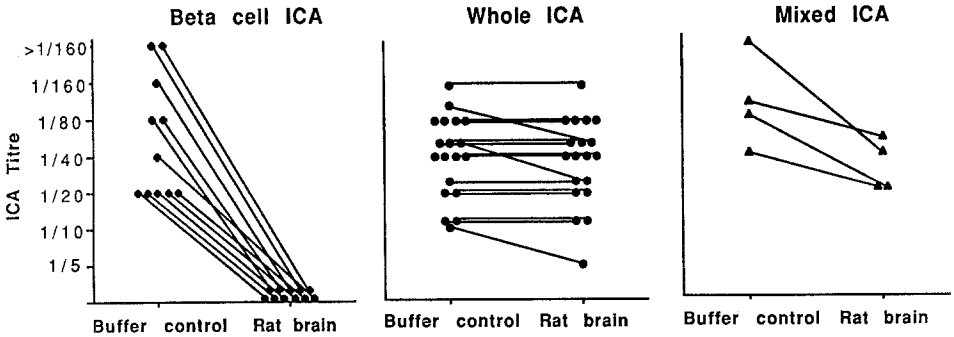

Fig. 2. Inhibiton of islet cell antibody (ICA) titres by rat brain homogenate and control buffer. The Beta cell 'selective' islet pattern was completely abolished by rat brain homogenate while 'whole' islet ICA remained unaffected. The 'mixed' islet pattern was only partially inhibited

cells): 'whole' islet pattern. The other pattern had a fine granular appearance and was Beta cell 'selective', being negative for the majority, although not all cells identified by the anti-glucagon antiserum (Fig. 1). A proportion of sera stained both Beta and Alpha cells, but the fluorescence was stronger in the Beta cells. This pattern was classified as 'mixed'.

Eleven sera with the Beta cell 'selective' pattern, 19 with the 'whole' islet pattern and four with a 'mixed' pattern were tested after incubation with rat brain homogenate (Fig. 2). Pre-incubation with homogenisation buffer did not affect the ICA titres in all samples, and pre-incubation with rat brain homogenate did not affect the titre of sera with the 'whole' islet pattern. In contrast, pre-incubation with rat brain homogenate completely abolished the staining in all sera with the Beta cell 'selective' islet pattern. Furthermore, pre-clearing of the rat brain homogenate with sheep anti-GAD Ig or a serum with a Beta cell 'selective' pattern, removed the inhibitory effect of rat brain homogenate. Pre-clearing with a 'whole' islet ICA did not remove the inhibitory effect of rat brain homogenate on the Beta cell 'selective' islet pattern. ICA titres of sera with a 'mixed' ICA pattern were reduced but not completely abolished.

Sera from 17 of 19 (89%) of the newly-diagnosed Type 1 diabetic patients gave the 'whole' islet pattern. The remaining two samples gave a 'mixed' pattern. None were only Beta cell 'selective'. In contrast, none of the sera from the 24 non-diabetic endocrine autoimmune patients had the 'whole' islet pattern. Sera from 19 of those (79%) gave a Beta cell 'selective' pattern only, and the remaining five contained a 'mixed' pattern. Of the nine endocrine autoimmune patients who developed Type 1 diabetes, sera from six (67%) gave the 'whole' islet pattern, two (22%) the Beta cell 'selective' pattern ($p < 0.0001$ vs non-diabetic patients), and one (11%) a 'mixed' pattern.

Discussion

This study confirms the existence of different antibody specificities of ICA detected by immunofluorescence. It reveals a novel type of ICA which has a reactivity restricted predominantly to the islet Beta cells. This is in contrast to the classically described immunofluorescence cytoplasmic ICA which stains also the Alpha, Delta and PP cells within islets [6]. The pattern of cytoplasmic staining also differs, the Beta cell 'selective' pattern giving a more granular appearance, suggesting that the respective autoantigens have different cytoplasmic distributions. Further, the Beta cell 'selective' pattern was completely blocked by pre-incubation with rat brain homogenate, while the 'whole' islet pattern remained unaffected. This inhibition was prevented by pre-clearing the rat brain homogenate with a sheep anti-GAD antibody.

These data indicate that the specificity of the antibody which gives the Beta cell 'selective' islet pattern is most likely directed against GAD, recently identified as the islet 64 kilodalton autoantigen in Type 1 diabetes [7]. GAD is predominantly localised in the Beta cells of islets, although it has been suggested that the enzyme is also present in some glucagon-cells (R. Pujol-Borrell, Barcelona, Spain; J. Petersen, Gentofte, Denmark; personal communications), a finding which can explain the weak staining of a few glucagon-cells in the Beta cell 'selective' pattern.

It is likely that the antibody we have described is similar to the anti-GAD antibody found associated with 'Stiff-man syndrome' [8]. Indeed, sera from a proportion of patients with 'Stiff-man syndrome' produced a similar 'selective' islet staining on sections of fixed rat pancreas [8]. It was suggested that this anti-GAD antibody is different either in titre or specificity to anti-GAD antibodies associated with Type 1 diabetes [7]. Interestingly, the novel Beta cell 'selective' islet pattern was found almost exclusively in individuals who have not developed Type 1 diabetes. In contrast, the 'whole' islet pattern was found only in newly-diagnosed Type 1 diabetic patients and in ICA-positive endocrine autoimmune patients who subsequently developed Type 1 diabetes. With reference to 'Stiff-man syndrome', it will be of interest to determine which islet pattern is given by sera from those patients who also had Type 1 diabetes.

Previous studies have shown that ICA do not always have very high specificity for disease progression [4, 9]. The data from this study indicate that subtyping the ICA may increase their specificity for disease. Indeed, in "The Polyendocrine Study" the predictive value of ICA greater than 80 JDF-units for progression to insulin dependency within 10 years was only 38% [4] but, clearly, if those with a Beta cell 'selective' islet pattern are excluded, the predictive value would increase considerably. In the current study, all the ICA-positive non-diabetic individuals studied also had other endocrine autoimmunities. Therefore, it remains to be established whether the Beta cell 'selective' islet pattern will also be found in other ICA-positive populations or if it

is merely associated with polyendocrinopathy. Studies in first degree relatives of Type 1 diabetic patients, and in particular, school age children are required.

The data, including the suggestion of the presence of a 'mixed' islet pattern in some sera, give further evidence that ICA-positive sera may contain several autoantibody specificities. The findings are further supported by the recent report that some ICA do not react on mouse pancreatic tissue. ICA-positive sera which do not stain mouse islets are Beta-cell 'selective' in the rat, and patients with this subgroup appear to have a low risk of progression to Type 1 diabetes [10]. Serum exchanges are being performed to determine if these ICA correspond to the Beta cell 'selective' pattern described here.

Several new islet autoantibody specificities have been recently identified, and we must now consider that the autoimmune response toward islet antigens is heterogeneous. Our results show that even within cytoplasmic ICA detected by immunofluorescence, there is heterogeneity, and that only some are likely to be predictive of Type 1 diabetes. With these, and other markers now available, predictive models of Type 1 diabetes can be further developed.

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References

1. Bonifacio E, Bottazzo GF (1991) Immunology of IDDM (Type I diabetes) – entering the '90s. In: Alberti KGMM, Krall LP (eds) The diabetes annual/6. Elsevier, Amsterdam, pp 20–47
2. Pilcher CC, Elliot RB (1990) A sensitive and reproducible method for the assay of human islet cell antibodies. J Immunol Meth 129: 111–117
3. Bonifacio E, Bingley P, Shattock M et al. (1990) Quantification of islet cell antibodies and prediction of insulin-dependent diabetes. Lancet 335: 147–149
4. Bosi E, Becker F, Bonifacio E et al. (1991) Progression to Type I (insulin-dependent) diabetes in autoimmune endocrine patients with islet cell antibodies. Diabetes 40: 977–984
5. Bottazzo GF, Lendrum R (1976) Separate autoantibodies to human pancreatic glucagon and somatostatin cells. Lancet II: 873–876
6. Bottazzo GF, Doniach D (1978) Islet cell antibodies (ICA) in diabetes mellitus: evidence of an autoantigen common to all the cells in the islet of Langerhans. Ric Clin Lab 8: 29–38
7. Baekkeshov S, Aanstoot H-K, Christgau S et al. (1990) Identification of the 64 K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347: 151–156
8. Solimena M, Folli F, Aparisi R, Pozza G, De Camilli P (1990) Autoantibodies to GABA-ergic neurons and pancreatic Beta-cells in stiff-man syndrome. N Engl J Med 322: 1555–1560
9. Landin-Olsson M, Karlsson A, Dahlquist G, Blom L, Lernmark Å, Sundkvist G (1989) Islet cell and other organ-specific autoantibodies in all children developing Type 1 (insulin-dependent) diabetes mellitus in Sweden during one-year and in matched control children. Diabetologia 32: 387–395
10. Gianani R, Pugliese A, Bonner-Wier S, Shiffrin AJ, Soelcher JS, Erlich H, Awdeh Z, Alper CA, Jackson RA, Eisenbarth GS (1992) Prognostically significant heterogeneity of cytoplasmic islet cell antibodies in relatives of patients with type 1 diabetes. Diabetes (in press)

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