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METHODS

DETERMINATION OF ISLET CELL ANTIBODIES USING AN ELISA SYSTEM WITH A PREPARATION OF RAT INSULINOMA (RIN A2) CELLS

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SUMMARY An enzyme-linked immunosorbent assay (ELISA) was established for the detection of islet cell antibodies in human sera. The antigen was prepared from rat insulinoma (RIN A2) cells. Cells were dissociated in lysis buffer and the lysate was centrifuged at 100,000 × g. The supernatant was used to coat microtiter ELISA plates (10 µg protein/ml in PBS pH 7.2). Non-specific binding sites on the plates were blocked with 2% PBS-BSA. Human test sera were preabsorbed on separate plates using 2% PBS-BSA and incubated on precoated plates at an optimal dilution of 1/10 in 60 mM PBS for 60 min at 37°C. Phosphatase-labeled anti-human IgG serum and phosphatase substrate were applied and the reaction was stopped by adding 3 M NaOH. Out of 90 sera from type I diabetic patients,

47 (52.2%) reacted in the new ELISA whereas none of 15 type II diabetics, 50 sera containing non-islet specific antibodies or 100 normal controls were positive. In the same group of patients, ICA were positive in 63.3%. When both, the ELISA and conventional ICA testing were applied, the number of positives was increased to 83%. The ICA-ELISA with the above described antigen preparation provides a well standardized and reproducible test method which is highly specific for type I diabetes. It may therefore be useful for large screening procedures.

Key words: Islet cell antibodies, ELISA, RIN cell preparation

INTRODUCTION

THE detection of islet cell antibodies (ICA) in the serum is a hallmark of insulitis and type I (insulin-dependent) diabetes mellitus (1, 2). Using the indirect immunofluorescence (IFL) test on unfixed cryostat sections of human pancreas, 60–90% of patients are positive at the onset of disease, whereas in normal individuals the antibodies occur in less than 1% of the cases (3–5). The presence in the serum of ICA points to an autoimmune form of diabetes even when the disease starts atypically at an older age or when the diabetes can be initially controlled by oral antidiabetic drugs (6–10). The detection of ICA

may therefore be relevant to the initial aetiological classification of diabetes (11).

Prospective family studies as well as the systematic investigation of non-diabetic, HLA identical twins and triplets of type I diabetics have shown that ICA may be present in the serum years before the clinical manifestation of diabetes and well before a deficiency of insulin secretion can be established by the sensitive i.v. glucose tolerance test (12, 13). Therefore, the determination of ICA has become increasingly important to identify individuals at risk to develop type I diabetes among predisposed individuals (14–19) or in population screening programs (20–22). Unfortunately, the standardization of the conventional immunofluorescence test for the detection of ICA has caused multiple problems and the results vary considerably when different donor organs are used as a substrate. We now describe a reproducible

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detection system for ICA using an enzyme-linked immunosorbent assay (ELISA) with a preparation of the RIN A2 cell line.

MATERIALS AND METHODS

Sera

The sera from 90 type I diabetics (32 males/58 females, mean age 19 yrs, range 17–45 yrs) were investigated. The duration of diabetes varied between onset of disease and a 17 yr course. Thirty sera could be gained within three months of diagnosis.

Fifteen sera from type II diabetics were tested (10 males/5 females, mean age 56 yrs, range 39–74 yrs). Only patients with more than 2 yrs of diabetes were studied. All patients were under treatment with diet and antidiabetic drugs. Cases of secondary diabetes were excluded.

To test the specificity of results, 50 sera from non-diabetic individuals were also investigated. Twelve of them had autoantibodies (Ab) to exocrine pancreatic tissue, five thyroid microsomal Ab, two adrenocortical Ab, six gastric parietal cell Ab, five dsDNA Ab, 10 other antinuclear Ab, five smooth muscle Ab, five anti-mitochondrial Ab.

One hundred sera from apparently healthy blood donors were also investigated. None of these normals had a family history of type I diabetes. The mean age of the control group was 24.5 yrs, range: 10–30 yrs.

All sera were stored at –20°C. Before testing in the cell assay, the sera were decomplemented by a 30 min incubation in a water bath at 56°C.

RIN A2 Cells

This insulin-producing rat insulinoma cell line RIN A2 derived from the subclone RIN m5F was kindly gained from Prof. Ammon, Tübingen. The cells were cultured in RPMI 1640 medium supplemented with 10% v/v heat-inactivated fetal calf serum, 5% glutamin, penicillin 100 U/ml and streptomycin 100 µg/ml at 37°C in an atmosphere containing 95% air and 5% CO₂.

The cells were fed every second day and passaged every four days. The cells were dissociated from the culture surface by incubation with trypsin-EDTA solution (0.5 mg/ml and 0.2 mg/ml respectively in calcium/magnesium-free Hank's balanced salt solution) for 4–8 min at 37°C. Cells were then washed twice in their original medium and resuspended. At the final harvesting, the cells were washed three times in RPMI 1640 without fetal calf serum.

Preparation of Pancreatic Islet Cells from Rat Pancreas

Wistar rats with a body weight of about 150 g were used throughout. Islets were prepared after the method of Lasy and Kostanovsky (22). After nembutal anesthesia, the pancreas was ballooned by an intra-arteric injection of a solution of neutral red. The pancreas was removed, mechanically minced with scissors and digested with collagenase (Sigma, type V, 7.5 mg/ml tissue) at 37°C. After microscopic control the digestion was stopped with ice cold Hank's solution. Exocrine and endocrine pancreas were separated using the discontinuous Ficoll density gradient centrifugation. The islets were selectively picked with a pipett under a stereo microscope. Picked islets were incubated in EGTA solution for 30 min at room temperature. The yield of islets was about 300/rat, and from these, about 200,000–300,000 islet cells were gained.

Antigen Preparation

RIN A2 cells were washed three times in phosphate buffered saline for 10 min each, and centrifuged at 200 × g in between. Lysis of cells was obtained by incubation with lysis buffer (Triton X-100 10 ml/L, Trasylol 50 ml/L, PMSF 0.17 g/L, NaCl 8.8 g/L, Hepes 11.9 g/L, pH 7.6) for 90 min at 4°C under constant stirring. The lysate was subsequently centrifuged at 100,000 × g for 90 min at 4°C.

The supernatant was designated as fraction A. For the isolation of the soluted membrane glycoproteins (23), the supernatant was further processed by affinity chromatography using Lentil-Lectin

Sepharose 4B (Pharmacia): the supernatant was mixed with the gel and incubated overnight in the roller at 4°C. It was then transmitted to the ice cooled chromatography column. The unbound fractions were washed with PBS + 1 M NaCl and eluted with PBS + 0.5 M methyl-alpha-D-mannopyranosid. This preparation was designated as fraction B. The protein concentration was determined according to the method of Bradford (24).

Coating of ELISA Plates

To determine the antibody binding of patients' sera to surface membranes of intact islet cells, 100 µl of suspension from freshly prepared viable rat islet cells and RIN A2 cells were submitted to microtiter plates ((a) Immulon M 129B, (b) Nunc-Immuno plate) at a density of 1 × 10⁵ cells/ml in RPMI 1640 medium. In different experiments, cells were seeded with or without preincubation of plates with poly-L-lysine (Sigma), respectively. The cell suspensions were primarily attached to the microtiter plates by centrifugation at 100 × g for 5 min. Suspended cells were cultured for 24 h and they were finally washed twice by gentle incubation with PBS pH 7.4. For their use in ELISA tests, the cells on coated plates either remained unfixed, or they were fixed with 4% paraformaldehyde or 0.25% glutaraldehyde in different experiments.

The coating of plates with islet cell preparations was performed with six different buffers. (a) 50 mM Carbonate buffer, pH 9.6. (b) 50 mM Carbonate buffer, pH 7.2. (c) 0.2 M Carbonate buffer, pH 8.3. (d) 0.2 M Carbonate buffer, pH 7.2. (e) 150 mM PBS, pH 7.2. (f) 150 mM PBS, pH 5.5.

ELISA Test Procedure

Coated plates were washed three times with PBS pH 7.4. Non-specific binding sites were blocked by incubation with 2% bovine serum albumin and subsequent washing with PBS. Human sera were diluted in 60 mM PBS buffer and applied in doubles. After incubation with sera for 60 min the plates were washed three times with Tris-Tween 0.1%, and incubated for 30 min with phosphatase-conjugated anti-human IgG (Tago) diluted 1/2,000 in PBS. After washing four times, 100 µl of substrate solution were added to each well. The reaction was stopped after visual control by adding 3 M NaOH. The coloured reaction product was measured at 410 nm with a Dynatech microELISA autoreader MR 600.

Detection of BSA Antibodies

Because all ELISA plates had been blocked with 2% BSA and the RIN cells had been originally cultured with BSA, the positive reaction of a serum could be due to the presence of albumin antibodies. Therefore, all positive sera were preabsorbed on ELISA plates coated with 2% BSA and they were then retested using the above ELISA system.

Indirect Immunofluorescence Test

For the detection of conventional ICA the indirect IFL test was applied using unfixed cryostat sections of human pancreas from a donor with blood group O (25).

RESULTS

Adhesion of Cells to the ELISA Microtiter Plates

The adhesion of viable RIN A2 cells and of freshly isolated rat islet cells to the microtiter plates was insufficient. It was only marginally improved by preincubation of plates with Poly-L-Lysine. Even after extremely cautious washing and incubation procedures there were large gaps in the originally dense cell layer (Figure 1a and 1b). The attachment of cells was not improved after fixation with 4% paraformaldehyde, but fixation with 0.25% glutaraldehyde resulted in a good adhesion throughout the ELISA procedure.

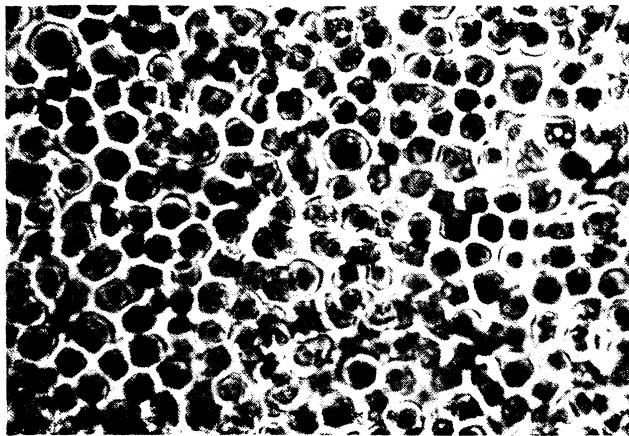


Figure 1(a). Phase contrast photography of an ELISA microtitre plate coated with unfixed RIN A2 cells before further testing.

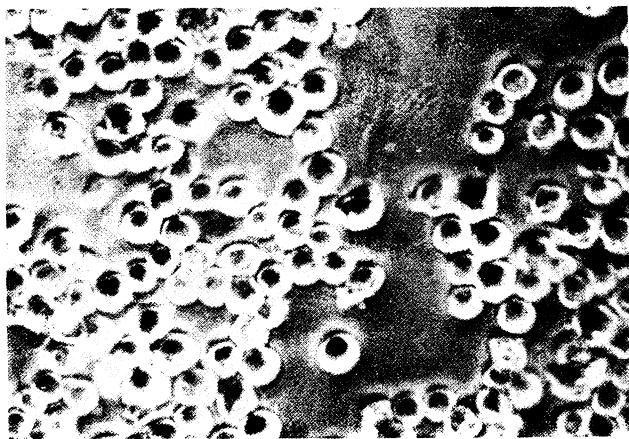


Figure 1(b). At the end of the ELISA procedure many RIN A2 cells were washed off from the bottom both with native plates as well as after preincubation with Poly-L-Lysin. These holes caused an unacceptable non-specific binding of sera.

ELISA-ICA Test Results with Viable Islet Cells

The large gaps in the coating cell layer observed with unfixed or formalin-fixed islets resulted in a significant nonspecific binding of serum components to the plates so that no reproducible results could be obtained. When glutaraldehyde-fixed RIN A2 cells were used, the individual ELISA test results were not consistent and they did not allow to distinguish type I diabetics from normal individuals. When freshly isolated glutaraldehyde-fixed rat islet cells were used, the mean binding of type I diabetic sera was higher in each test than the one obtained with control sera (Figure 2). However, the individual reproducibility of positive and negative test results was insufficient. The intra assay variance was 9.4%, the inter assay variance was as high as 31%. This system was therefore abandoned.

ELISA with Fraction A from RIN A2 Cells

Evaluation of the optimal coating buffer Figure 3 shows the ELISA test results in relation to the sort and pH of

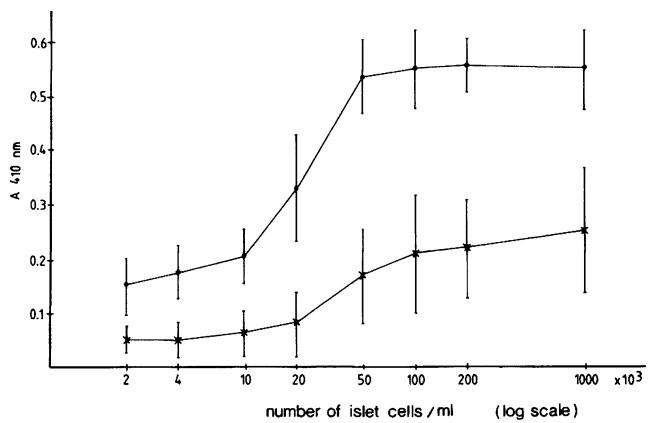


Figure 2. Results of an ELISA using freshly isolated rat islet cells fixed with 0.25% glutaraldehyde. The sera from type I diabetics gave an increased overall binding as compared to controls. However, the individual test results were very variable and not clearly reproducible. (●), Type I diabetics; (X), normal controls.

coating buffer used for the dilution of the antigen. The antigen (50 µl/well) was incubated overnight at 4°C. The further test conditions were as described below (Figure 6). The highest binding of diabetic sera and the best discrimination between diabetics and normal controls was achieved with a phosphate buffer pH 7.2.

Optimal antigen concentration for coating The RIN A2 cell fraction A was used at dilutions 1/50–1/10,000. It is evident from Figure 4 that the best discrimination between diabetic and normal human sera was obtained at a dilution of 1/200 which contained 10 µg protein/ml.

Optimal serum dilution The highest antibody binding was obtained with a serum dilution of 1/5 (Figure 5). The absorption values of both patients and controls

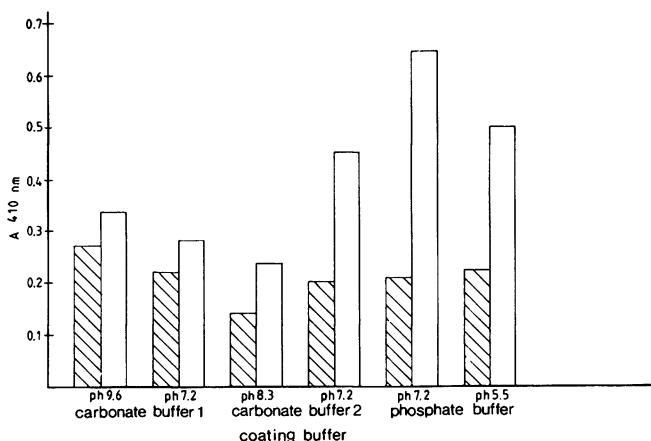


Figure 3. Influence of various coating buffers upon the ICA-ELISA with the RIN A2 cell preparation (fraction A). The mean values of results obtained with 10 ICA-positive type I diabetic patients and with 10 controls are given in the figure. (□), Type I diabetics; (▨), normal controls.

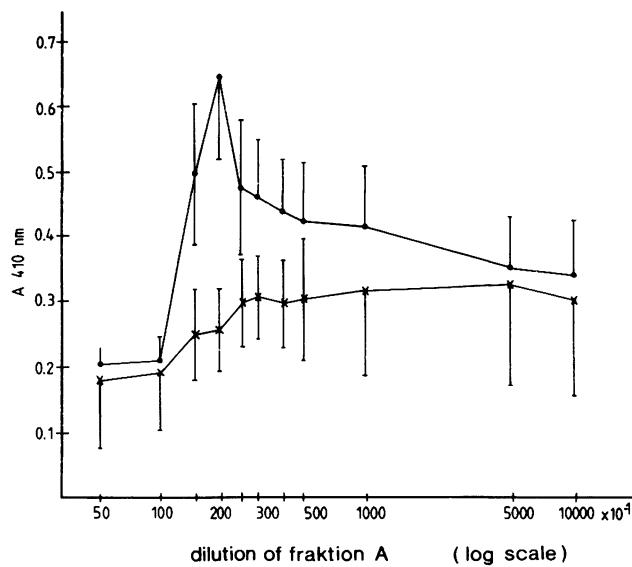


Figure 4. Influence of the concentration of coating antigen upon ICA-ELISA test results. Fraction A from RIN A2 cells was diluted in PBS pH 7.2 as a coating buffer and 100 µl/well were used. The figure gives the mean values of results obtained with 10 sera from type I diabetics and from 10 normal controls. The best discrimination between the two groups was obtained at a serum dilution of 1/200 (= 10 µg protein/ml). (●), Type I diabetics; (X), normal controls.

decreased with increasing serum dilutions used in the assay. The best discrimination between patients and controls was obtained with sera applied 1/10. At this dilution the mean value of patients versus mean value of controls was 2.72.

ELISA with Fraction B from RIN A2 Cells

When the membrane glycoprotein fraction was used for coating, diabetic sera did not produce an increased

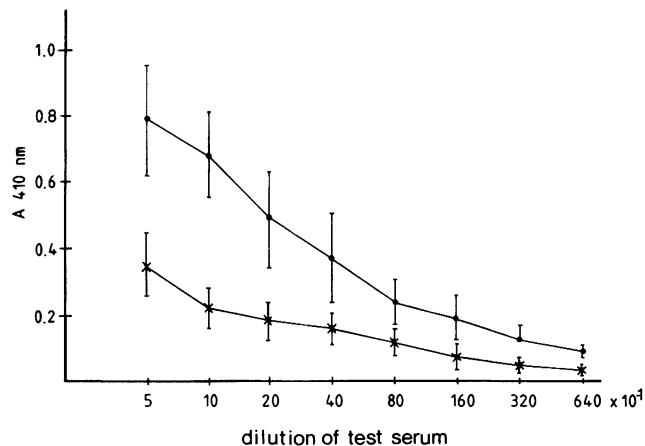


Figure 5. Influence of the concentration of test serum upon the specific binding in the ICA-ELISA. Fraction A from RIN A2 cells was used at 1/200. PBS pH 7.2 as a coating buffer. The best discrimination between type I diabetic patients and controls was achieved at a serum concentration of 1/10. (●), Type I diabetics; (X), normal controls.

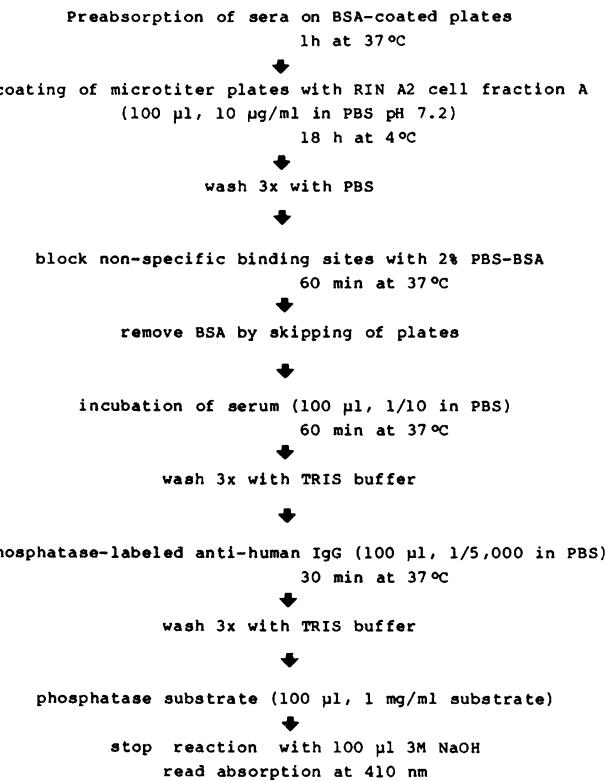


Figure 6. Schematic description of the optimal ICA-ELISA using fraction A from the RIN A2 cell preparation.

binding with any of the procedures applied for fraction A. Therefore, fraction B was not used for further testing.

On the basis of the results above described the ELISA for ICA testing was applied as indicated in Figure 6.

Specificity of the islet cell antibody—ELISA and its correlation with conventional ICA testing Forty-seven of the 90 sera (52.2%) from type I diabetic patients were positive in the new ELISA. Fifty-seven (63.3%) were ICA-positive as measured by the indirect immunofluorescence test. None of the sera from type II diabetics or from patients with non-islet cell specific autoantibodies or controls was positive in the ELISA. The results obtained with the ELISA and with the IFL test on 30 sera from type I diabetics gained within three months of diagnosis are given in Table 1. ICA were positive in 20 (67%),

Table 1 Results of the ICA-ELISA using fraction A from RIN A2 cells compared with the results obtained with conventional ICA determination using the indirect immunofluorescence test on unfixed cryostat sections of human pancreas. The 30 sera were obtained within the first three months of diagnosis of type I diabetes

	ICA (IFL-Test)	
	positive	negative
ELISA	positive	14
ELISA	negative	6

ELISA-ICA reacted in 19 (63%) of the cases. With a combination of the two methods the number of positives was increased to 25 (83%).

Antibodies to bovine serum albumin (BSA) Eleven out of 80 sera (19%) from type I diabetics contained BSA antibodies. After preabsorption with BSA, eight of these sera remained positive in the ICA-ELISA indicating that they contained both BSA antibodies and islet cell antibodies. None of the sera from type II diabetics, four of the sera from patients with associated autoantibodies and three out of 100 normal human sera were positive for BSA antibodies.

DISCUSSION

The feasibility of ICA determination in large population screening programs depends on an easily reproducible and objective test system. Following a standard protocol with the conventional IFL test on unfixed cryostat sections of human pancreas even specialized laboratories recorded a wide variability of titres ranging from negative to 128. With low titre sera the interlaboratory concordance for a positive result was only 52–79% (20). This is mainly due to the subjective reading of the IFL test and the variance of human pancreases used as an antigen.

The new ELISA using a rat insulinoma (RIN A2) cell preparation for the detection of islet cell antibodies provides the advantage of easy standardization and objective measurement of ICA. Not only antibodies to membrane proteins but also reactivities directed to soluble islet cell proteins can be measured in the assay. This may explain part of the positive ELISA test results in ICA-negative sera from type I diabetics. In contrast to the immunofluorescence test, the ELISA may also detect low affinity antibodies. The negative ELISA-ICA results with sera containing ICA as determined by indirect immunofluorescence may be explained by the limited cross-reactivity of human sera with the RIN cell antigens.

The disease specificity of ELISA-ICA is shown by the fact that patients and normal individuals as well as patients with non-diabetes-specific antibodies were constantly negative in the new assay. Like conventional ICA the ELISA-ICA can therefore be considered as a marker of autoimmune insulitis. With the ELISA system here described the lower limit of detection of islet cell specific autoantibodies in the serum of type I diabetics was clearly elevated. The two tests may be used complementary since biologically false positive results are very rare in the IFL (3–5) and they were not detected in our ELISA.

Some authors have tried to perform an ELISA for the detection of islet cell surface antibodies (ICSA) by coating microtitre plates with fixed primary rat islet cells (26) or with RIN m5F cells (27). The promising first reports,

however, were not reproduced by other authors. In our own experiments, the attachment of native cells to the precoated plates was insufficient to resist the ELISA procedure. Even with gentle washing cell-free holes appeared which produced an unacceptable increase of non-specific binding to the plates. Sufficient attachment of cells was achieved by fixation with 0.25% glutaraldehyde, but this resulted in an increase of false negative and false positive results. The change of islet cell antigens by fixatives has also been documented in previous reports (28, 29).

The presence of BSA antibodies in human sera is well explained by the ingestion of cow milk or beef (30), but it may disturb the interpretation of a positive ICA-ELISA result so that a preabsorption of sera with BSA is recommended. BSA antibodies have also been detected by other authors (31, 32). Using an immunoprecipitation method, Colman *et al.* (32) were able to discriminate the BSA reactivity from ICA. In contrast to the above mentioned report, we found an increased prevalence of BSA antibodies in the sera from type I diabetic. Although this finding may be due to a selection of our patients it is worth while to study BSA antibodies in a larger group of type I diabetic patients.

The ICA-ELISA here described is well standardized and highly specific for type I diabetes. It may therefore be considered as a valuable tool for islet cell antibody testing in large screening investigations. Further research should be aimed at the isolation of the islet cell antigen which will allow to establish the optimal test for ICA determination.

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