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A two-colour immunofluorescence test with a monoclonal human proinsulin antibody improves the assay for islet cell antibodies

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Summary. The conventional indirect immunofluorescence assay for islet cell antibodies was compared with a two-colour immunofluorescent assay to detect both islet cell antibodies with fluorescein isothiocyanate-labeled rabbit anti-human IgG and pancreatic B cells with a monoclonal human proinsulin antibody and Texas red-labeled sheep anti-mouse IgG. Determinations of end-point titres showed a correlation between the new two-colour immunofluorescent assay and the conventional indirect immunofluorescent assay in 1) selected sera positive for islet cell antibodies and insulin autoantibodies $r_s = 0.93$ ($p < 0.01$) or for islet cell antibodies alone $r_s = 0.99$ ($p < 0.005$) and 2) sera from children or young adults with newly diagnosed Type 1 (insulin-dependent) diabetes $r_s = 0.95$ ($p < 0.0001$). No interference between the monoclonal human proinsulin antibodies and islet cell antibodies with or without insulin autoantibodies or between the two second fluorescent antibodies was detected. It is concluded that the two-colour immunofluorescence assay is advantageous since a) it is possible to mix the reagents to avoid a more time-consuming and technically complicated assay, b) the presence of B cells can be confirmed in each section to permit detection of B cell cytoplasmic antibodies and c) microscopic evaluation is easier and more accurate, particulary in islet cell antibody negative samples.

Key words: Islet cell antibody assay, two-colour assay, human proinsulin antibody.

The conventional method for studying islet cell antibodies (ICA), first described by Bottazzo et al. in 1974 [1], is based on an indirect immunofluorescent assay with sections of frozen human pancreas to visualize ICA by a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG. The precision and accuracy of the ICA vary between different pancreas and even in the same pancreas; the quality and frequency of islets may differ from one part to another [2]. The possibility of detecting B cells in frozen sections of human pancreas [3] with the human proinsulin specific mouse monoclonal antibody GS9A8 [4] implies that a combined assay based on simultaneous indirect immunofluorescent analysis of both ICA and proinsulin would positively identify B cells and abolish the risk for falsely negative ICA reactions. The purpose of this study was to establish a two-colour immunofluorescence assay, to exclude any possible interaction between the reagents and to compare ICA end-point titres in the conventional assay with the two-colour indirect immunofluorescent assay.

Materials and methods

Reagents and conventional ICA assay

Frozen pancreas from four individuals of blood group 0 were used. One pancreas was obtained at autopsy 29 h after death from a 65 year old female suffering from myelofibrosis. The other three were taken from cadaver kidney donors with only cold ischaemia varying between 0.5 to 4 h. The preparation of the tissue, sections and the conventional ICA assay were as described [2].

The second fluorescent antibody was a fluorescein isothiocyanate (FITC)-conjugated rabbit antihuman IgG (Code F 202, Dako, Copenhagen, Denmark). In the two-colour ICA test this second antibody was first absorbed to mouse IgG to remove rabbit antibodies cross reactive with mouse immunoglobulins. Mouse immunoglobulin conjugated agarose (Jackson, Immuno Reasearch, Avondale, PA, USA) was first washed 4 times in phosphate-buffered saline (PBS) and mixed with FITC-conjugated anti-human IgG. After 1 h incubation with shaking at 4 °C in the dark, the mixture was centrifuged twice and the supernatant was collected.

The human proinsulin monoclonal antibody GS9A8, previously characterized in detail [4], was used as an ascites preparation to detect B cells in frozen sections of human pancreas [3]. Texas red-conjugated anti-mouse IgG from sheep (N 2031, species specific, Amersham, Buckinghamshire, U. K.) was used to visualize the GS9A8 antibody.

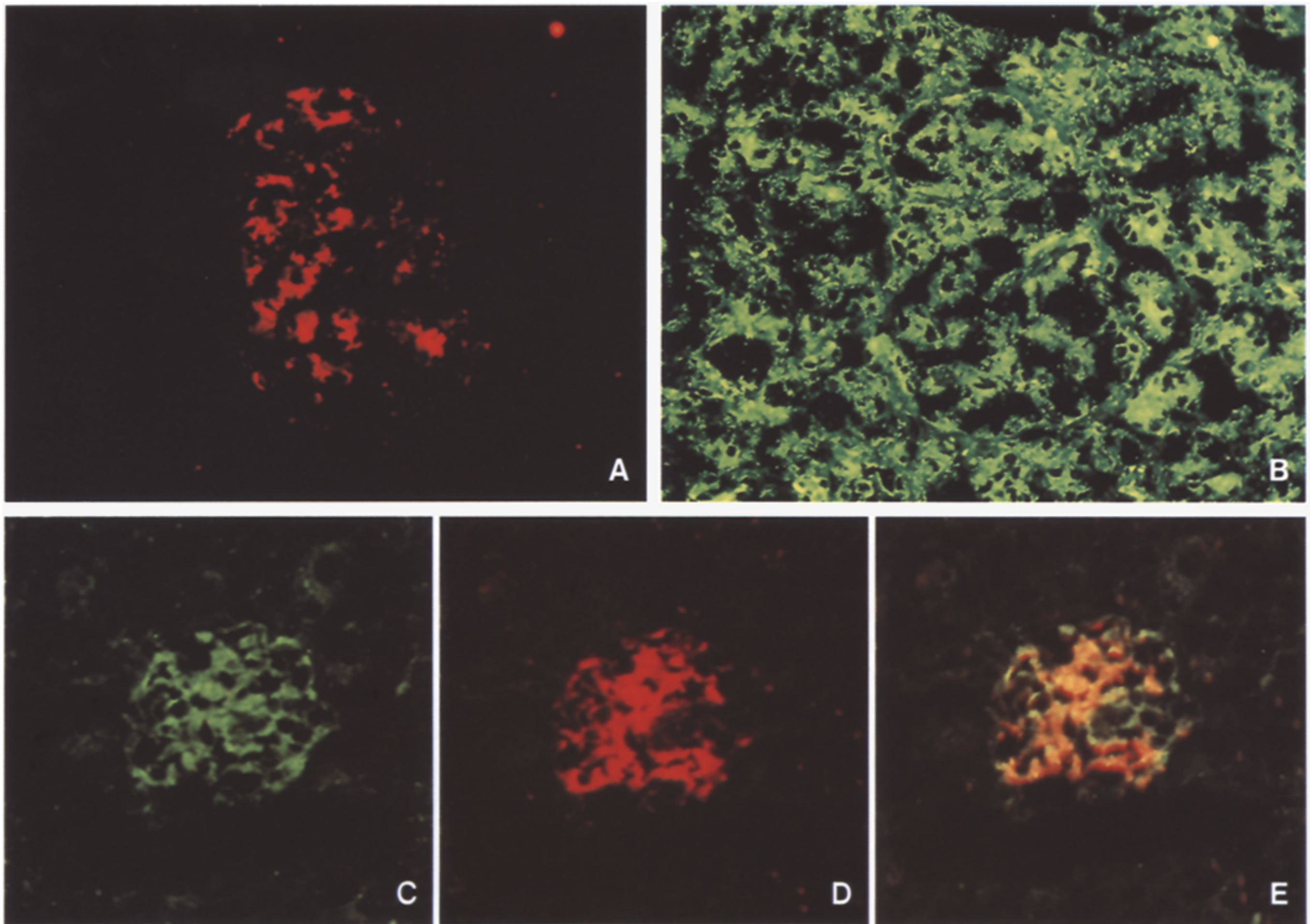

Fig. 1. Two-colour immunofluorescence in frozen sections of human pancreas (**A**). Visualization of B cells after incubation with the human proinsulin mouse monoclonal GS9A8 antibody diluted in 1:200 in phosphate buffered saline (**B**). In the conventional assay no islets are distinguishable after incubation with a ICA negative serum. In the two-colour assay FITC-labeled islets are visualized in an ICA positive serum (**C**). With simultaneous indication of proinsulin-containing cells stained with Texas red (**D**) and in double exposure (**E**), both colours can be seen. Note that green fluorescence occurs in additional cells than those stained for proinsulin (495 ×).

The optimal dilutions of the two antibodies were determined in checkerboard dilutions, demonstrating that B cells could still be detected at dilutions as high as 1:6000 with GS9A8.

Two-colour ICA assay

Slides with sections of pancreas were dried for 30 min by a fan and then incubated in a moist chamber at room temperature for 30 min with a mixture of serum and PBS containing the GS9A8 antibody diluted 1:200–1:1000. PBS-GS9A8 buffer was used to dilute all sera, either as 1:2, 1:4, 1:8, etc., or 1:3, 1:9, 1:27, etc. After the first incubation, the slides were washed 3 times each for 5 min in PBS. After washing, excess PBS was wiped off, the slides again placed in the dark moist chamber and the sections incubated with the mixture of the two second fluorescent antibodies for another 30 min. The slides were washed in the same way as before and then mounted with TRIS glycerol HCl (pH 8,4).

The slides were evaluated in an Olympus BH2 microscope with epi-illumination. Each slide was coded and evaluated by at least two independent observers. The end-point titre was defined as the maximal dilution at which fluorescence could be detected.

Serum samples

Two groups of samples were analyzed:

a) Based on previous analyses, sera from newly diagnosed Type 1 diabetic patients were selected to represent 9 individuals positive for ICA and insulin autoantibodies (IAA) with an ^{125}I-insulin binding varying between 1.7 and 12.4% [5, 6]. Also selected were 10 individuals positive for ICA but negative for IAA and 8 individuals negative for ICA and IAA but positive for islet cell surface antibodies (ICSA) in an ^{125}I-protein A assay [7]. In addition, sera from 10 healthy controls previously found to be ICA, IAA and ICSA negative were tested.

b) Sera from 31 newly diagnosed Type 1 diabetic patients were consecutively collected. The mean age at onset was 9.4 years (range 2–16 years), and there were 20 females and 11 males.

Statistical evaluation

Statistical analysis was done by calculating the Spearman's rank correlation coefficient.

Results

The GS9A8 monoclonal proinsulin antibody effectively detected pancreatic B cells in frozen sections of human pancreas. Red fluorescent cells in islets of varying size or even at the single cell level were easily detected (Fig. 1 A). ICA negative serum samples did not permit

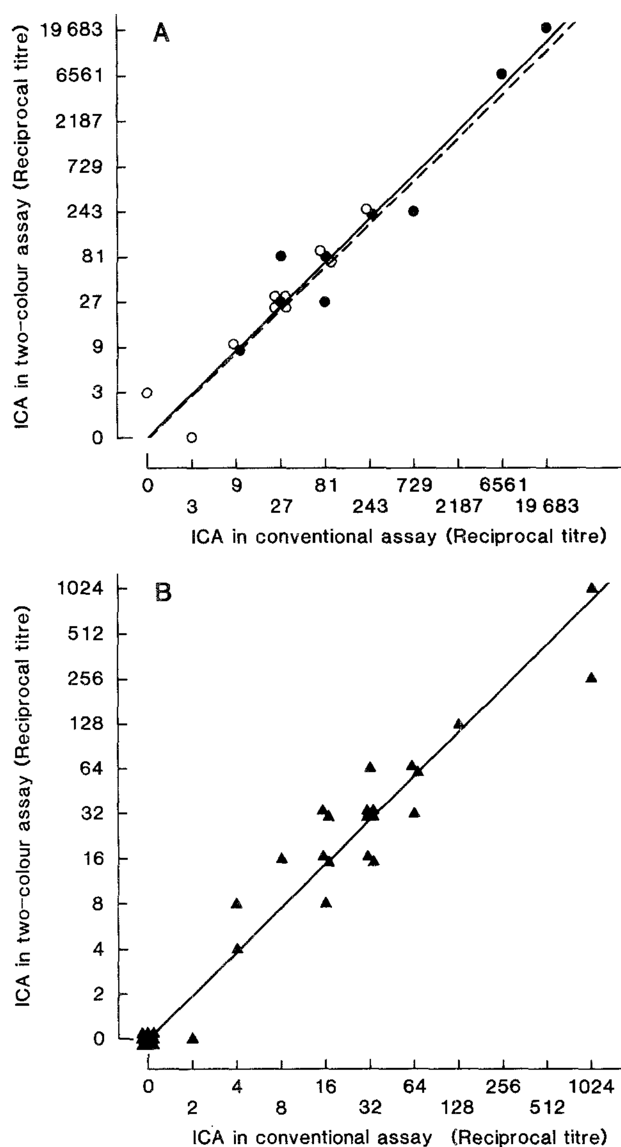

Fig. 2. Reciprocal ICA titres comparing the conventional with the two-colour immunofluorescence assay (**A**). The two assays correlated well in 9 samples selected positive for ICA and insulin autoantibodies (IAA) (●, solid line) ($r_s = 0.93$; $p < 0.01$), in 10 serum samples positive for ICA only (O, stippled line) ($r_s = 0.99$; $p < 0.005$) and in (**B**). 31 consecutively collected patients with Type 1 diabetes (▲). The regression lines are indicated. The two assays were correlated ($r_s = 0.95$; $p < 0.0001$).

the localization of either B cells or islets in the conventional assay (Fig. 1 B). However, in ICA positive sera the entire islet may be seen (Fig. 1 C). Since the GS9A8 monoclonal proinsulin antibody labeled the B cells in the two-colour assay (Fig. 1 D), the double exposure showed that ICA reacted not only with B cells, but with other islet cells as well (Fig. 1 E). The autopsy pancreas of expected inferior quality had a distinctly positive reaction with the GS9A8 antibody but did not react with ICA (data not shown).

A close correlation was found between the conventional ICA assay and the new two-colour immunofluorescence method (Fig. 2). The correlation coefficients for the selected sera were $r_s = 0.93$ ($n = 9$; $p < 0.01$) for ICA and IAA positive sera, and $r_s = 0.99$ ($n = 10$; $p < 0.005$) for sera positive only for ICA (Fig. 2 A). The sera selected for ICSA positivity and ICA negativity were all ICA negative in the two-colour assay, as were the control sera. Among the newly diagnosed Type 1 diabetic patients, 22 out of 31 (71%) were ICA positive (Fig. 2 B). The titres ranged from 1:2 to 1:1024. Again the two-colour method correlated to the conventional assay, $r_s = 0.95$ ($n = 31$; $p < 0.0001$). The interobserver variation in the conventional ICA assay was 1.6% (8 out of 497 slides were read differently by two observers) compared to 0.9% (4 out of 465 samples) in the two-colour assay. The inter-assay variation between the conventional and the two-colour assay was 1.5% (7 out of 480 samples were scored differently in the two assays).

Discussion

Our two-colour indirect immunofluorescence assay was found to correlate well to the conventional ICA assay in titrating ICA and to improve assay reliability by visualizing the B cells in each section. A similar approach was recently reported [8], but this monoclonal antibody was not B cell specific and the method of procedure was more elaborate than the one described here. The easy detection of the B cells makes the microscopy more convenient than in the conventional one, especially when screening population-based samples where many negative samples are expected.

For technical reasons it was desirable to keep only two steps of incubation, which was possible since no interference between the different antibodies was demonstrated. However, it is imperative to absorb the FITC-conjugated rabbit anti-human IgG to mouse IgG in solid phase to eliminate non-specific binding. The addition of antibody to the PBS at a fixed concentration will result in a varying concentration of the GS9A8 antibody when this PBS buffer is used for titration of sera. However, this variation is of little importance since the monoclonal antibody detects B cells in dilutions ranging as widely as 1:200 to 1:6000. The proinsulin positive immunofluorescence in the autopsy pancreas suggest that the prohormone is a more stable antigen than that of ICA.

IAA may occur prior to insulin treatment among 30% of recent onset Type 1 diabetic patients [5, 6]. In our selected samples with and without IAA, the ICA titres were not influenced by the presence or absence of the GS9A8 antibody. The antigenic site for the IAA would therefore seem to differ from the GS9A8 binding-site. It should also be noted that the two-colour fluorescence assay provides a better differential analysis of ICA and detects cells other than the B cells. Cells on the periphery of islets and not staining for red fluorescence are easily seen by a green fluorescence in many ICA positive sera, suggesting the presence of A, D or PP cell

autoantibodies. The prevalence (71%) of ICA among newly diagnosed Type 1 diabetic children is in accordance with numerous previous investigations.

In conclusion, our two-colour assay provides an important improvement of ICA determinations, without any additional incubation step, compared to the conventional method. The two ICA assay systems correlate well, and a suspected interference between the GS9A8 and the ICA antibody was not observed.

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