Quantitative Assay for Human Cytoplasmic Islet Cell Antibodies

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SUMMARY

An assay for human islet cell antibodies (ICAs) in serum yielding numerical values and amenable to statistical evaluation has been developed utilizing fluorescence spectrophotomicroscopy (FSPM). The results of the blinded trials by FSPM were compared with those by standard indirect immunofluorescence (IFL). No false-positive or false-negative readings were obtained in 258 observations when the results by FSPM were compared with those by IFL. The intra- and interassay variabilities encountered were not enough to misclassify a specimen. The presence of anti-thyroid, anti-adrenal, or anti-nuclear antibodies did not produce false-positive readings. Twenty-eight additional specimens from diabetic children were also analyzed via three blood group type O pancreases. There was complete agreement concerning the presence or absence of ICA between IFL and FSPM analyses. Analysis of the three pancreases yielded different numbers of results positive for ICA (14/28 vs. 22/28 vs. 15/28, $P = .008$) in both assays. Thus, selection of pancreatic substrate may influence the outcome of assays for ICA. A matrix of fluorescent microspheres has been devised that allows calibration of the FSPM system. Now, reproducible and comparable readings for ICA values can be obtained from the various reporting laboratories. Should an international reference serum for ICA become available, the remaining problem in the ICA assay, that of substrate (pancreas) variability, should be resolved.

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The presence of human cytoplasmic islet cell antibodies (ICAs) in the sera of diabetic subjects was reported in 1974 (1,2) and has been documented in most individuals with type I diabetes mellitus (3). Although the true pathogenetic implications of ICAs remain unclear, their presence as demonstrated by indirect immunofluorescence has been used to predict the failure of oral hypoglycemic agents in adult diabetes (4), subserve the distinction between type I and type II diabetes, and allow the classification of coexistent endocrine disorders into recognizable syndromes (5). Of greater potential significance is the fact that ICAs can be detected before the onset of diabetes (6,7). The presence of ICAs may therefore serve to identify individuals in the process of becoming diabetic and allow appropriate epidemiological studies as to the cause, or causes, of type I diabetes. An assay for ICA that can be standardized and reproduced in various laboratories is critical to this undertaking. At the first meeting of the international workshop on the standardization of cytoplasmic ICAs, the participating laboratories disagreed on the endpoint titers of many of the test sera and disagreed as to whether some sera were positive or negative (8). I therefore report a quantitative assay for ICA that can be calibrated with a readily made fluorescent matrix that allows for intra- and interlaboratory standardizations.

MATERIALS AND METHODS

Fluorescence spectrophotomicroscopy system (FSPM). The method uses acetone-fixed, blood group type O human pancreas sections in an indirect immunofluorescence (IFL) assay (3) with a goat anti-human IgG-FITC second antibody (1:40 dilution, 2.27 F/P ratio, Kallestad, MN). An epi-illuminated Zeiss standard microscope with a 450- to 490-nm excitation filter, a 520- to 560-nm filter in the beam splitter, and a 50-W mercury lamp generates the fluorescent signal. To quantitate the fluorescence intensity (FI) from each sample, a photomultiplier tube (Zeiss 474282) is attached to the trinocular microscope head. A 1.0-mm pinhole stop in the microscope head serves as a beam collimeter (Zeiss 476005). The output of the photomultiplier tube is fed into a

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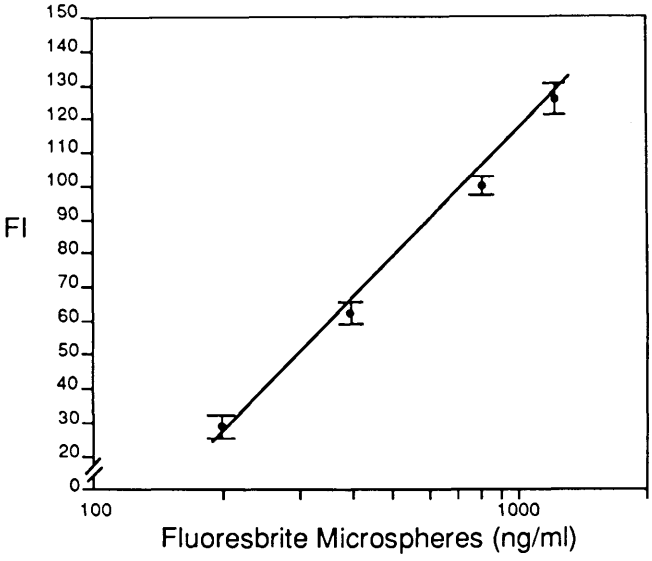

FIG. 1. Standard curve for fluorescence intensity (FI) generated by calibration of the fluorescence spectrophotomicroscopy sytem with matrix of Fluoresbrite microspheres. Values are means of triplicate readings ± SD.

digital display meter equipped with separate verniers for setting electrical zero and adjusting the gain of the system (Zeiss 477414). A ×25 planachromat (NA 0.45) objective is used to observe the specimen. The field size is controlled by an iris diaphragm in the epi-illuminator. The right ocular (KPL 10X/18) contains a reticle of concentric circles to monitor the field size.

The output of the FSPM system is calibrated before use. With no light incident of photomultiplier, the digital display is set to electrical zero. The fluorescent matrix is made by adding 40 μl of various phosphate-buffered saline dilutions of Fluoresbrite carboxylate microspheres (1.09 μm, 2.5 g solids/dl, Polysciences, Warrington, PA) with 40 μl of 2% agarose PBS (Seakem, ME FMC, Rockland, ME) at its boiling point and allowed to solidify in a well of a 96-well flat-bottomed plate (Nunc Immulon II). The plate is inverted onto the microscope stage, and viewed with the field setting at 4.0 with a ×10 objective. The output of the photomultiplier is adjusted with the gain vernier to 100 U utilizing the 1:512 dilution of Fluoresbrite microspheres. Sections of human pancreas, prepared for IFL, are viewed with a ×25 objective with the iris diaphragm at the 4.0 setting on the ocular reticle. Islets that fill this field size (150 μm) are read for FI, and the background FI is obtained from adjacent acinar tissue at the same field size. The difference between the FI (ΔFI) from islet and acinar tissue is the reading for that specimen. When taking background readings, it is important to avoid blood vessels, ducts, or tissue folded on itself because spurious results are obtained.

TABLE 1
Mean difference in fluorescence intensity (ΔFI) and range in FI by fluorescence spectrophotomicroscopy system

Group	*n*	ΔFI (mean ± SD)	Range
Diabetes			
Positive	95	25.8 ± 22	5 to 121
Negative	33	−4.1 ± 2.5	−10 to 0.5
Control	47	−2.5 ± 2.3	9 to 1.0
Antibodies			
Thyroid microsomal	10	−3.3 ± 3.1	−10 to 3
Adrenocortical	10	0.5 ± 0.8	0.7 to 2
Antinuclear	10	−9.4 ± 8.0	−28 to −1

Subjects. Multiple observations were made by FSPM and IFL with sera from 67 individuals with type I diabetes, 32 control sera from individuals with no known endocrine disease, and 10 specimens each of sera positive only for antinuclear (ANA), adrenocortical (ACA), or thyroid microsomal (TMA) antibodies. I coded and read all sera without knowledge of the subjects' clinical conditions.

In all studies, bias was avoided by first rendering the subjective interpretation (negative or 1–4+ positive) of the IFL and then taking the objective reading by FSPM from the digital display. Intra- and interassay variabilities were assessed with sera duplicated within or between assays.

Three human, blood group type O pancreases (P) were used to assess the variability inherent in pancreatic substrates. The HLA types of the pancreases (P) were: PI-A3, 7 B31,Y CW7 DR2; PII-A1,2 B27,35 CZ, DR4,8; and PIII-A2, 3 B7;17 CW4,6 DR3,7. Twenty-eight additional sera from diabetic children (mean age 12.4 yr, SD = 5.8 yr; mean disease duration 1.8 yr, SD = 1.4 yr) were used on each pan-

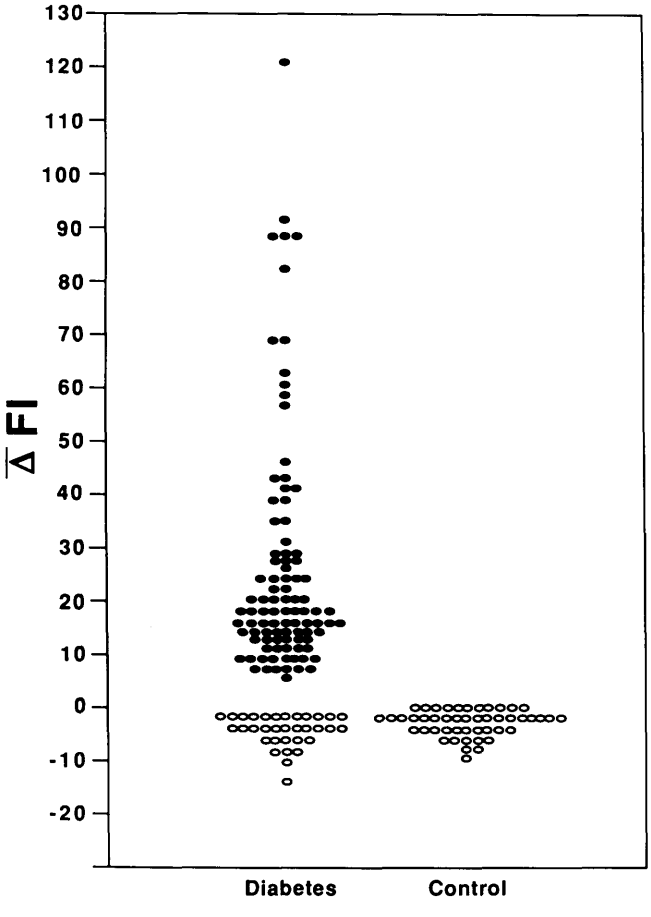

FIG. 2. Difference in fluorescence intensity (ΔFI) from 175 observations of coded sera from diabetic and control subjects. For comparison, results from standard immunofluorescence assay are presented as positive (●) or negative (○).

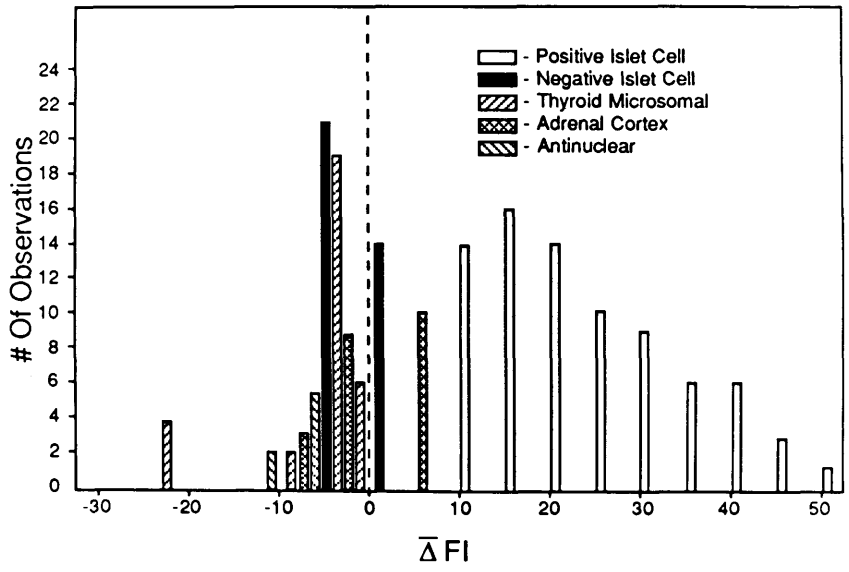

FIG. 3. Difference in fluorescence intensity (ΔFI) from 181 specimens of coded sera. No overlap in ΔFI is observed between specimens containing ICA (positive islet cell) and specimens from normal controls (negative islet cell) or sera containing other anti-endocrine antibodies.

creas. Results of the trials of the three pancreases were analyzed by Cochran's *Q* test (9).

Variability (V) within and between assays is assessed from readings of FI of paired specimens. Where FI_p is the mean reading of a pair of observations and FI_i an individual observation, the variability is expressed as a percentage of the readings where $V = (FI_p - FI_i/FI_p) \times 100$.

RESULTS

A typical standard curve is demonstrated in Fig. 1. It was produced by setting the gain of the FSPM to 100 with a 1:512 dilution of the fluorescent matrix. Between ~200 and 1250 ng/ml of fluorescent material, the FI was a linear function of concentration. Below 200 ng/ml, the FI asymptotically approaches zero, whereas concentrations >1250 ng/ml saturate the photomultiplier (data not shown).

A sample is assigned as positive for ICA if the ΔFI is greater than +3SD of the mean ΔFI of the negative controls. The mean ΔFI (±SD) from control specimens (n = 47) was −2.5 ± 2.3. A specimen with a ΔFI of +4.4 is therefore considered positive for ICA. Negative FIs exist when the background FI reading is greater than the FI from the islet.

No overlap in ΔFI was observed by FSPM among diabetic subjects positive and negative for ICA (25.8 vs. −4.1) or between positive diabetic and control sera (25.8 vs. −2.5; Table 1; Fig. 2). No negative control or sera containing ACA, TMA, or ANA read greater than +3SD of the mean negative

TABLE 2
Variability data from paired readings of background (acinar) tissue, positive islet tissue, and negative islet tissue

	Mean variability (% ± SD)	n
Background		
Intra-assay	11 ± 11	28
Interassay	14 ± 13	48
Positive islet		
Intra-assay	11 ± 8	44
Interassay	13 ± 12	26
Interislet	6 ± 7	36
Negative islet		
Intra-assay	9 ± 6	22
Interassay	5 ± 3	24

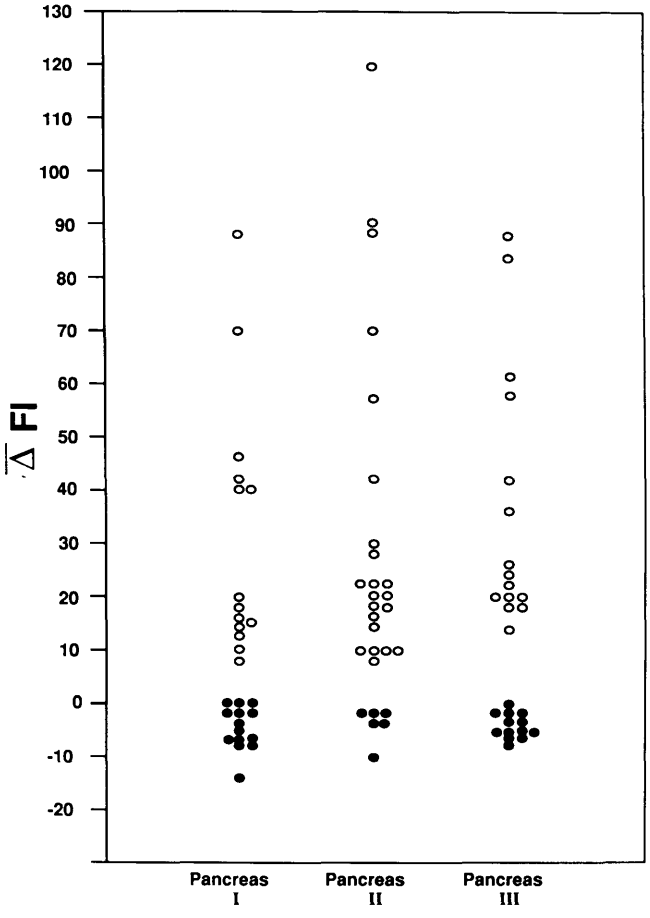

FIG. 4. Difference in fluorescence intensity (ΔFI) from 28 specimens of coded sera from diabetic subjects applied to 3 type O human pancreases. Mean ΔFI from negative control sera (n = 30) for these pancreases (P) were: PI, 3.3 ± 2.6; PII, 2.3 ± 1.5; and PIII, 1.6 ± 2.4. ○, Visually positive by indirect immunofluorescence; ●, visually negative by indirect immunofluorescence.

control (Fig. 3). Because no overlap in ΔFI occurred, the statistical significance is extreme. There was complete concordance in ICA readings by FSPM when compared with IFL (Figs. 2 and 3). No false-positive readings occurred among specimens of anti-adrenal, anti-thyroid microsomal, or anti-nuclear antibodies (Fig. 3), thus indicating a high degree of specificity for ICA by FSPM.

The variability of FI from paired specimens is presented in Table 2 for background (acinar), positive islet, and negative islet readings. The range of individual FI readings was 6–41 background, 11–95 positive islet, and 13–41 negative islet. The large overlap of FI readings indicates the need for expressing the value for an individual specimen as ΔFI. The intra- and interassay measurements are included in Table 2. The variability encountered did not misclassify any specimen as a false positive or false negative.

The results of the assays performed on three type O pancreases are presented in Fig. 4. With each pancreas, a cutoff point exists (ΔFI = 4.4) to separate the samples into positive and negative populations. The agreement of positivity and negativity of specimens between the IFL and FSPM assays was complete. The pancreases yielded 14/28, 22/28, and 15/28 positive specimens, respectively. Analyses of results for these pancreases by Cochran's *Q* test reject the null hypothesis of similarity among pancreases ($P = .008$).

DISCUSSION

Fluorescence spectrophotomicroscopic analysis of human pancreas prepared for conventional IFL provides an objective and numerical value for each specimen and can be considered a quantitative assay for ICAs. Because the system can be calibrated (e.g., with Fluoresbrite microspheres) before each assay, the technique may be standardized within and between each laboratory. With specimens also characterized by IFL, the assay yielded no false-positive or false-negative results, nor did it misread specimens containing anti-adrenal, anti-thyroid microsomal, or anti-nuclear antibodies.

The true predictive value of this and other ICA assays is unknown. Knowledge of the predictive value of a test presumes agreement on both positive and negative reference points, in this case reference sera. There are no international reference sera for ICA. The readings for FI in this assay are therefore arbitrary but defined by the standard curve. A reference standard is being evaluated by an international workshop for the standardization of the ICA. With assays such as FSPM, the instrument can be calibrated with both an agreed on positive sera (gain) and independently with negative sera (zero), thus generating standard curves to allow valid interlaboratory comparisons.

This study also indicates that some blood group type O pancreatic tissue may be more suitable for ICA assays than others. This problem can be resolved by testing only pancreatic tissue with favorable fluorescence signal (islet) to noise (acinar) characteristics when stained with the endpoint dilution of an international reference serum.

In a previous study, Srikanta et al. (10) used a spectrophotometric assay for ICA with FITC–protein A. In contrast to their analyses where multiple islet and acinar readings are taken, my variability data suggest that a single reading of each will suffice, making the assay less laborious. Although a monoclonal antibody may be used to detect ICA, my data indicate that the more generally available polyclonal antibodies may also be used, because complete separation of positive ICA from negative ICA specimens was achieved in this study.

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