Quantitative Determination of Islet Cell Surface Antibodies Using ^{125}I-Protein A

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SUMMARY

A quantitative method to measure islet cell surface antibodies in human patients has been developed using ^{125}I-protein A. Isolated, dispersed, viable rat islet cells prepared by collagenase digestion were fixed in 4% paraformaldehyde to allow storage for up to 7 wk at 4°C. Human sera, heat inactivated and adsorbed with rat liver and kidney powder (100 mg/ml), were incubated with the fixed cells (50 × 10^3) for 60 min at 37°C. Thereafter the cells were washed and exposed to 5 × 10^5 cpm ^{125}I-protein A, which binds to IgG attached to the cell surface. Assay precision (14%) and reproducibility (16%) were established by repeated analysis of pooled sera from healthy individuals and IDDM patients using pooled batches of islet cells. Using this method, islet cell surface antibodies were detected in 35% of insulin-dependent diabetic patients. DIABETES 32:460–465, May 1983.

Islet cell antibodies have been detected in the sera of many patients with insulin-dependent (type I) diabetes (IDDM).[1–3] Using sections of frozen human pancreas, autoantibodies against cytoplasmic components of islet cells can be detected by indirect immunofluorescence. These cytoplasmic antibodies are organ-, but not pancreatic β-cell-specific, and are present in more than 75% of recent onset IDDM. A second type of antibody reacts with cell surface determinants of islet cells derived from rats or mice.[4,5] These cell surface antibodies also appear to be organ-specific, but not species-specific. Recent studies suggest that the islet cell surface antibodies react mainly with β-cells and can mediate a complement-dependent cytotoxic reaction.[4–10] It is possible that these antibodies are an indicator of progressive β-cell destruction. The assay of islet cell surface antibodies by immunofluorescence has limitations, since this technique is semiquantitative and the potential for observer bias is significant. We have therefore developed a radioassay which employs dispersed, fixed rat islet cells and staphylococcal ^{125}I-protein A to detect cell surface bound antibodies. The results demonstrate that this radioligand assay provides a quantitative and reproducible method to detect islet cell surface antibodies in diabetic patients.

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MATERIALS AND METHODS

Preparation of islet cell suspensions. Islets of Langerhans were isolated from the pancreas of 6–8 male Sprague-Dawley rats by collagenase digestion (type IV, Worthington Biochemical Corp., Freehold, New Jersey). The islets were separated from the digest by Ficoll gradient centrifugation and washed repeatedly by sedimentation in Swims' S-77 medium, supplemented as described in detail elsewhere.[5] The islets, individually chosen with the aid of a stereomicroscope, were dispersed by mechanical shaking.[6] The cells were washed by centrifugation (5 min at 50 × *g*) through 10 ml 40 g/L bovine serum albumin (BSA) in Swims' medium[4,6] and the cell pellet carefully resuspended in 0.5–1 ml Swims' medium with 40 g/L BSA. Approximately 2 × 10^6 dispersed islet cells, comprising approximately 75–80% β-cells,[3,11] were obtained in each preparation.

Fixation of islet cells. Islet cells were fixed (60 min at room temperature) in 4% (w/v) paraformaldehyde in 0.9% NaCl, 1% BSA and 10 mmol/L N-2-hydroxyethylpiperazine-N'2 ethane-sulphonic acids (Hepes) (NaCl-Hepes buffer, pH 7.4). The fixed cells were washed 2-3 times by centrifugation (5 min at 50 × *g*) in NaCl-Hepes buffer further supplemented with 0.5% sodium azide. A preparation of cells from 6–8 rats usually resulted in 1–2 × 10^6 fixed cells. The fixed cells were stored at 4°C in NaCl-Hepes-NaN_3 buffer for up to 7 wk.

Preparation and adsorption of serum. Serum or plasma samples from patients or normal controls was kept frozen at −20°C. One milliliter of each sample was heat inactivated (56°C for 20 min) and incubated at room temperature with

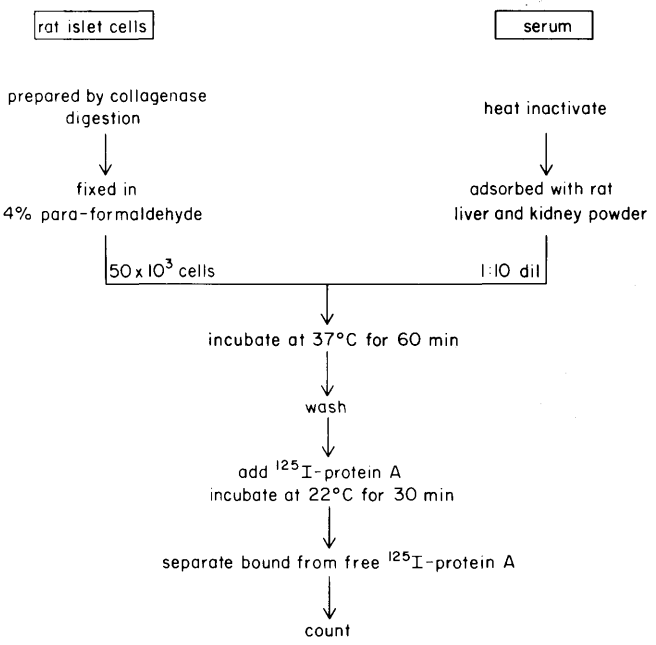

FIGURE 1. Sequential steps in the ^{125}I-protein A assay for the detection of islet cell surface antibodies in human serum.

100 mg of rat liver tissue powder and, following centrifugation (2000 × *g* for 20 min), subsequently with 100 mg rat kidney powder, each for 60 min. After centrifugation each sample was filtered through a 0.45-μm Millipore filter. The tissue powders were obtained by acetone precipitation[12] and washed with 40 g/L BSA/Swims' medium immediately before use.

Immunofluorescent test. Suspensions of rat islet cells (250 × 10^3 cells) were incubated in 100 μl Swims' medium with 40 g/L BSA containing 50 μl adsorbed serum. After incubation at 22°C for 60 min, the cells were washed by adding 10 ml Swims' medium with 4% BSA and centrifuged for 5 min at 50 × *g*. The cell pellet was resuspended in 100 μl fluorescein isothiocyanate (FITC) conjugated rabbit anti-human IgG (Miles Research Products, Elkhart, Indiana), diluted 1:15 in Swims' medium and incubated for 30 min at 22°C. Following a wash in 10 ml Swims' medium containing 4% BSA, the cell pellet was resuspended in 10 μl Swims' medium with 4% BSA and mounted on slides. The cells were evaluated by phase contrast and fluorescence microscopy using a Zeiss microscope equipped with an epifluorescence condenser, as described in detail elsewhere.[3]

Iodination of protein A. Staphylococcal protein A was obtained from Pharmacia Fine Chemicals (Piscataway, New Jersey) and Na ^{125}I (specific activity = 1000 mCi/ml) from Industrial Nuclear Co. (St. Louis, Missouri). Protein A (50 μg) was incubated in 50 μl 0.3 mol/L sodium phosphate buffer, pH 7.4, with 2 mCi Na ^{125}I and 20 μl 0.1 mmol/L chloramine T for 3 min at room temperature.[13] The iodination reaction was stopped by adding 5 μl 1.0 mmol/L sodium metabisulphite. The reaction mixture was then placed on a 0.9-×-12-cm superfine Sephadex G-75 column equilibrated with 130 mmol/L sodium borate buffer (pH 8.0) containing 0.5% BSA. The ^{125}I-protein A eluting in the void volume had a specific activity of 20–30 mCi/mg, and was stored in aliquots at −20°C for up to 6 wk.

^{125}I-protein A assay. The radioligand assay, schematically shown in Figure 1, was carried out in siliconized 15 ml tapered test tubes. Fifty microliters of fixed islet cells (50 × 10^3 per tube) were aliquoted into each test tube and to this was added 50 μl adsorbed serum diluted 1:5 (final 1:10) in Swims' medium containing 40 g/L BSA. Fifty thousand cells per tube were chosen because the ratio of the binding of ^{125}I-protein A between ICSA-positive sera and normal control sera was greatest with this number of cells, as compared with 100 × 10^3, 25 × 10^3, and 10 × 10^3 cells. After incubation at 37°C for 60 min, the cells were mixed and washed by centrifugation (50 × *g*, 10 min) in 10 ml Swims' medium with 4% BSA. The cell pellet was resuspended in 100 μl buffer containing ^{125}I-protein A (5 × 10^5 cpm) and incubated at 22°C for 30 min. Cell surface bound ^{125}I-protein A was separated from the unbound ^{125}I-protein A by rapid centrifugation (Beckman Microfuge, Beckman Instruments, Fullerton, California) of the cells through oil (Versilube silicone F-50 oil, General Electric). The tip of the test tube containing the pellet was cut from the top of the tube and counted in an automatic gamma counter. Each sample was run in duplicate.

Patients and controls. Plasma or serum samples were obtained from 19 (males:females = 11:8) healthy individuals (19–45 yr); 6 (males:females = 2:4) IDDM patients treated with insulin for 4 mo to 17 yr; 20 (males:females = 8:12) IDDM patients with recent onset diabetes treated with insulin for only 2–7 days; and 7 (males:females = 4:3) adult patients with miscellaneous disorders (4 NIDDM; 1 multinodular goiter; 1 obesity; and 1 postpancreatectomy for chronic pancreatitis).

RESULTS

Dependency of binding on serum concentration and adsorption of serum with rat tissue. The binding of ^{125}I-protein A to fixed islet cells increased with increasing concentrations

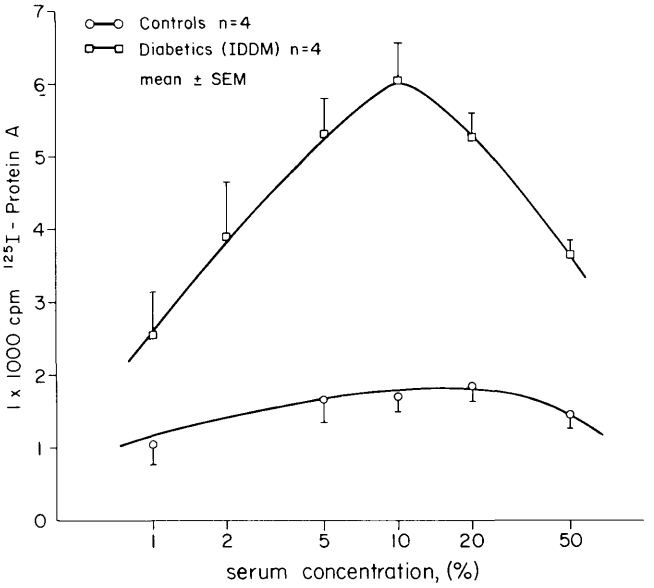

FIGURE 2. Effect of increasing concentrations of adsorbed serum on the binding of ICSA (as detected by ^{125}I-protein A) to islet cells. Positive sera from four (□—□) insulin-dependent diabetics and negative sera from four (○—○) healthy controls were selected for this experiment.

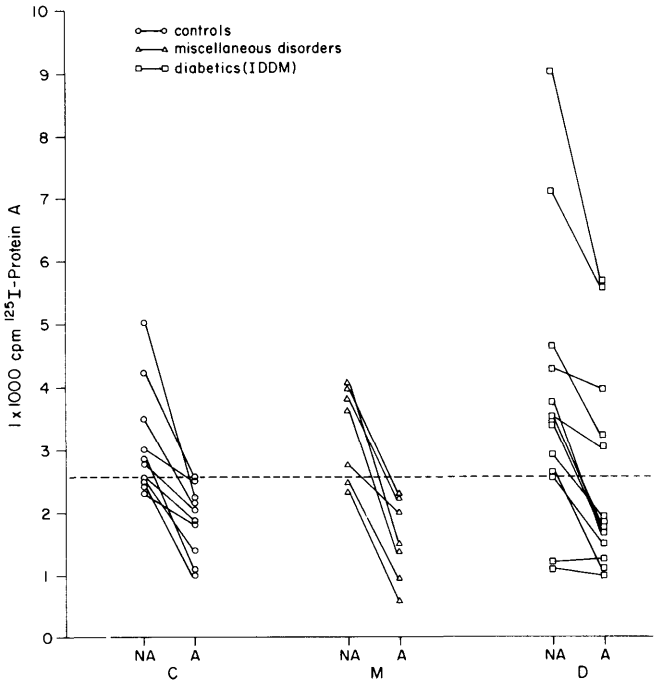

FIGURE 3. Effect of adsorption of serum with rat liver and kidney powder (100 mg) on the binding of ICSA (as detected by ^{125}I-protein A) to islet cells. In preliminary experiments exposure of sera to 100 mg rat liver and kidney powder (compared with 25 and 50 mg) resulted in maximal differences in binding between positive and control samples. Repeated adsorptions did not change this result. NA denotes the result for sera which were not adsorbed with rat liver and kidney powder, while A indicates the result with adsorbed sera (○) controls, (△) patients with miscellaneous disorders, and (□) patients with diabetes.

of adsorbed control or IDDM sera (Figure 2). Maximum binding was achieved in the range of 5–20% (vol/vol), while an apparent decrease in binding was observed at 50%, the highest concentration tested. A standard 10% (vol/vol) concentration was used in subsequent experiments.

The effect of adsorbing sera with rat liver and kidney powder was studied in three groups of patients: normal controls (C), a group of patients with miscellaneous disorders (M), and patients with diabetes (IDDM) (Figure 3). The binding of ^{125}I-protein A decreased after adsorption in all three groups. The dashed line indicates the highest value of ^{125}I-protein A binding to fixed islet cells incubated in adsorbed sera from controls or patients with miscellaneous disorders.

Assay precision and reproducibility. In our preliminary studies, we observed marked variability between absolute counts bound after exposure of aliquots of the same serum to different batches of islet cells (Figure 4). The variability of a pooled normal control serum was studied over a 6-mo period representing 19 assays (Figure 5). The interassay coefficient of variation was 34%. Nevertheless, the binding of ^{125}I-protein A to rat islet cells exposed to the pooled normal control serum correlated closely in each assay with the binding of the protein A to an aliquot of the same batch of islet cells exposed to a positive IDDM sample (Figure 6). It thus seemed likely that the reactivity between the cell surface antigen and human antibodies could be affected by the degree of collagenase digestion of the pancreas and islets, as well as other variables occurring during the isolation of each batch of cells. Because of this interassay variability, we (1) included aliquots of a pool of adsorbed control sera (pooled from 10 control subjects) in each analysis, and (2) pooled several batches of islet cells which had been fixed in paraformaldehyde, and stored them in NaCl-Hepes-NaN_3 buffer at 4°C for several weeks. To test the effects of storage on the cell surface binding characteristics, one batch of fixed cells was kept at 4°C for 2 wk and another for 7 wk, respectively. The binding of ^{125}I-protein A to six IDDM sera (expressed as the ratio of cpm bound with each IDDM serum to the cpm bound to the pooled control sample) was 1.18 ± 0.40 for 2-wk-old cells and 1.14 ± 0.47 (mean ± SD) for 7-wk-old cells. Because the binding to the cells was stable during 2–7 wk storage, assays were subsequently carried out using pooled, fixed cells. The interassay coefficient of variation of the pooled normal control serum was 16% (30 assays), while the intraassay coefficient of variation was 14%.

Islet cell surface antibodies in IDDM patients. When sera from 19 healthy adults and 7 patients with miscellaneous disorders were compared with the pooled control sample, none bound ^{125}I-protein A in amounts exceeding $\bar{x}$ + 2 SD of the pooled control sample (Figure 7). In 26 IDDM patients, 9 (35%) showed protein A binding higher than the $\bar{x}$ + 2 SD of the pooled control sample.

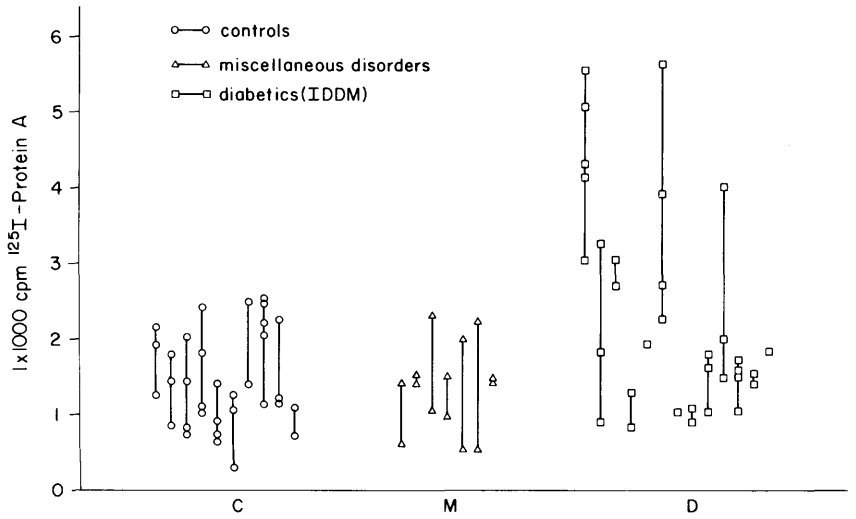

FIGURE 4. Variability of binding of serum ICSA (as detected by ^{125}I-protein A) to islet cells. The points on each vertical line represent the binding of aliquots from the same serum sample in assays using different batches of islet cells.

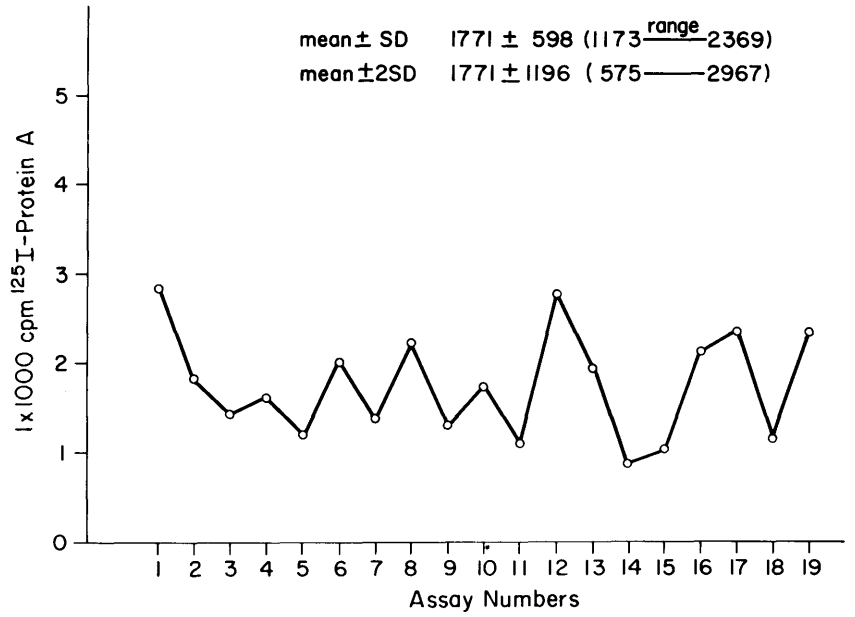

FIGURE 5. Variability of a pooled control serum sample on the binding of ICSA (as detected by ^{125}I-protein A) to islet cells over a 6-mo period representing 19 assays.

Correlation of ^{125}I-protein A binding with immunofluorescence. We examined the correlation of ^{125}I-protein A binding to sera with their surface immunofluroescence reaction. Sera from 19 controls and 7 patients with miscellaneous disorders showed low protein A binding (less than 2600 cpm in each sample), but sera from 2 (8%) of these normal individuals had positive immunofluorescence (Figure 7). Among the 26 insulin-dependent diabetic samples, 9 had protein A counts higher than $\bar{x} \pm 2$ SD of the pooled control sample. Five of these samples were found to be immunofluroescence (IF) positive and two were considered to be borderline. Three samples, which were considered to be IF-positive, fell within the control values of the protein A binding.

DISCUSSION

Indirect immunofluorescence is the method in current use to detect islet cell surface antibodies. This assay is relatively insensitive and relies on a subjective diagnostic decision by microscopy. We have developed a radioligand assay for the detection of human islet cell surface antibodies using ^{125}I-protein A and fixed rat islet cells. Protein A, isolated from the cell wall of *Staphylococcus aureus*, binds specifically to the F_c portion of most subclasses of human IgG. Protein A was iodinated to high specific activity without apparent alteration of its binding characteristics and the tracer could be stored frozen for several weeks. The protein A radioligand assay has also proved useful to detect islet cell surface antibodies in the serum of spontaneously diabetic BB rats,[14] in xenogeneic antisera against islet cells[6] as well as in screening for monoclonal islet cell antibodies.[15] Recent experiments also demonstrate that detergent-solubilized, radiolabeled islet cell antigens from human or rodent islets can be complexed to antibodies isolated from the sera of diabetic patients, and detected by gel electrophoresis after specific absorption to protein A-Sepharose.[16]

The use of paraformaldehyde-fixed islet cells is advantageous since islet cells can be prepared in bulk on consecutive days, pooled, and stored at 4°C for later assays.

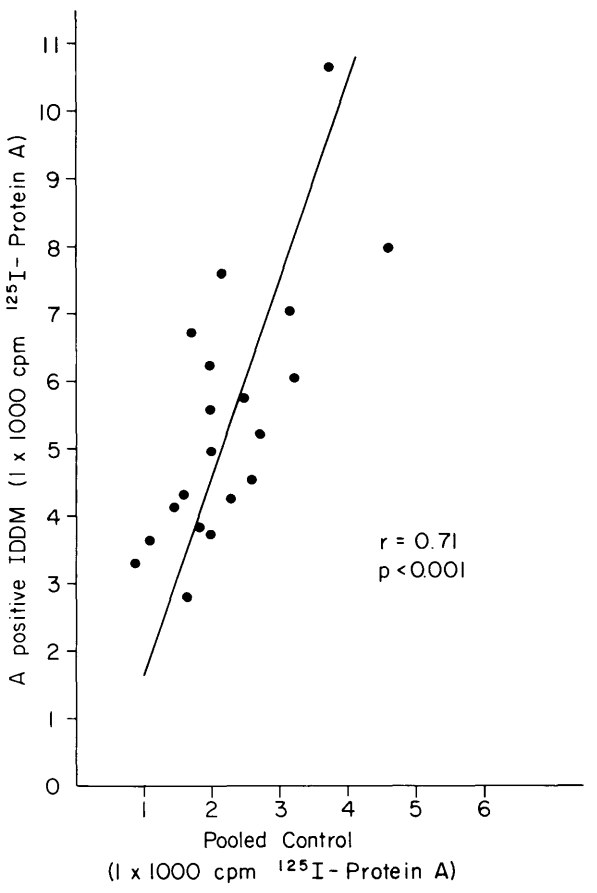

FIGURE 6. Correlation between a pooled control serum sample and a positive serum sample from a patient with IDDM. Each point represents the results of the two sera in individual assays.

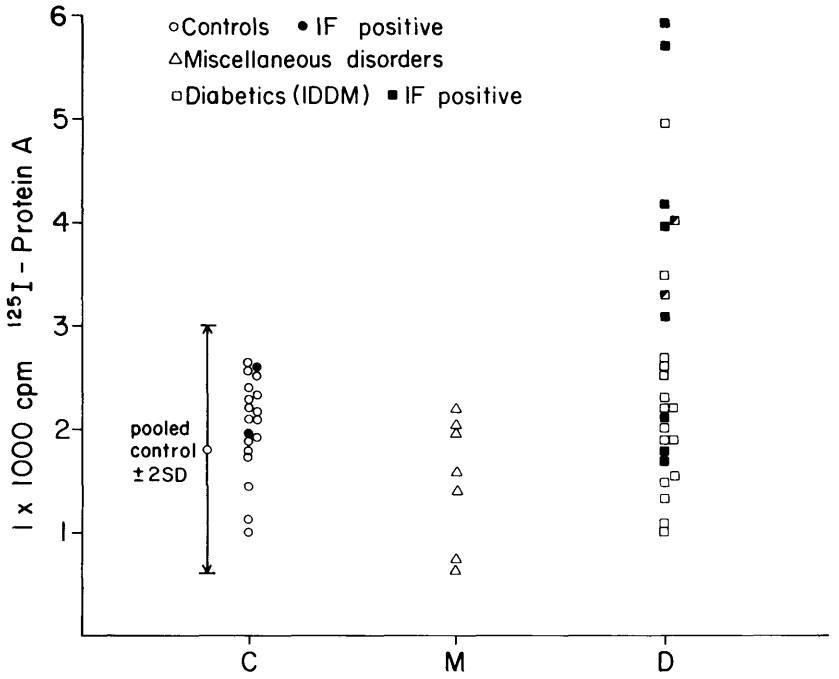

FIGURE 7. Comparison of results of the ^{125}I-protein A and immunofluorescent assays. ●■ Immunofluroescent positive. ◪ Immunofluorescent—borderline positive. ○□ Immunofluorescent negative.

Our results indicate that the fixed islet cells can be kept for up to 7 wk without alteration of their binding characteristics. Different fixation techniques were tested initially, but the present paraformaldehyde technique was superior to glutaraldehyde or osmium fixation, since the integrity and morphology of the cells were well preserved. Our data also indicate that it should be possible to determine the concentration of islet cell antibodies once an antigen standard becomes available. At present, it appears that adsorption of human sera on rat tissue powders provides the means by which falsely positive samples are effectively excluded. This is important since we have detected antibodies against specific rat antigens among several laboratory workers who often donate blood as controls. Following double adsorption against rat liver and kidney powder and a 1:10 dilution of each sample, we clearly detected samples from diabetic individuals with a binding activity higher (mean + 2 SD) than the pool of adsorbed control sera, which were routinely included in each assay. Recently, we found two additional individuals, both laboratory workers, whose levels of positive ^{125}I-protein A binding did not decrease below the level of $\bar{x}$ + 2 SD of the controls (not shown) after adsorption. Presumably these individuals have developed multiple antibodies to rat antigens and we are now carrying out experiments to characterize these antibodies.

The present assay provides a simple and convenient method to determine the presence of islet cell surface antibodies. When applied to sera from 26 insulin-dependent diabetic patients, 35% were found to have these antibodies. This prevalence is lower than observed with the conventional fluorescence assay on living cells.[5,6] The discrepancy may be explained by the fact that protein A does not react with the IgG_3 subclass of immunoglobulin G or IgM. These antibodies may thus be detected by immunofluorescence, but not by protein A. This discrepancy may be particularly important at the onset of the disease, when IgM antibodies predominate. A further possible problem inherent in the method is that collagenase digestion and/or paraformaldehyde fixation might damage some islet antigens and reveal others which are not normally expressed. The use of cultured human beta-cells might eventually provide a solution to these difficulties. Nevertheless, despite these limitations, this study demonstrates that islet cell surface antibodies can be quantitatively determined in human sera.

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