Expression of Cytoplasmic Islet Cell Antigens by Rat Pancreas

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SUMMARY
A major problem in standardization of the islet cell cytoplasmic antibody (ICA) assay is variation in sensitivity of the different human pancreas substrates used in individual laboratories. To circumvent this problem, we have developed an assay that utilizes Wistar-Furth rat pancreas as substrate, an anti-islet monoclonal antibody (A2B5) to identify islets and fluorescein-conjugated protein A to identify patient autoantibodies. Sera from 85 control subjects, 27 type I diabetics, and 17 subjects at high risk for developing type I diabetes were assayed in parallel with our standard ICA assay on human pancreas substrate and with Wistar-Furth rat pancreas as substrate. Two sera from control subjects (2 of 85) were ICA positive with rat pancreas compared to 1 of 85 with human pancreas substrate. Sera from 11 of 27 type I diabetics and 15 of 17 sera from high-risk subjects were ICA positive with either rat or human pancreas substrate. A correlation between the specific islet fluorescence readings on human and rat pancreas sections was found with sera from high-risk and control subjects. Furthermore, endpoint titers of an ICA-positive serum were identical with both assays. Finally, incubation of an ICA-positive serum with glycolipids, extracted from either human or Wistar-Furth rat pancreas, blocked subsequent ICA binding. These findings suggest that Wistar-Furth rat pancreas expresses an identical or similar autoantigen to human pancreas.

DIFFICULTY IN OBTAINING FROZEN HUMAN PANCREAS

Difficulty in obtaining frozen human pancreas is a serious limitation to studies of cytoplasmic islet cell antibodies (ICAs). In a few countries, religious considerations further restrict access to frozen human tissue. Recent studies have suggested that primate pancreas may substitute for human pancreas (1), but again access to such tissue is limited. Several studies have demonstrated limited cross-reactivity of ICA on rodent pancreas (2–4), whereas another study showed the islets of rat, mouse, guinea pig, sheep, and pig did not fluoresce significantly above background (1). In yet another recent study, ICAs were assayed with fresh frozen mouse pancreas as substrate and reportedly gave identical results to human pancreas for 40 sera from type I diabetics (5). In our studies of anti-islet monoclonal antibodies (MoAbs), we observed that anti-ganglioside anti-islet antibodies reacted with rat tissue and that a small subset of human serum anti-islet autoantibodies reacted with mouse pancreas (6). In addition, we found examples of human anti-islet antibody containing sera that showed a marked antibody-binding "prozone" on sections of human pancreas, giving positive reactions only when diluted (7). Consequently, we studied binding of serum autoantibodies from high-risk individuals (i.e., ICA positive and/or developed diabetes on follow-up) on frozen sections of rat pancreas with two different immunofluorescent assays.

MATERIALS AND METHODS
Sera. Sera were obtained from 19 individuals (aged 6–68 yr) identified to be at high risk for development of type I diabetes by virtue of having a relative with type I diabetes and being cytoplasmic ICA positive with our standard assay (8). Of this group, 5 have subsequently developed overt type I diabetes, and 7 others had no first-phase insulin secretion in response to intravenous glucose. All individuals were studied as outpatients. Sera from 21 normal individuals (aged 13–62 yr) without diabetes and from 14 negative controls from ICA workshops were utilized as controls. In a further set of experiments, sera from 50 subjects from the offspring cohort of the Framingham heart study and 27 type I diabetics were assayed in parallel with rat and human pancreas substrate. Interassay variation was assessed by including the same positive and negative control at a final dilution of 1:2 in each assay.

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ICA assays. Our standard assay used frozen sections of normal human pancreas. Sections were covered first with 30 μl of Trasylol (FBA, New York) (diluted 1:2 in phosphate-buffered saline; PBS) and then with 30 μl of serum and incubated for 12 h at 4°C. They were then washed with PBS and incubated with 40 μl of fluorescein-conjugated protein A (1 μg/ml; Sigma, St. Louis, MO) (FITC-pA). After a 20-min incubation, 10 μl of rhodamine-conjugated MoAb BISL-32 (Boehringer, Mannheim, FRG) was added, and 10 min later, sections were again washed before incubation with rhodamine-conjugated anti-mouse Ig (Dako, Santa Barbara, CA) for 30 min. Finally, sections were washed with PBS, mounted with AFT Systems mounting medium (Behring), and read through a Leitz fluorescence microscope (×25 and ×10 oil immersion lens). All sections were read without knowledge of the serum sample tested.

Preliminary studies compared frozen rat pancreas sections with and without acetone fixation, various serum dilutions, and absorption of sera. For the rat FITC-pA assay, frozen sections of 6-wk-old Wistar-Furth rat pancreas were acetone fixed (5 min at 20°C) immediately after sectioning. Sera were absorbed overnight at 4°C with acetone and methanol-extracted rat liver powder and diluted 1:1.5 (vol/vol) in PBS plus 1% bovine serum albumin (BSA) before use. Sections were covered first with 20 μl of Trasylol (diluted 1:2 in PBS) and then 20 μl of absorbed serum and were incubated overnight at 4°C. They were washed with PBS and incubated for a further 30 min at room temperature with FITC-pA. After further washing with PBS, they were incubated with MoAb A2B5 (diluted 1:100 in PBS–1% BSA) for 30 min at room temperature, washed with PBS, incubated with a 1:20 dilution of rhodaminated anti-mouse Ig, washed, mounted, and evaluated.

An alternative immunofluorescent method utilizing a mouse anti-human IgG MoAb was also studied. For this assay, rat pancreas sections were not acetone fixed, and sera were not absorbed. Rat pancreas sections were incubated with sera overnight at 4°C (usually at a 1:50 final dilution), washed with PBS, incubated with anti-human IgG MoAb ascites (HB43) (diluted 1:100 in PBS–1% BSA) for 30 min at room temperature, washed, incubated with FITC anti-mouse Ig (Dako) (diluted 1:20 in PBS–1% BSA) for 30 min at room temperature, washed with PBS, mounted, and evaluated.

To further compare the fluorescence intensity with rat or human sections in the FITC-pA assay, specific islet fluorescence was quantitated, as previously described, by means of a Leitz MPV compact photometer (8), for sera from 10 high-risk and 9 control subjects. At least 12 paired readings from islets and adjacent acinar tissue were obtained for each sample on both human and rat pancreas sections, and the mean specific islet fluorescence was obtained by subtracting the acinar background fluorescence from the islet fluorescence. The mean intra-assay coefficient of variation for a standard ICA-positive serum was 14%.

ICA-binding blockade by pancreatic glycolipid extracts. Pancreatic glycolipid extracts were prepared from human and Wistar-Furth rat pancreas by a modification of the technique of Svensson and Fredman (9). Briefly, pancreas was extracted twice in chloroform:methanol:water (4:8:3), and the glycolipids were recovered from an aqueous phase by Folch partition. Sep-Pak C18 cartridges (Waters, Milford, MA) were used to separate lipid from nonlipid components as described by Williams and McCluer (10). Increasing amounts of glycolipids eluted from Sep-Pak cartridges (related to starting weight of pancreas) were evaporated to dryness on polypropylene tubes. Two hundred microliters of serum from a high-titer ICA-positive "prediabetic" (diluted 1:5 in PBS–1% BSA) was added to the tubes containing the dried test extract. Tubes were vortexed and then incubated for 12 h at 4°C on a rocking table, after which tubes were centrifuged at 10,000 × g for 5 min and the supernatant saved. Supernatants were then applied to rat pancreas sections, and a standard ICA assay was performed. Again, specific islet fluorescence was measured by use of a photometer. In previous studies a dose-dependent reduction in fluorescence was observed after incubation of ICA-positive serum with human pancreas glycolipid extracts before ICA assay on human or mouse pancreas (6).

RESULTS

When sera at 1:2 final dilution from 5 individuals known to be ICA positive by our standard (human pancreas) ICA

**FIG. 1. Islet cell antibody assay with Wistar-Furth rat and human pancreas as substrate.** Serum autoantibodies were detected with mouse anti-human IgG monoclonal antibody and then fluorescein-conjugated anti-mouse antibody. *Left panel:* dilution of sera from 5 control (X) and 5 high-risk (●) subjects. *Middle panel:* qualitative islet fluorescence for serum from high-risk and control subjects (assayed at 1:50 dilution). *Right panel:* dilution of strongly ICA-positive serum assayed with rat (●) and human (C) pancreas.
assay (FITC-pA) were assayed with rat pancreas and anti-
human IgG MoAb. 3 of the 5 individuals were found to be
ICA negative or indeterminant (Fig. 1, left panel). With
dilution (e.g., 1:10 or 1:50), these 3 sera became strongly
positive. At a 1:10 dilution, 2 control sera were also positive,
whereas at a 1:50 dilution, all 5 controls were negative and
all 5 ICA-positive sera were read as +w or greater. Figure
1, right panel, illustrates ICA readings at varying serum di-
lutions for 1 of the ICA-positive individuals whose serum had
a prozone. Readings via the same detecting MoAb with both
rat and human pancreas sections are compared. Except for
the marked prozone with rat pancreas at the 1:2 dilution,
the results were identical, with the greatest positivity at a
dilution of 1:50. With this assay at a 1:50 serum dilution, 15
sera from high-risk individuals (ICA positive by standard as-
say) and 29 normal controls were studied. Thirteen of 15
(86%) high-risk individuals were +w or greater, but 5 of 29
(17%) normal individuals were also +w or greater (Fig. 1,
center panel). Qualitatively, 1 (3%) of the controls was 2+
or greater, whereas 20 (60%) of the high-risk patients were
2+ or greater. Despite the high dilution of sera (1:50) that
could be utilized with this assay format, the high percentage
of positive readings with sera from normal individuals led us
to evaluate an alternative detection system (FITC-pA).
The FITC-pA assay was less sensitive for end-point titer with
both rat and human pancreas compared with the anti-IgG
MoAb assay (cf. Fig. 2, left panel, with Fig. 1, right panel).
After the initial serial dilution studies, sera from control and
high-risk individuals were studied at a 1:5 dilution via rat
pancreas and assayed in parallel with our standard assay
with human pancreas at a 1:2 dilution. Fifteen of 17 sera
(88%) from high-risk individuals were positive with the rat
pancreas compared to only 1 of 35 (2.8%) of sera from
normal*controls (Fig. 2, right panel). The 1 positive normal
control was subsequently read as negative on repeated as-
says, whereas high-risk positives were repeatedly positive.
With human pancreas, the same number of sera from the
high-risk individuals were read as +w or greater and 1 of
22 controls as +w or greater (Fig. 2, right panel). In contrast
to the anti-human IgG MoAb technique, we did not detect a
prozone effect with dilution of the sera studied with the FITC-
pA assay and rat pancreas.
After developing the FITC-pA assay with rat pancreas substrate, we studied 27 type I diabetics and 50 controls from the offspring cohort of the Framingham heart study. Eleven of 27 (41%) type I diabetics were weak positive or stronger with both human and rat pancreas (Fig. 3, left panel). Only 1 sample was weakly positive with human but negative with rat pancreas; similarly 1 sample was weakly positive with rat but negative with human pancreas. Of 50 Framingham controls, 1 was +w with rat pancreas; all were negative with human pancreas (Fig. 3, right panel).

Interassay variation was evaluated by assaying the same positive and normal sera in multiple assays; the positive control was always read as positive (2–3+ on 1 occasion, 2+ on 3 occasions, and 1+ on 1 occasion). The negative control was always read as less than +w (± on 1 assay and negative on 6 assays).

To quantitatively compare the fluorescent readings with rat versus human pancreas, 9 control and 10 sera from high-risk patients were studied via FITC-pA to detect antibody binding with determination of specific islet fluorescence by means of a photometer. Readings on rat and human pancreases were significantly correlated throughout the entire range of specific islet fluorescence ($r = .66, P < .01$). The lowest control subject's sera gave a photometer reading of $2.1 \pm 1.2$ U (mean ± SE) on human and $5.6 \pm 1.5$ U on rat pancreas, whereas the highest ICA-positive subject's sera gave a reading of $23.6 \pm 2.8$ U on human and $35.2 \pm 4.2$ U on rat pancreases. Furthermore, incubation of an ICA-positive serum with upper-phase glycolipid extracts of either human or Wistar-Furth rat pancreas blocked subsequent ICA binding to Wistar-Furth rat pancreas. The photometer reading for a standard ICA-positive serum was $15.9 \pm 0.7$ U and after absorption with partially purified human pancreatic glycolipids was $16.6 \pm 1.4$ U (0.2 g starting wet wt of pancreas) $9.8 \pm 0.9$ U (0.4 g), $7.9 \pm 0.9$ U (0.6 g), and $8.9 \pm 2.0$ U (0.8 g).

**DISCUSSION**

These studies indicate that 1) most sera that are ICA positive with human pancreas can also be detected with rat pancreas, 2) end-point titers with two different assay formats (FITC-pA and anti-human IgG MoAb with FITC anti-mouse Ig) are similar with rat and human pancreas substrates, 3) specific islet fluorescence quantitated by photometer for sera from controls and high-risk subjects on human and rat pancreas sections are highly correlated, and 4) glycolipid antigens extracted and partially purified from human and rat pancreas when incubated with sera block ICA binding to the rat pancreas.

It is not clear why most earlier investigators found the rat pancreas to be an unsuitable substrate in ICA assay. Two major factors in this study may be the absorption of sera with rat liver powder to reduce the background fluorescence and the use of FITC-pA as the detecting reagent. When we evaluated an assay via FITC-conjugated rabbit anti-human antibody, a very high background fluorescence was found, making interpretation of sections impossible. In addition, awareness of the existence of a prozone phenomenon with the FITC anti-IgG assay may be crucial in interpreting earlier studies. In this study, with FITC-pA as detecting reagent, we could not demonstrate a prozone with any sera. This is analogous to the lower frequency of prozone interference with FITC-pA versus anti-human IgG and human pancreas (7).

These observations suggest that Wistar-Furth rat pancreas expresses autoantigen(s) with similar immunocytochemical properties to the autoantigen(s) of human pancreas. This information should facilitate biochemical characterization of cytoplasmic antigen(s) and comparison between surface (isolated rat islet cells) and cytoplasmic (frozen sections of rat pancreas) assays. A larger group of controls and ICA-positive individuals need to be studied to determine whether rat pancreas can substitute for human pancreas in screening all populations at risk for type I diabetes.

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