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### Authors

Dyrberg, T

Baekkeskov, S

Lernmark, A

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# Specific Pancreatic $\beta$ -Cell Surface Antigens Recognized by a Xenogenic Antiserum

THOMAS DYRBERG, STEINUNN BAEKKESKOV, and ÅKE LERNMARK  
*Hagedorn Research Laboratory, Niels Steensensvej 6, DK-2820 Gentofte, Denmark*

**ABSTRACT** An antiserum (R4) from a rabbit immunized with suspensions of C57BL/6J ob/ob mouse islet cells contains antibodies which in a  $^{125}\text{I}$ -protein A radioligand assay can be demonstrated to bind to single cell suspensions of normal Naval Medical Research Institute (NMRI) mouse islet cells. The binding of  $^{125}\text{I}$ -protein A to islet cells was about four times that of normal rabbit serum (NRS) after incubation at a 1/600 dilution of R4 antiserum quantitatively absorbed to mouse spleen lymphocytes (R4A antiserum) and hepatocytes. Subsequent absorption of the R4A antiserum to islet cells significantly reduced the binding of  $^{125}\text{I}$ -protein A to islet cells incubated with the doubly absorbed serum. Immunoprecipitation of radiolabeled islet cell lysates followed by SDS polyacrylamide gel electrophoresis and autoradiography suggested that the R4A antiserum recognized a  $M_r$  40,000 glycoprotein. This glycoprotein was not detected in spleen lymphocytes. Electron microscope detection of gold-protein A complexes suggested that the binding of islet cell surface antibodies was cell specific. Islet cell suspensions incubated with R4A antiserum and gold-protein A showed that  $86 \pm 3$  gold particles were bound per 100  $\beta$ -cells (mean  $\pm$  SE for six experiments). In contrast, the number of gold particles per 100 endocrine non- $\beta$ -cells was  $8 \pm 1$  which was similar to the number achieved with NRS ( $3 \pm 1$ ) on all endocrine islet cells. Our observations suggest that the pancreatic islet cells, in particular the  $\beta$ -cells, express a specific antigen.

The mammalian endocrine pancreas is composed of clusters of cells distributed throughout the exocrine parenchyma. Endocrine cells appear early during embryogenesis, and in the adult pancreas at least four endocrine cells have been identified. Disregarding connective tissue and endothelial cells, immunocytochemistry and electron microscopy have shown that the pancreatic islet is composed of ~70% cells producing insulin ( $\beta$ -cells), of 15–20% cells producing either glucagon ( $\alpha$ -cells) or pancreatic polypeptide (PP-cells), and of 5–10% cells producing somatostatin (D-cells) (23, 30). Although hormone biosynthesis and secretion in isolated islets are studied extensively, little or no information exists about the individual cell types, their course of differentiation and possible expression of cell-specific determinants. Many cell types are, for example, found to express specific cell membrane components during embryogenesis or functional differentiation. Such membrane components are often identified as cell-specific surface antigens by xenogenic or allogenic antisera, autoantibodies or monoclonal antibodies.

We have previously demonstrated that rabbits immunized with suspensions of mouse or rat islets develop islet cell surface antibodies (19). In the present study a rabbit antiserum raised

against cell suspensions from  $\beta$ -cell-rich C57BL/6J ob/ob mouse islets was subjected to quantitative absorption with mouse spleen lymphocytes. The absorbed serum was used to test whether remaining antibodies (a) recognize islet cell antigens in a radioligand assay or by immunoprecipitation and (b) are able to discriminate between the different endocrine islet cells using immunocytochemical electron microscopy. The results suggest that the absorbed xenogenic antiserum is specific for an antigen mainly detectable on the surface of the pancreatic  $\beta$ -cells.

## MATERIALS AND METHODS

### Materials

Crude collagenase (type I), HEPES, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO.). Hanks' balanced salt solution (HBSS) and RPMI 1640 tissue culture medium were supplied by Flow Laboratories (Irvine, Ayrshire, Scotland). Crystallized bovine serum albumin, fraction V, was purchased from Miles Laboratories (Elkhart, IN). Percoll, Ficoll, protein-A, and molecular mass ( $M_r$ ) standards were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Chloramine-T, gold ( $\text{HAuCl}_4$ ) were obtained from Merck (Darmstadt, W. Germany) and polyethylene glycol ( $M_r$  20,000) from Fluka AG (Buchs, Switz-

erland). Minisorb centrifuge tubes were obtained from Nunc (Roskilde, Denmark) and polypropylene micro-test tubes were supplied by Milian Instruments SA (Geneva, Switzerland). Na-<sup>125</sup>I and <sup>35</sup>S-methionine were obtained from The Radiochemical Center (Amersham, Buckinghamshire, England). All chemicals for gel electrophoresis were obtained from Bio-Rad Laboratories (Irvine, CA), except acrylamide, which was from Serva (Heidelberg, W. Germany).

EN<sup>3</sup>HANCE and Aquasol II were obtained from New England Nuclear (Dreiech, W. Germany). Nonidet P-40 (NP-40) was from Particle Data Laboratories (Elmhurst, IL). Phenylmethylsulfonylfluoride (PMSF) was from Boehringer Mannheim GmbH Biochemica (Mannheim, W. Germany); trasylol (Aprotinin) from Novo Laboratories (Bagsvaerd, Denmark); *Lens culinaris* from the Wallenberg Laboratory (Uppsala, Sweden); and cyanogen bromide activated Sepharose 4B was from Pharmacia Fine Chemicals.

### Preparation of Rabbit Anti-mouse Islet Cell Serum

Rabbit anti-mouse islet cell antiserum was obtained as previously described (19). In short, islet cells, prepared from female C57BL/6J ob/ob mice (Jackson Laboratory, Bar Harbor, ME) were injected into a lymphnode on each hind-leg of three New Zealand rabbits, each animal receiving  $0.5$  to  $1 \times 10^6$  cells. Complete Freund's adjuvant was injected intracutaneously at multiple sites on the hind-legs and abdomen. Two intravenous booster injections were given 2 wk apart, each with  $0.5$  to  $1 \times 10^6$  islet cells. The rabbits were bled 2 wk after the last booster injection, and the serum was removed after centrifugation and kept at  $-20^\circ\text{C}$ . The serum (R4 antiserum) was heat-inactivated ( $56^\circ\text{C}$  for 30 min) and centrifuged ( $10^5 \times g$  for 30 min) at  $4^\circ\text{C}$  before use. Preimmune serum served as the normal rabbit serum (NRS) control.

### Preparation of Islet Cells

Male Naval Medical Research Institute (NMRI) mice (Gl. Bomholtgård, Ry, Denmark), 20–25 g, were fasted overnight and killed by cervical dislocation before serving as donors for isolation of pancreatic islets (20). The pancreas was excised, and two intact glands were incubated with shaking (180 cycles/min) for 15 min at  $37^\circ\text{C}$  in HBSS with 1.5 mg/ml collagenase. Several 5-min incubations with 0.75 mg/ml collagenase followed until the exocrine tissue was completely digested. The islets, freed from the exocrine parenchyma at each incubation, were pooled and rinsed three to four times by sedimentation for 2–5 min in 50-ml centrifugation tubes containing ice-cold HBSS with 1% BSA. The islets were separated from the digest by centrifugation on a discontinuous Percoll gradient (3). The islets were individually selected at room temperature into RPMI 1640 supplemented with 1% BSA and 2 mmol/l EGTA (RPMI-EGTA), allowing the islets to remain in this medium for a total of 30 min. The islets were dissociated into single cells in 1 ml of RPMI-EGTA by aspiration five to ten times through a Pasteur pipette with a blunt-ended constricted tip. Unbroken islets or islet remnants were allowed to sediment and the supernatant containing free cells was transferred to 2.5 ml of RPMI with 4% BSA. Single cells were separated from cell debris and free nuclei by centrifugation at 50 g for 10 min (15). The cell pellet was resuspended in 0.5 ml of RPMI 1640 with 4% BSA and the cell number determined in a hemocytometer.

In 35 experiments using a total of 294 mice,  $148 \pm 4 \times 10^3$  islet cells were obtained per mouse pancreas. The proportion of single cells was  $92 \pm 1\%$  (mean  $\pm$  SE for 35 experiments).

### Preparation of Spleen Lymphocytes and Liver Cells

Spleens from male NMRI-mice were pressed through a nylon filter into ice-cold HBSS, and the resulting cell-suspension was layered onto Ficoll-Hypaque and centrifuged (800 g for 15 min) at  $4^\circ\text{C}$  (2). The lymphocytes were removed from the interface and washed twice by centrifugation (100 g for 10 min) at  $4^\circ\text{C}$  in HBSS containing 1% BSA.

Liver slices of NMRI-mice were pressed through a nylon filter into ice-cold,  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free HBSS supplemented with 2 mmol/l EGTA. The cells were washed twice by centrifugation in HBSS with 1% BSA before being resuspended in RPMI 1640 supplemented with 4% BSA.

### Absorption of Serum

Aliquots of NRS and antiserum were absorbed to different cell types by carefully resuspending the cells in serum, followed by incubation at  $4^\circ\text{C}$ . The serum was retrieved by centrifugation (10,000 g for 5 min) at  $4^\circ\text{C}$ . The R4 antiserum absorbed with spleen lymphocytes will be referred to as the R4A antiserum.

### Preparation of <sup>125</sup>I-Protein A

Protein A was iodinated using chloramine-T as the reducing agent and free iodine separated from the labeled protein by polyacrylamide electrophoresis (21). Briefly, 20  $\mu\text{g}$  of protein A dissolved in 50  $\mu\text{l}$  of 0.3 mol/l phosphate buffer (pH 7.4) were incubated with  $3.7 \times 10^7$  Becquerels (Bq) Na <sup>125</sup>I and 20  $\mu\text{l}$  of chloramine-T (0.033 mg/ml) for 3 min. The reaction was stopped by adding 5  $\mu\text{l}$  of sodium disulfite (0.3 mg/ml) and 100  $\mu\text{l}$  of 130 mmol/l sodium borate buffer with 0.5% BSA (pH 8.0). The iodination mixture was subjected to polyacrylamide electrophoresis at pH 8.9 (21). The gel slices containing the iodinated protein were eluted overnight in the borate BSA buffer. Aliquots of labeled protein A were kept at  $-20^\circ\text{C}$  and used within 3 mo.

### <sup>125</sup>I-Protein A Assay

Islet cell surface bound antibodies were determined by a <sup>125</sup>I-protein A assay essentially as described (19). Briefly, 100  $\mu\text{l}$  of islet cell suspension ( $5 \times 10^5$  cells/ml in RPMI 1640 with 4% BSA) were added to 100  $\mu\text{l}$  of rabbit serum and incubated for 60 min at  $4^\circ\text{C}$  in a Minisorb tube. Each tube was gently shaken every 10–15 min. The cells were then washed by centrifugation (50 g for 2 min) in 4 ml of RPMI 1640 with 4% BSA. Approximately 200  $\mu\text{l}$  of medium were left in the test tube and 50  $\mu\text{l}$  of <sup>125</sup>I-protein A ( $\sim 2$  to  $2.5 \times 10^5$  cpm/tube) added before the cells were incubated for 30 min at  $4^\circ\text{C}$ , shaking the tubes gently every 10 min. The cell suspension was finally transferred to a 500- $\mu\text{l}$  polypropylene micro-test tube containing a bottom layer of 40  $\mu\text{l}$  of 0.12 mol/l phosphate buffer supplemented with 4% (wt/vol) Ficoll (pH 7.4) which was overlaid with 200  $\mu\text{l}$  of immersion oil to separate the cells from the medium as described (19). The micro-test tubes were centrifuged (10,000 g for 2 min) at room temperature in a Beckman microcentrifuge (Beckman Instruments, Spinco Div., Palo Alto, CA). The radioactivity in the cell-pellet was determined by counting the tip of the micro-test tube in a gamma counter. Results are given as counts per min per  $5 \times 10^4$  cells. Control experiments showed that the binding of <sup>125</sup>I-protein A to antibody-incubated cells was linear relative to the cell number (25 to  $200 \times 10^4$  cells/tube).

### Radioactive Labeling and Immunoprecipitation

Biosynthetic labeling of islets and lymphocytes with <sup>35</sup>S-methionine (4) and lactoperoxidase catalyzed cell surface iodination (13) was carried out as described. The viability of islet cells and lymphocytes after iodination remained  $>95\%$  as determined by trypan blue exclusion. Labeled islets and lymphocytes were lysed in 20 mmol/l Tris HCl (pH 7.4) containing 150 mmol/l NaCl, 5 mmol/l methionine, 2 mmol/l PMSF, 1% NP-40, and 1,000 KIE trasylol/ml (NP-40 buffer). In some experiments the cells were lysed by boiling in 0.1–0.5 ml 20 mmol/l Tris HCl (pH 7.4) containing 3% SDS followed by immediate dilution in 10 vol of NP40 buffer. Insoluble material was removed by centrifugation ( $10^5$  g for 30 min) and the supernatant used for immunoprecipitation either after absorption to normal rabbit serum (4) or after purification of glycoproteins on a lentil lectin-Sepharose 4B affinity column (9). Aliquots were incubated with 10  $\mu\text{l}$  of antiserum at  $4^\circ\text{C}$  for 3 h followed by addition of 100  $\mu\text{l}$  of 10% solution of formalin-fixed heat-inactivated *Staphylococcus aureus*. Cowan I strain (SAC). The SAC was washed four times in 0.5% NP-40 buffer, once in the same buffer containing 0.4 M NaCl and once with water. SAC-precipitates of cell extracts prepared by boiling in 3% SDS were washed by centrifugation six times in 10 mmol/l Tris HCl (pH 7.4) containing 2 mmol/l EDTA, 5 mmol/l methionine, and 0.1% SDS.

### SDS PAGE

The antigen antibody complexes bound to SAC were solubilized by boiling for 3 min in 80 mmol/l Tris HCl (pH 6.8) containing 3% SDS, 15% sucrose, 5%  $\beta$ -mercaptoethanol, and 0.001% bromphenol blue and analysed by PAGE on a 10% slab gel in a discontinuous buffer system (14). The gels were processed for autoradiography as described previously (4).

### Preparation of Gold-Protein A

Gold-protein A (25) was prepared from colloid gold which was obtained by heating 100 ml of 0.01% (wt/vol) HAuCl<sub>4</sub> to boiling in a siliconized Ehrleumeyer flask before adding 4 ml of 1% Na-citrate (7). The procedure was carried out to give a monodisperse gold-particle solution, the diameter being  $\sim 60$  nm. The mixture was boiled for 15 min and cooled to room temperature before pH was adjusted to 6.9 with 0.2 mol/l K<sub>2</sub>CO<sub>3</sub>. Protein A, 0.5 mg in 0.1 ml H<sub>2</sub>O, was stirred for 2–3 min in 10 ml of colloid gold-solution before 1 ml of 1% (wt/vol) polyethylene glycol (PEG) (*M*, 20,000) in 10 mmol/l HEPES with 140 mmol/l NaCl (HEPES-NaCl buffer) was added. The resulting mixture was allowed to

stand for 15 min at room temperature, and the gold-protein A particles were isolated by centrifugation (60,000 g for 1 h) at 4°C. The sediment, resuspended in 1.5 ml of HEPES-NaCl buffer with 0.2 mg/ml PEG (*M<sub>w</sub>* 20,000), was stored at 4°C before being used within 2 wk.

### Electron Microscopical Immunocytochemistry

Dispersed islet cells ( $2$  to  $3 \times 10^6$ ) were incubated with 25% (vol/vol) rabbit serum as described above ( $^{125}\text{I}$ -protein A assay). After washing by centrifugation (50 g for 2 min) the cell suspension was incubated for 30 min at 4°C with gold-protein A and transferred to the micro-test tubes. The cells were centrifuged (10,000 g for 5 s) through oil into a bottom layer of 0.12 mol/l phosphate buffer (pH 7.3) with 4% (wt/vol) Ficoll and 2% (vol/vol) glutaraldehyde. Each tube was cut above the bottom layer and the cells were allowed to remain in the glutaraldehyde for 60 min at 4°C. The cell pellet was then kept overnight at 4°C in 10 ml of 0.12 mol/l phosphate buffer (pH 7.3) before postfixation in 1%  $\text{OsO}_4$ , dehydration in ethanol, and embedding in Epon 812.

The specimens were cut on a LKB Ultratome III (LKB Instruments, Inc., Rockville, MD) (500–800 Å), mounted on 300-mesh coppergrids, poststained with uranyl acetate and lead-citrate, and finally examined in a JEM 100B electron microscope.

### Morphometric Analysis

The number of gold particles bound to the surface of endocrine cells was determined in six separate experiments. In each experiment a total of 100  $\beta$ -cells and ~50 non- $\beta$ -cells was examined. The mean number of particles per 100 cells in each experiment was entered as one observation in the statistical evaluation.

## RESULTS

### Binding of $^{125}\text{I}$ -Protein A

The relationship between the binding of  $^{125}\text{I}$ -protein A to mouse islet cells and the concentration of the rabbit anti-mouse islet cell serum (R4) was linear when the dilution was 1/12,800–1/400 (Fig. 1). The binding of  $^{125}\text{I}$ -protein A reached an apparent maximum at a dilution of 1/400, being seven times larger than that of the NRS.

Absorption of the R4 antiserum to mouse spleen or islet cells showed that the protein A binding decreased in proportion to an increasing number of absorbing cells (Fig. 2). The absorption curve for spleen lymphocytes reached an apparent maximum with  $20 \times 10^6$  cells/ml. The quantitative absorption was verified in separate experiments allowing further absorptions of the R4A antiserum first to spleen cells ( $5 \times 10^7$  cells/ml for 15 h at 4°C) and then to hepatocytes ( $5 \times 10^7$  cells/ml for 8 h at 4°C). The resulting absorbed serum, when tested at a dilution of 1/600, induced a binding of  $^{125}\text{I}$ -protein A which amounted to 2,625 (mean of duplicate determination) cpm/ $10^6$  spleen cells and 898 cpm/ $5 \times 10^4$  mouse islet cells. The absorbed serum was finally absorbed to islet cells ( $12.5 \times 10^6$  cells/ml for 15 h at 4°C). This serum preparation bound 2,811 cpm/ $10^6$  spleen cells but decreased to 640 cpm/ $5 \times 10^4$  mouse islet cells.

Direct absorption of the R4 antiserum to as many as  $2 \times 10^6$  islet cells (the maximum number of islet cells obtained in a large-scale islet isolation in 1 d) resulted in a steep decrease in  $^{125}\text{I}$ -protein A binding to islet cells (Fig. 2). The effects of absorbing the R4 antiserum to  $50 \times 10^6$  spleen cells were documented in additional two series of experiments (Figs. 1 and 3). In both series the R4A  $^{125}\text{I}$ -protein A binding activity to both spleen cells (not shown) and islet cells decreased but remained in islet cells about four times or more that of NRS absorbed to spleen cells or not (Figs. 1 and 3). Subsequent absorption of the R4A antiserum to  $2 \times 10^6$  islet cells/ml resulted in a decreased binding as compared to R4A ( $P < 0.005$ ). NRS was noted to consistently induce some binding of  $^{125}\text{I}$ -protein A as compared to tissue culture medium alone ( $P < 0.001$ ) (Fig. 3).

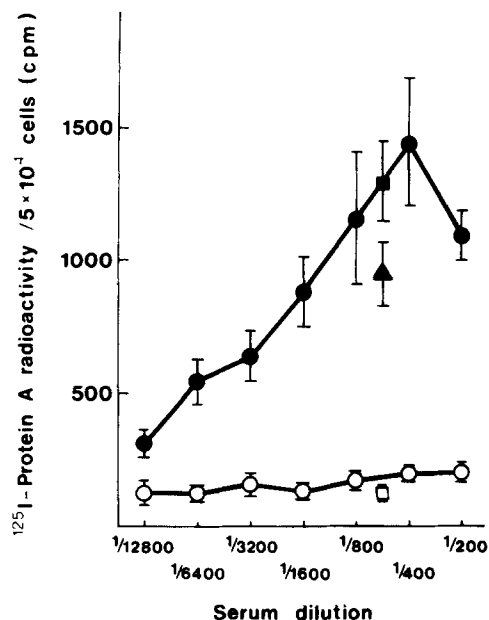


FIGURE 1 Binding of  $^{125}\text{I}$ -protein A by mouse islet cells incubated with increasing concentrations of normal rabbit serum (O) or rabbit anti-mouse islet cell serum (●). The cells were washed by centrifugation after incubation with serum and incubated with  $^{125}\text{I}$ -protein A before they were separated from the incubation medium by centrifugation through oil. In one series of experiments, the binding of  $^{125}\text{I}$ -protein A to islet cells was determined after incubation at various dilutions of normal rabbit serum (O) or rabbit anti-mouse islet cell serum (●) and in a second series at a 1/600 dilution of normal rabbit serum (□), rabbit anti-mouse islet cell serum, without absorption (■) or absorbed to spleen lymphocytes (▲). Mean values  $\pm$  SE for four to five experiments.

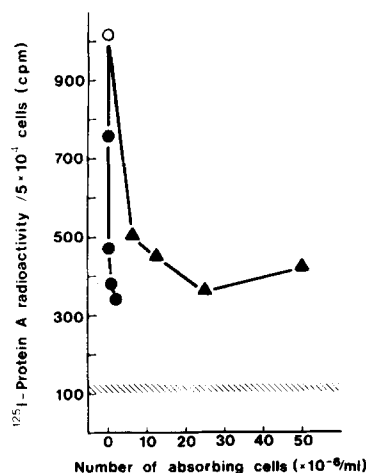


FIGURE 2 Quantitative absorption of the rabbit anti-mouse islet cell serum (R4) with increasing numbers of (●) mouse islet cells or (▲) mouse spleen lymphocytes. After incubation with the absorbed serum preparations, the islet cells were washed by centrifugation, incubated with  $^{125}\text{I}$ -protein A and finally separated from the medium by centrifugation through oil. The binding of  $^{125}\text{I}$ -protein A to islet cells after incubation with unabsorbed rabbit anti-mouse islet cell serum (O) or by normal rabbit serum (hatched area) is also shown. All sera were tested at a dilution of 1/600. Mean values for two to three experiments.

### SDS PAGE Antigen Analysis

SDS PAGE antigen analysis was used in three series of experiments to test the ability of the R4 and R4A antisera to recognize labeled islet cell antigens (Figs. 4 and 5): (a) analysis of total lysates of  $^{35}\text{S}$ -methionine-labeled islets (Fig. 4); (b) analysis of a  $^{35}\text{S}$ -methionine-labeled glycoprotein fraction (Fig. 4); and (c) analysis of lysates of surface-iodinated islet cells and spleen lymphocytes (Fig. 5).

In the first series of experiments, the R4 and R4A antisera,

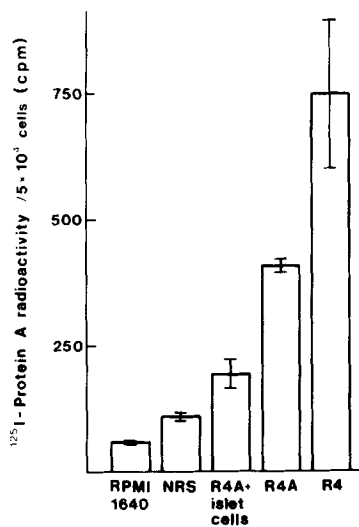


FIGURE 3 Binding of <sup>125</sup>I-protein A to islet cells after incubation with rabbit anti-mouse islet cell serum (R4), rabbit anti-mouse islet cell serum absorbed to 50 × 10<sup>6</sup> mouse spleen lymphocytes/ml (R4A), rabbit anti-mouse islet-cell serum absorbed to spleen lymphocytes and subsequently absorbed to 2 × 10<sup>6</sup> mouse islet cells/ml (R4A + islet cells), NRS, or tissue culture medium (RPMI 1640). All sera were tested at a dilution of 1/600 and <sup>125</sup>I-protein A binding determined as described in Fig. 1. Mean values ± SE for six experiments.

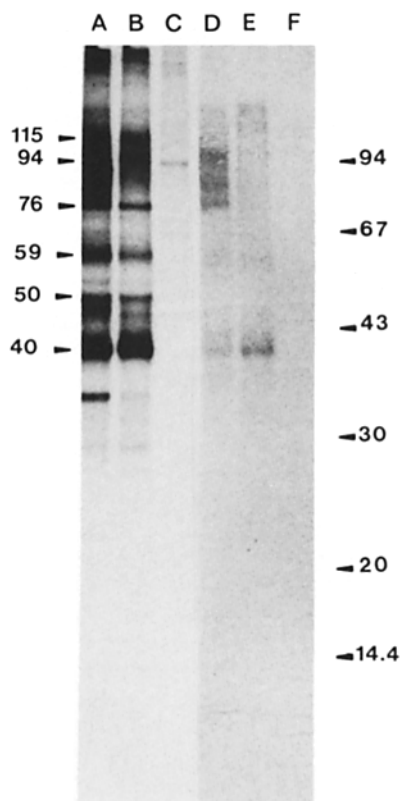


FIGURE 4 Analysis by SDS PAGE and autoradiography of [<sup>35</sup>S]methionine-labeled islet cell lysates immunoprecipitated with rabbit anti-mouse islet cell serum (R4) (lanes A and D), R4 absorbed to spleen lymphocytes (R4A) (lanes B and E) and NRS (lanes C and F). 1,500 labeled islets were lysed by boiling for 3 min in 100 μl of 20 mmol/l Tris-HCl (pH 7.4) with 3% SDS and subsequently diluted 1:10 in buffer with NP-40 detergent. After centrifugation at 100,000 g for 30 min the supernatant was used for immunoprecipitation either after after preabsorption with NRS (lanes A, B, and C) or after *L. culinaris* affinity chromatography (lanes D, E, and F) as described in Materials and Methods. Measurements, × 10<sup>-3</sup>.

expected also to react with intracellular antigens not readily absorbed to intact lymphocytes, precipitated multiple bands from lysates of <sup>35</sup>S-methionine-labeled islets (Fig. 4, lanes A and B). One prominent band (*M<sub>r</sub>* 40 kdaltons) and five weaker bands (*M<sub>r</sub>* 115, 94, 76, 59, and 50 kdaltons) were observed in immunoprecipitates of R4A (Fig. 4, lane B). Although some bands observed in the R4 immunoprecipitates were clearly reduced in the R4A immunoprecipitates, the latter antiserum immunoprecipitated bands at 94 and 76 kdaltons from <sup>35</sup>S-methionine-labeled lymphocyte lysates (not shown). These proteins may represent antigens common to lymphocytes and islet

cells, not accessible when absorbing the R4 antiserum to lymphocytes.

In the second series of experiments, a glycoprotein fraction of islet cell proteins was isolated by passing the islet cell lysates over a *Lens culinaris* lectin affinity column (Fig. 4, lanes D-F). The lectin bound material which could be eluted with α-methyl-mannoside represented ~3% of the total lysate radioactivity. Despite the considerable dilution of antigen induced by this affinity chromatography step, the R4 antiserum precipitated bands at 94-76 kdaltons and 40 kdaltons while the R4A antiserum precipitated a major band at 40 kdaltons. A very faint band at a *M<sub>r</sub>* position of ~59 kdaltons was seen in immunoprecipitates with both R4 and R4A.

In the third series of experiments, dispersed islet cells and spleen lymphocytes were labeled by lactoperoxidase catalysed iodination to determine whether any of the proteins recognized by R4 were accessible at the cell surface (Fig. 5). Both R4 and R4A antisera precipitated the 40-kdalton band from iodinated islet cell lysates (Fig. 5, lane A and B). The bands at 94, 76, and 67 kdaltons observed after R4A immunoprecipitation of islet cell lysates (Fig. 5, lane B) were also present in the lymphocyte immunoprecipitates (Fig. 5, lanes D and E), some of them even with NRS (Fig. 5, lane F). However, although the presence of a 40-kdalton band cannot be ruled out in R4 immunoprecipitates of iodinated lymphocytes (Fig. 5, lane D), it was not detectable in the R4A immunoprecipitate (Fig. 5, lane E).

Both islets and lymphocytes were prepared in 4% bovine serum albumin (BSA) and washed only once in PBS before iodination to avoid islet cell damage. The 67-kdalton band therefore most likely represents iodinated BSA not successfully removed by washing of cells and immunoprecipitates. The

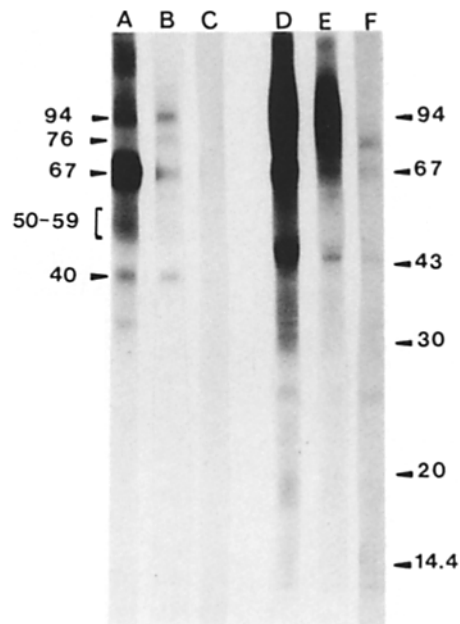


FIGURE 5 Analysis by SDS PAGE autoradiography of immunoprecipitates of surface iodinated mouse islet cells (lanes A, B, and C) and lymphocytes (lanes D, E, and F) with rabbit anti-mouse islet cell serum (R4) (lanes A and D), R4 serum absorbed to spleen lymphocytes (R4A) (lanes B and E) and NRS (lanes C and F). Islets and lymphocytes were lysed in buffer with NP-40 detergent, centrifuged at 100,000 g for 30 min and the supernatant was used for immunoprecipitation after preabsorption to NRS.

iodination experiments thus indicate that at least the 40-kdalton protein is specific for islet cells and present at the cell surface.

### Immunocytochemical Electron Microscopy

To ensure a specific binding of gold-protein A, islet cells were first incubated with the R4A antiserum and then with either gold-protein A or medium alone. After washing by centrifugation the cells were finally incubated with  $^{125}\text{I}$ -protein A. The gold-protein A complex inhibited the subsequent binding of radioactive protein A to antibody-treated islet cells ( $321 \pm 86$  cpm vs.  $1,238 \pm 114$  cpm [ $\bar{x} \pm \text{SEM}$ ] in cells not incubated with gold-protein A,  $P < 0.05$ ,  $n = 3$ ).

Gold particles were bound to the plasma membrane of  $\beta$ -cells in cell suspensions incubated with R4A antiserum and gold-protein A (Fig. 6). The number of gold particles bound to islet cells incubated with R4A antiserum or NRS is shown in Table I. The difference between the number of gold particles bound to  $\beta$ -cells and non- $\beta$ -cells in the R4A preparations was highly significant ( $P < 0.001$ ), whereas no difference between these cell types was seen with NRS. Note, however, that the number of gold particles bound to non- $\beta$ -cells incubated with the R4A serum was higher than the number bound to all endocrine cells in the NRS-preparation ( $P < 0.01$ ).

### DISCUSSION

Our results suggest that the pancreatic islet cells may express cell-specific surface membrane proteins. This conclusion is based on (a) the presence of islet cell antibodies after extensive absorption of the antiserum to spleen lymphocytes and hepatocytes, (b) a significant decrease in binding of  $^{125}\text{I}$ -protein A to islet cells incubated with R4A antiserum absorbed to islet cells, (c) the demonstration of an islet cell glycoprotein immunoprecipitated with the R4A antiserum, and (d) a marked binding of gold-protein A particles to  $\beta$ -cells compared to endocrine non- $\beta$ -cells in islet cells incubated with the R4A antiserum. Although the reactivity of the absorbed antiserum

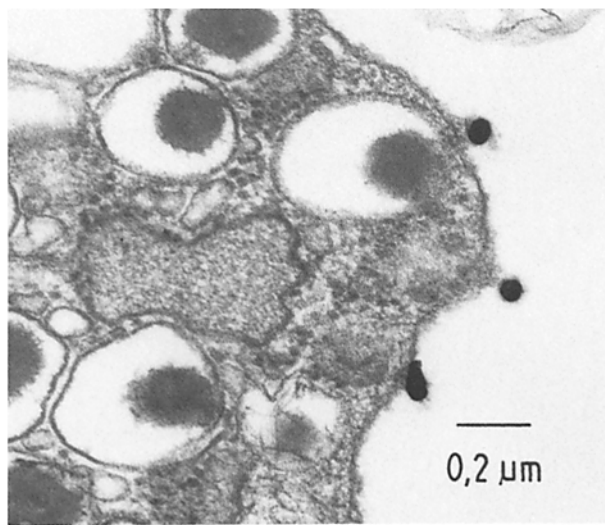


FIGURE 6 Electron micrograph of a mouse  $\beta$ -cell with gold-protein A particles attached to the plasma membrane. Islet cells were incubated with rabbit anti-mouse islet cell serum absorbed to spleen lymphocytes at a final concentration of 25% (vol/vol). After washing by centrifugation the cells were incubated with gold-protein A solution before they were centrifuged through oil into 2% glutaraldehyde as described in Materials and Methods. Bar  $0.2 \mu\text{m}$ .  $\times 48,200$ .

TABLE I  
Distribution of Gold-Protein A Particles in Islet Cells Incubated with Islet Cell Antiserum

Serum	No. of gold-protein A particles per 100	
	$\beta$ -cells	Non- $\beta$ -cells
Absorbed rabbit anti-mouse islet cell serum	$86 \pm 3$	$8 \pm 1$
NRS	$3 \pm 1$	$1 \pm 1$

Cells were submitted to electron microscope morphometric analysis after incubation firstly with either rabbit anti-mouse islet cell serum absorbed to spleen cells or normal rabbit serum and secondly with gold-protein A after washing by centrifugation. Cells were determined as  $\beta$ -cells or as endocrine non- $\beta$ -cells based on the morphology of the secretion granules. The number of gold-protein A particles on intact cells was determined as described in the text. Mean values  $\pm \text{SE}$  from four to six experiments.

could be decreased by an additional absorption to islet cells, complete removal of antibody was not accomplished. We were unable to obtain a larger number of islet cells since the living islet cells could not be pooled from day to day. However, the absorption curve with islet cells was steep and did not seem to level off (Fig. 2), and, when multiple absorptions were carried out, the islet cells caused a relatively larger decrease per cell than spleen lymphocytes and hepatocytes.

The major protein detected in the three series of experiments in which immunoprecipitates of the R4A antiserum were analyzed had a  $M_r$  of 40 kdaltons determined by SDS PAGE and autoradiography. The immunoprecipitation with R4A of labelled proteins eluted from the *L. culinaris* lectin column suggests that the 40-kdalton band is a glycoprotein. The observation that the R4A antiserum immunoprecipitates the 40-kdalton band after iodination of viable islet cells suggests that this protein is accessible at the cell surface. The 40-kdalton protein was detected in neither  $^{35}\text{S}$ -methionine-labeled (not shown) nor surface iodinated lymphocytes (Fig. 5). This is in agreement with the inability of lymphocytes to absorb out the 40-kdalton activity from the R4 serum. It is therefore concluded that the 40-kdalton band may represent an islet cell specific plasma membrane antigen. This conclusion is supported by recent immunoprecipitation experiments demonstrating that an extensively absorbed rabbit antiserum against rat islet cells (19) immunoprecipitates a 40-kdalton protein from  $^{35}\text{S}$ -methionine-labeled rat islets and tumor  $\beta$ -cells but not from rat spleen lymphocytes (authors's unpublished observations).

Bands at 50, 59, and 115 kdalton were also recognized by R4 and R4A in  $^{35}\text{S}$ -methionine-labeled islet cells but not in lymphocytes (not shown). R4 precipitated a smear of bands in the  $M_r$ -range 50–59 kdalton from surface iodinated islet cells; however, they were not detected in R4A immunoprecipitates and their possible cellular specificity needs to be investigated. While immunoprecipitation experiments of labeled glycoproteins and surface iodinated cells resulted in a limited number of bands, the total labeled islet lysates were more complex (Fig. 4). For example, the 50- and 115-kdalton proteins (Fig. 4, lanes A and B, and Fig. 5, lane A) were not detected in surface iodinated islet cells with the R4A serum and may therefore represent intracellular proteins. Antibodies present in the R4 serum directed against intracellular antigens are likely to escape absorption to living spleen lymphocytes.

The islets of Langerhans represent a complex mixture of endocrine cells. Electron microscopical immunocytochemistry with gold-protein A was therefore used to test whether the

R4A antiserum would react differently with the different endocrine cell types. A specific binding of the gold-protein A to islet cells was first established by demonstrating a decrease in  $^{125}\text{I}$ -protein A binding to antiserum incubated islet cells previously incubated with gold-protein A. The increased binding of gold-protein A to the  $\beta$ -cells (Table I) cannot be explained by a disproportionate low number of other endocrine cells in the electron microscopical preparations since ~35% of the examined cells were non- $\beta$ -cells (not shown). The proportion of  $\beta$ -cells to non- $\beta$ -cells in our cell preparations was in fact similar to that reported for intact islets (23, 30), and at least four endocrine cell types were seen. Occasional nonendocrine cells were seen, none of which bound gold-protein A. Although the R4A antiserum induced binding of gold particles in particular to  $\beta$ -cells, the binding to non- $\beta$ -cells was increased compared to all endocrine cells incubated with NRS. It cannot be excluded therefore that the R4A antiserum may contain additional islet cell surface antibodies or that a common antigen is expressed, but at a lower density, on the non- $\beta$  endocrine islet cells.

In addition to the systematically distributed surface antigens such as the histocompatibility antigens, many cells have been shown to express organ- or cell-specific antigens in the plasma membrane. The use of antibodies to probe cell surface determinants has revealed antigens present, e.g., in the immature cell only, certain antigens in the mature state and some present permanently throughout the lifespan of the cell. Studies with xenogenic antisera raised against embryonic chick skeletal muscle revealed the presence of surface antigens specific to the pre-fusion myoblast but not to later stages of muscle cell differentiation (8). Specific surface antigens have been detected in a variety of organs and cells, e.g., platelets (29), B-lymphocytes (27), liver (10), and exocrine pancreas (22), but such antigens have not previously been described in the endocrine pancreas. Although the functional role of cell-specific antigens has not been determined, these structures unique to a given cell type may be important in cell growth and differentiation.

Antibodies directed against cellular antigens are often found in autoimmune diseases. Insulin-dependent diabetes demonstrates a high incidence of antibodies directed against the pancreatic islet cells (1, 16, 18). It is controversial whether such antibodies have a pathogenetic role or whether they merely reflect cellular damage. If antibodies against cell-specific antigens play a role in the pathogenesis of an autoimmune disease, one would expect the antigen to be present on the surface of the target cell. Antibodies against the acetylcholine receptor can, for example, cause myasthenia gravis (24, 28), and a syndrome of severe insulin resistance is associated with antibodies to the insulin receptor (6, 11). Although islet cell surface antibodies have been shown to mediate complement-dependent cytotoxicity (5, 12, 26), it will be important to identify and isolate  $\beta$ -cell specific antigens in order to fully understand the phenomenon of autoimmunity in insulin-dependent diabetes. We have recently found that diabetic sera may contain auto-antibodies against at least two proteins ( $M_r$  ~64 and 38 kdalton) in human islets (17). A possible identity between these islet cell antigens and the antigens recognized by our xenogenic antiserum remains to be established.

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## REFERENCES

- Bottazzo, G. F., R. Pujol-Borrell, and D. Doniach. 1981. Humoral and cellular immunity in diabetes mellitus. *Clinics in Immunology and Allergy*. 1:139-159.
- Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21:77-89.
- Brunstedt, J. 1980. Rapid isolation of functionally intact pancreatic islets from mice and rats by Percoll gradient centrifugation. *Diabete Metab.* 6:87-89.
- Baekkeskov, S., T. Kanatsuna, L. Klarekog, D. A. Nielsen, P. A. Petersen, P. A. Rubenstein, D. F. Steiner, and Å. Lernmark. 1981. Expression of major histocompatibility antigens on pancreatic islets cells. *Proc. Natl. Acad. Sci. U. S. A.* 78(10):6456-6460.
- Doberson, M. J., J. E. Scharff, F. Ginsberg-Fellner, and A. L. Notkins. 1980. Cytotoxic autoantibodies to beta-cells in the serum of patients with insulin-dependent diabetes mellitus. *N. Engl. J. Med.* 303:1493-1498.
- Flier, J. S., C. R. Kahn, D. B. Jarrett, and J. Roth. 1976. Characterization of antibodies to the insulin receptor. *J. Clin. Invest.* 58:1442-1449.
- Frens, G. 1973. Controlled nucleation for the regulation of the particle size in monodisperse gold solutions. *Nature Physical Science.* 241:20-22.
- Friedlander, M., and D. A. Fischman. 1979. Immunological studies of the embryonic muscle cell surface. *J. Cell Biol.* 81:193-214.
- Hayman, M. J., and M. J. Crumpton. 1972. Isolation of glycoproteins from pig lymphocyte plasma membrane using *Lens culinaris* phytohemagglutinin. *Biochem. Biophys. Res. Commun.* 47:923-930.
- Hopf, U., K. H. Meyer zum Büschenfelde, and J. Freudenberg. 1974. Liver-specific antigens of different species. *Clin. Exp. Immunol.* 16:117-124.
- Kahn, C. R., J. S. Flier, R. S. Bar, J. A. Archer, P. Gorden, M. M. Martin, and J. Roth. 1976. The syndromes of insulin resistance and acanthosis nigricans. *N. Engl. J. Med.* 294:739-745.
- Kanatsuna, T., Å. Lernmark, A. H. Rubenstein, and D. F. Steiner. 1981. Block in insulin release from column-perfused pancreatic  $\beta$ -cells induced by cell surface antibodies and complement. *Diabetes.* 30:231-234.
- Kulczycki, A., Jr., V. Krause, C. C. Killon, and J. P. Atkinson. 1980. Improved cell surface radioiodination of macrophages. *J. Immunol. Methods.* 37:133-138.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Lernmark, Å. 1974. The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. *Diabetologia.* 10:431-438.
- Lernmark, Å., V. Bonnevie-Nielsen, S. Baekkeskov, T. Dyrberg, T. Kanatsuna, and J. Scott. 1981. Islet cell antibodies. In *Etiology and Pathogenesis of Insulin-dependent Diabetes Mellitus*. J. M. Martin, R. M. Ehrlich, and F. J. Holland, editors. Raven Press, New York. 61-67.
- Lernmark, Å., and S. Baekkeskov. 1981. Islet cell antibodies—theoretical and practical implications. *Diabetologia.* 21:431-435.
- Lernmark, Å., Z. R. Freedman, C. Hofmann, A. H. Rubenstein, D. F. Steiner, R. L. Jackson, R. J. Winter, and H. S. Traisman. 1978. Islet cell surface antibodies in juvenile diabetes mellitus. *N. Engl. J. Med.* 299:375-390.
- Lernmark, Å., T. Kanatsuna, C. Patzelt, K. Diakoumis, R. Carroll, A. H. Rubenstein, and D. F. Steiner. 1980. Antibodies directed against the pancreatic islet cell plasma membrane. *Diabetologia.* 19:445-451.
- Lernmark, Å., A. Nathans, and D. F. Steiner. 1976. Preparation and characterization of plasma membrane-enriched fractions from rat pancreatic islets. *J. Cell Biol.* 71:606-623.
- Linde, S., B. Hansen, and Å. Lernmark. 1980. Stable iodinated polypeptide hormones prepared by polyacrylamide gel electrophoresis. *Anal. Biochem.* 107:165-176.
- Metzgar, R. S. 1980. Pancreas specific alloantigens. *Transplant Proc.* 12(Suppl. 1):123-128.
- Orci, L., D. Baetens, C. Rufener, M. Amherdt, M. Ravazzola, P. Studer, F. Malaisse-Lagae, and R. H. Unger. 1976. Hypertrophy and hyperplasia of somatostatin-containing D-cells in diabetes. *Proc. Natl. Acad. Sci. U. S. A.* 73:1338-1342.
- Richman, D. P., C. M. Gomez, P. W. Berman, S. A. Burres, F. W. Fitch, and B. G. W. Arnason. 1980. Monoclonal anti-acetylcholine receptor antibodies on cause experimental myasthenia. *Nature (Lond.)*. 286:738-739.
- Roth, J., M. Bendayan, and L. Orci. 1978. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. *J. Histochem. Cytochem.* 26:1074-1081.
- Soderstrom, W. K., Z. R. Freedman, and Å. Lernmark. 1979. Complement-dependent cytotoxic islet cell surface antibodies in insulin-dependent diabetes. *Diabetes.* 28:397.
- Stashenko, P., L. M. Nadler, E. Hardy, and S. F. Schlossman. 1980. Characterization of a human B lymphocyte-specific antigen. *J. Immunol.* 125:1678-1685.
- Toyka, K. V., D. B. Drachman, D. E. Griffin, A. Pestronk, J. A. Winkelstein, K. H. Fischbeck, and I. Kao. 1977. Myasthenia Gravis. Study of humoral immune mechanisms by passive transfer to mice. *N. Engl. J. Med.* 296:125-131.
- Van der Weerd, Ch. M., L. E. Veenhoven von Riesz, L. E. Nijenhuis, and J. J. van Loghem. 1963. The Zw blood group system in platelets. *Vox Sang.* 8:513-530.
- Wellman K. F. and B. W. Volk. 1977. Histology, cell types and functional correlation of islets of Langerhans. In *The Diabetic Pancreas*. B. W. Volk, and K. F. Wellmann, editors. Plenum Press, New York.