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Expression of receptors for tetanus toxin and monoclonal antibody A2B5 by pancreatic islet cells

(ganglioside/amine precursor uptake and decarboxylation/melanoma/neuroblastoma)

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ABSTRACT **Studies of the reaction of antibody A2B5 and tetanus toxin with pancreatic islet cells, islet cell tumors, and other human amine precursor uptake and decarboxylation (APUD) tumors are described. By indirect immunofluorescence, antibody A2B5 and tetanus toxin were shown to specifically bind to the plasma membrane of human, rat, chicken, and mouse islet cells. The binding of antibody A2B5 to the cell surface of living islet cells has allowed isolation of these cells from a suspension of pancreatic cells by using a fluorescence-activated cell sorter. In studies designed to determine whether tetanus toxin and antibody A2B5 bound to the same surface antigen, A2B5 and tetanus toxin did not compete for binding to normal islet cells, a human islet cell tumor, or a rat islet cell tumor. In addition to binding to islet cell tumors, antibody A2B5 reacts with frozen sections, isolated cells, and cell lines of neural, neural crest, and APUD origin.**

The acronym APUD (amine precursor uptake and decarboxylation) was attached in 1968 to a series of cells that have common cytochemical properties, in particular their amine-handling characteristics (1). The APUD series includes pancreatic islet, adrenomedullary, enterochromaffin, anterior pituitary, and thyroid C cells. It was hypothesized that these diverse cell types were all of neural crest origin. Although some controversy persists (1), recent evidence, including grafts of quail neural primordium and chicken embryos (2–4), demonstration of APUD cells in the gut groove at 16- to 18-somite stages (5), and transplantation studies (6, 7), indicates that pancreatic islet cells are not of neural crest origin but arise from the endoderm. It is noteworthy that these same techniques confirm a neural crest origin for avian thyroid C cells (8). Despite this difference in embryonic derivation, neurons and pancreatic islet cells share many metabolic pathways (e.g., APUD), release a number of identical peptides (e.g., somatostatin and glucagon), and express neurone-specific enolase (9). Little is known, however, concerning the expression of similar cell surface differentiation antigens by islet cells and neurons. Recently, the techniques developed by Kohler and Milstein for the production of lymphocyte hybridomas (10) have been used to produce monoclonal antibodies reacting with a variety of cell surface differentiation antigens (11). In the present study, we use two "neuronal" markers that react with specific gangliosides, monoclonal antibody A2B5 and tetanus toxin, to test the hypothesis that islet cells and neurons express similar cell surface antigens.

METHODS AND MATERIALS

Production of Monoclonal Antibody A2B5. Antibody F12 A2B5 was produced in ascites fluid of BALB/c mice by clone 105, which has been donated to the American Type Culture Collection (12).

Cells. RINm5f and RINm14B rat insulinoma cell lines were kindly supplied by A. Gazdar, H. Oie, and W. Chick (13). Viable islet cells were isolated from chicken pancreas by mincing the pancreas with a fine scissor followed by collagenase digestion (type IV, 10 mg/ml, 37°C, 15 min; Worthington) followed by Ficoll gradient centrifugation (25/20% Ficoll 400, Pharmacia) to enrich for islet cells and then cultured in RPMI 1640 medium/10% heat-inactivated fetal calf serum (56°C, 30 min)/0.1 mM 3-isobutyl-1-methylxanthine (Sigma). Human cell lines (H series) were kindly provided by D. Bigner and pancreatic carcinoma cell lines were provided by R. Metzgar.

^{125}I-Labeled Protein A Radioassay. RINm5f cells (1×10^6) were incubated with 50 μl of a 1:500 dilution of antibody A2B5 ascites (0.32 μg of antibody protein) in Dulbecco's phosphate-buffered saline (lacking Ca^{2+} and Mg^{2+}; P_i/NaCl)/1% albumin in glass test tubes, and unbound antibody was then removed by washing three times with P_i/NaCl/1% albumin. Fifty microliters of cell suspension was placed in microtiter wells and incubated with 50 μl of ^{125}I-labeled protein A (0.012 μCi, 10 mCi/mg of protein; 1 Ci = 3.7×10^{10} becquerels) in P_i/NaCl/1% albumin. After a 30-min incubation at room temperature, cells were washed three times with 200 μl of P_i/NaCl/1% albumin. Wells were then cut from the plate and ^{125}I associated with the cell pellet was determined.

Cytotoxicity and Absorption Assay. RINm5f cells were cultured as monolayers in 96-well microtiter U plates (Flow Laboratories, McLean, Va). Culture medium was removed, to each well was added 100 μl of RPMI 1640 medium supplemented with 10% heat-inactivated bovine serum (56°C for 30 min) and 0.4 μCi of $Na^{51}CrO_4$ (0.048 μM), and the cells were incubated at 37°C for 4 hr in 95% air/5% CO_2. Cells were then washed three times with 200 μl of RPMI 1640 medium, incubated with 5 μl of rabbit serum as a source of complement and 50 μl of diluted antibody for 30 min at 37°C, and sedimented at 1,000 $\times$ g; the supernatant solution was harvested by using Titertek harvesting filters (Flow Laboratories) and the radioactivity in the supernatant was determined. For absorption studies, diluted antibody A2B5 was incubated for 30 min at room temperature with tissue extracts, the mixture was centrifuged, and the resultant supernatant was added to microtiter wells to assay cytotoxicity against RINm5F cells.

Antibody A2B5 Indirect Immunofluorescence. Pelleted cells ($1–50 \times 10^6$) were incubated with 50 μl of a 1:100 dilution of monoclonal antibody A2B5 ascites (1.1 μg of antibody protein) in P_i/NaCl/1% albumin for 30 min at room temperature, washed three times with P_i/NaCl, incubated for 30 min at room temperature with 50 μl of a 1:10 dilution of either fluorescein- or rhodamine-conjugated rabbit anti-mouse antibody (Cappel Laboratories, Cochranville, PA) in P_i/NaCl/1% albumin, and

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Abbreviations: APUD, amine precursor uptake and decarboxylation; P_i/NaCl, Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+}.

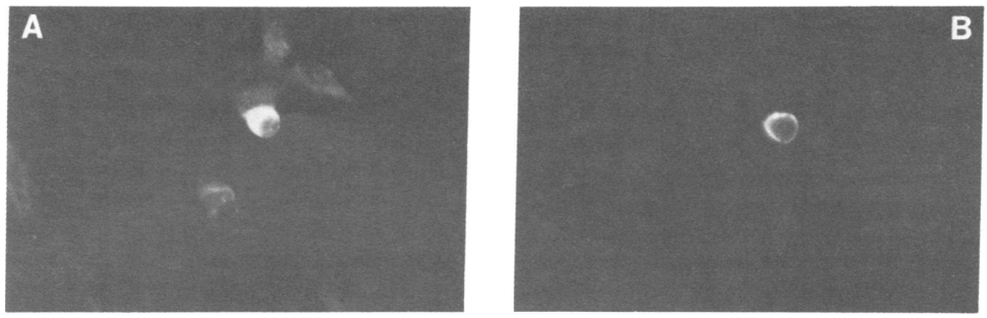

FIG. 1. Distribution of antibody A2B5 binding (*B*) and intracytoplasmic insulin (*A*) in a monolayer culture of day 21 embryonic chicken pancreas. (*A*) A single insulin-containing cell is present as well as fibroblasts that have a low level of nonspecific fluorescein binding. (*B*) The single beta cell shown in *A* reacts brightly with antibody A2B5. The distribution of fluorescence of the beta cell (fluorescein, cytoplasmic, rhodamine, rim staining) shows that neither rhodamine nor fluorescein fluorescence is visible with the opposite excitation filter. (×350.)

washed three times with P_i/NaCl. They were then studied with a Leitz Diavert epifluorescence microscope using an H-2 blue light excitation filter and a K480 barrier filter for fluorescein or an N2 filter for rhodamine fluorescence (×250). Tumors and pieces of pancreas were rapidly frozen in liquid nitrogen, air dried on gelatin-coated slides, fixed for 5 min with acetone at −30°C, and processed for immunofluorescence as described above. When cells or sections were stained for insulin content and A2B5 binding, insulin was detected by using a guinea pig anti-insulin serum (Cappel Laboratories) followed by a fluorescein-conjugated anti-guinea pig antibody (diluted 1:10 in P_i/NaCl/albumin; Cappel Laboratories).

Tetanus Toxin Immunofluorescence. Tetanus toxin was kindly supplied by R. O. Thomsen of The Wellcome Research Laboratories, England. Tissue sections or pelleted cells were incubated with tetanus toxin (0.1 μg/ml, 0.5 mM) in P_i/NaCl/1% albumin for 30 min at room temperature, washed three times with P_i/NaCl, incubated with a 1:10 dilution of rhodamine-conjugated anti-rabbit antibody, washed three times with P_i/NaCl, and viewed with a Leitz Diavert microscope equipped for rhodamine epifluorescence.

Flow Cytometry. Approximately 10^6 chicken pancreatic cells dissociated with collagenase were incubated with a 1:100 dilution of antibody A2B5 in P_i/NaCl/1% albumin for 30 min at room temperature, washed three times with RPMI 1640 medium/20% heat-inactivated calf serum, incubated with 50 μl of a 1:10 dilution of ultracentrifuged (1 hr at 100,000 × *g*) fluorescein-conjugated rabbit anti-mouse antibody (Cappel Laboratories) for 30 min at room temperature, and then washed three times with P_i/NaCl/1% albumin. Flow cytometry was performed using a FACS II (Robert Mittler, Becton Dickinson, Research Triangle, NC). Fluorescence-positive cells were col-

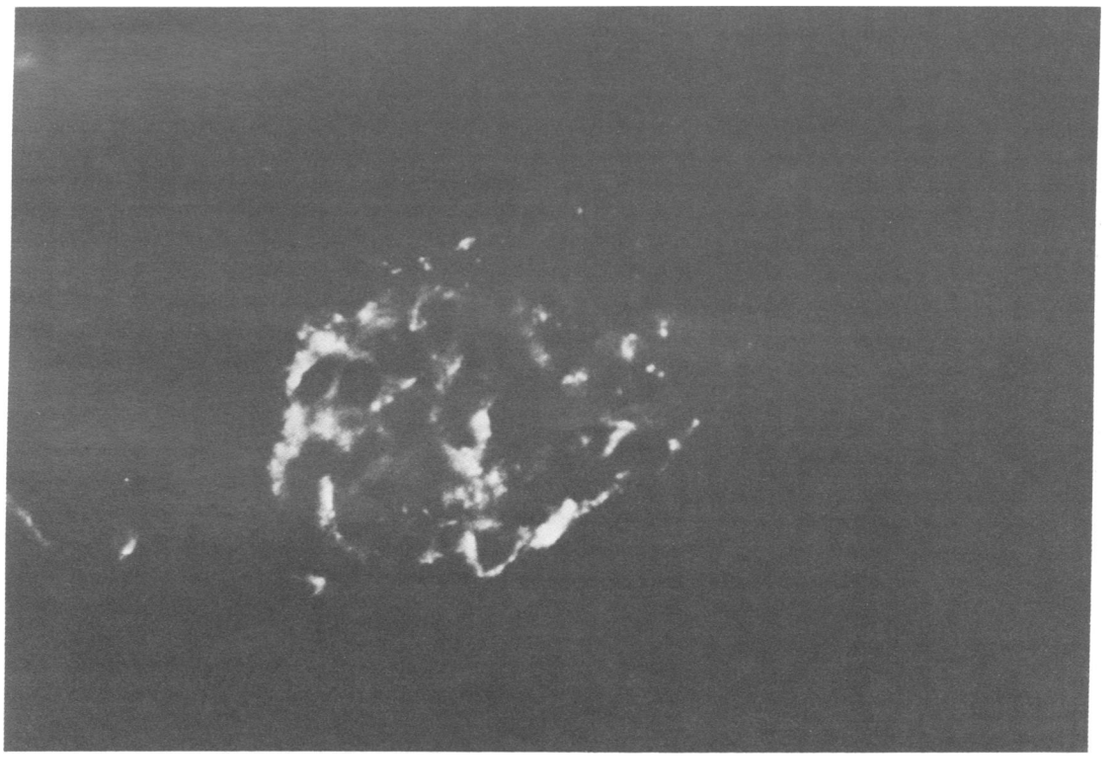

FIG. 2. Distribution of antibody A2B5 binding on sections of rat pancreas. Sprague–Dawley rat pancreata were frozen in liquid nitrogen, and 5-μm sections were fixed with acetone, incubated with a 1:100 dilution of monoclonal antibody A2B5 ascites, washed, incubated with fluorescein-conjugated rabbit anti-mouse antibody, washed, and viewed with a Leitz Diavert microscope. The islet is surrounded by acinar cells that are not fluorescent.

Table 1. Properties of antigen A2B5 of RINm5F cells

Addition	Residual cytotoxicity, %
None	100 ± 5
Pellet of RINm5F homogenate	28 ± 1
Residual pellet after extraction with chloroform/methanol (2:1) for 5 min	69 ± 14
Material extracted from pellet into chloroform/methanol	35 ± 3

Antibody A2B5 (0.16 μg of protein per assay) was treated with various fractions from RINm5F target cells. The pellet fraction was derived from 5×10^6 RINm5F cells. Release of ^{51}Cr from RINm5F cells was determined. Residual cytotoxicity = (cpm released due to antibody A2B5 after absorption/cpm released due to unabsorbed antibody A2B5) × 100. One hundred percent cytotoxicity corresponded to 2,169 cpm. Results represent mean ± SEM of three determinations.

lected, extracted with acetic acid, lyophilized, and suspended in P_i/NaCl, and the insulin content was determined with a standard double-antibody radioimmunoassay.

RESULTS

Binding of Antibody A2B5 to Islet Cells. Chicken pancreatic cells were cultured as a monolayer, fixed with acetone, and evaluated for intracellular insulin and binding of antibody A2B5 by using a double immunofluorescence technique (Fig. 1). As shown, beta cells (intracellular insulin) react with antibody A2B5 while fibroblasts do not. Intense rim fluorescence is characteristic (Fig. 1*B*) of the reaction of antibody A2B5 with cells. Similarly, the indirect immunofluorescence technique shows that antibody A2B5 reacts with islet cells but not with acinar cells of sections of rat pancreas (Fig. 2). Antibody A2B5 binds to islet cell plasma membranes of all vertebrate species we have studied (chicken, mouse, rat, and human). The ability of antibody A2B5 to label viable islet cells was evaluated by using fluorescence-activated cell sorting with a FACS II sorter. Pancreata from day 21 embryonic chickens were dissociated by incubating with collagenase and the number of cells reacting with antibody A2B5 was determined. By using the sorting capabilities of the FACS II, fluorescence-positive cells were isolated and the insulin content of 27,000 A2B5-positive cells was compared with the insulin content of 100,000 unsorted cells. A2B5-positive cells contained 100 pg of insulin per cell and the initial pancreatic cells (A2B5-positive and -negative) contained 10 pg of insulin per cell.

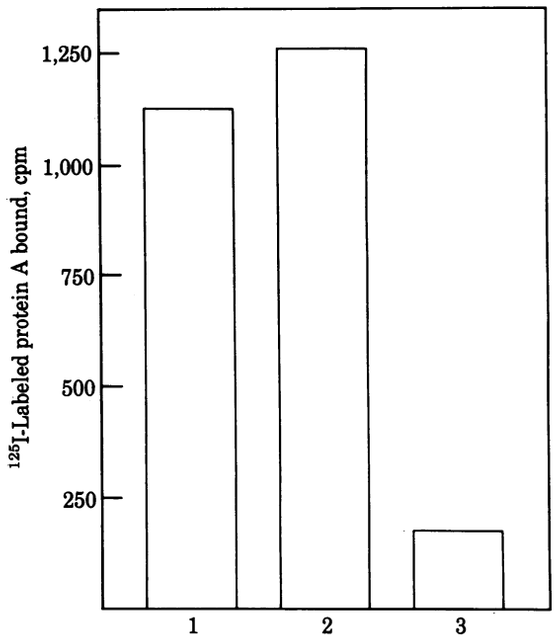

FIG. 4. Absence of tetanus toxin inhibition of antibody A2B5 binding to RINm5F cells. RINm5F cells were incubated first with either tetanus toxin at 0.1 μg/ml in P_i/NaCl/1% albumin or with P_i/NaCl/1% albumin only for 15 min at room temperature and then with 50 μl of a 1:500 dilution of monoclonal antibody A2B5 ascites in P_i/NaCl/1% albumin for 45 min, washed three times with P_i/NaCl/1% albumin, suspended in 250 μl of P_i/NaCl, transferred to microtiter wells, and assayed for ^{125}I-labeled protein A bound. Bars: 1, A2B5 only; 2, tetanus toxin/A2B5; 3, tetanus toxin only. Results represent mean for four determinations.

Previous studies of antigen A2B5 expressed on neuronal cells indicated that the antigen was a glycolipid having the solubility and chromatographic properties of a GQ ganglioside (14). Several studies were carried out to determine whether the RINm5F antigen reacting with antibody A2B5 is similar to the antigen of neurons. Identical to studies with retina neurons, trypsinization (50 μg of protein/ml, 217 units/mg of protein, 15 min, room temperature; Worthington) of RINm5F cells did not decrease A2B5 binding while incubation of these cells with neuraminidase (type IX from *Clostridium perfringens*, 100 μg/ml, 47 units/mg of protein, 37°C; Sigma) blocked subsequent binding of A2B5 as detected by indirect immunofluorescence (not

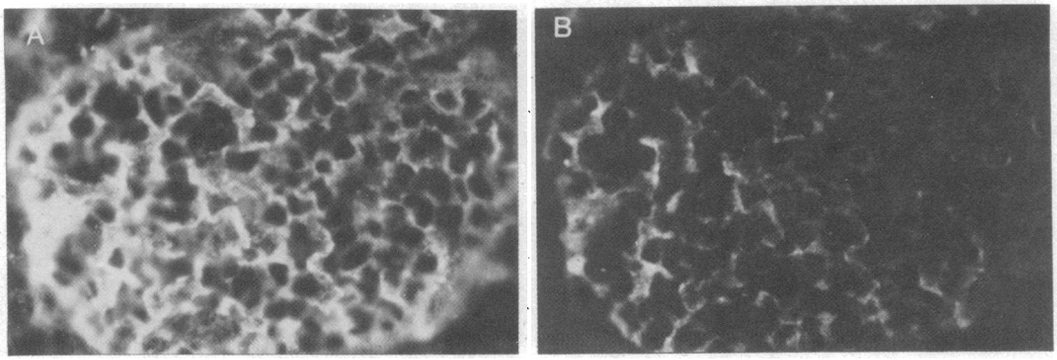

FIG. 3. Tetanus toxin immunofluorescence was determined as follows: A 5-μm section of human pancreas was fixed with acetone, incubated with tetanus toxin at 0.1 μg/ml, washed, incubated with rabbit anti-tetanus toxin antibody A2B5, washed, incubated with rhodamine-conjugated goat anti-rabbit antibody, and washed. Antibody A2B5 immunofluorescence was determined as follows: After reaction with tetanus toxin, antitoxin, and rhodamine-conjugated anti-antitoxin, the section was incubated with fluorescein-conjugated rabbit anti-mouse antibody and viewed with a Leitz Diavert microscope equipped for rhodamine and fluorescein epifluorescence. (*A*) Rhodamine staining of tetanus toxin binding. (*B*) Fluorescein staining of antibody A2B5 binding. (×220.)

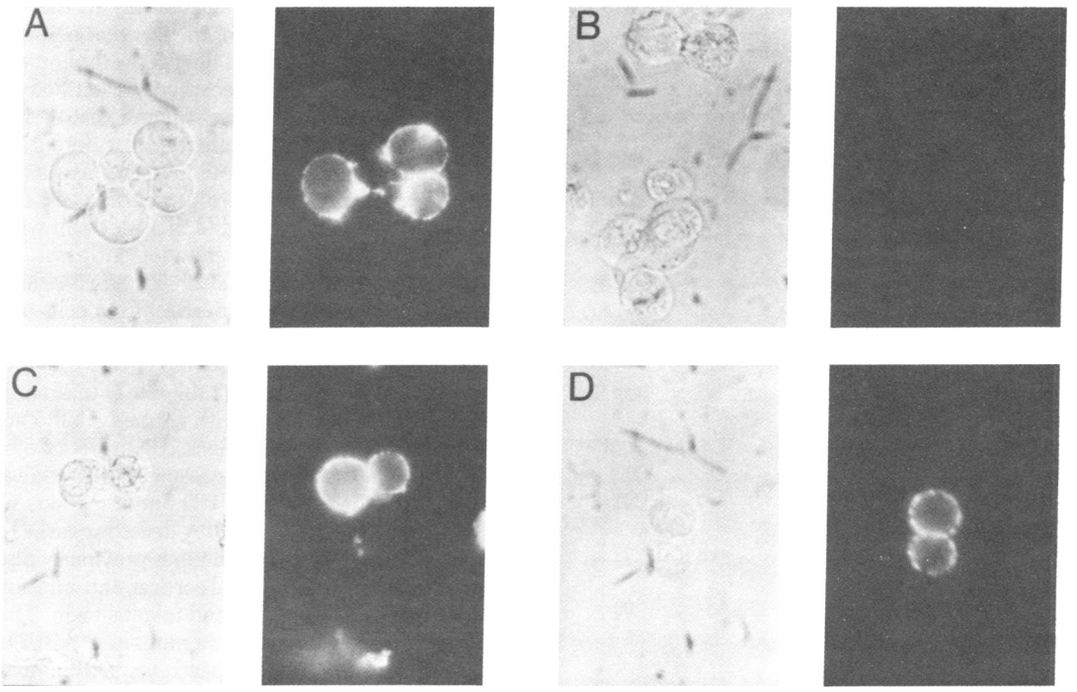

FIG. 5. Distribution of tetanus toxin and antibody A2B5 binding to RINm5F and RINm14B cells. Antibody A2B5 binding was studied as follows: Cells were incubated with monoclonal antibody A2B5 and then with fluorescein-conjugated rabbit anti-mouse antibody and observed by phase-contrast (*Left*) and fluorescence (*Right*) microscopy. Tetanus toxin binding was studied as follows: Cells were incubated with tetanus toxin at 0.1 μg/ml, with rabbit anti-tetanus toxin, and then with rhodamine anti-rabbit antibody; washed; and observed by phase-contrast and fluorescence microscopy. (*A* and *C*) RINm5F cells. (*B* and *D*) RINm14B cells. (×350.)

shown). A ^{51}Cr complement-dependent cytotoxicity assay using antibody A2B5 and RINm5F target cells was developed to further study the antigen. After homogenization of RINm5F cells in water, antigenic activity was found to pellet at 8,000 × *g*. The activity could be extracted from the pellet with chloroform/methanol (2:1) and, after removal of chloroform/methanol, be resuspended in water (Table 1). Again, these properties are identical to those of the antigen on neuronal cells.

Binding of Tetanus Toxin to Islet Cells. We found that tetanus toxin binds specifically to islet cells in sections of human (Fig. 3*A*) and rat pancreas (not shown). Because of the striking similarity of binding of tetanus toxin and antibody A2B5 (Fig. 3*B*), we sought to determine whether tetanus toxin and antibody A2B5 bound to the same molecules. As shown, prior binding of tetanus toxin did not block A2B5 binding, and prior incubation with antibody A2B5 did not block subsequent tetanus toxin binding (not shown). The effect of tetanus toxin on the binding of A2B5 to living cells was further studied by using a ^{125}I-labeled protein A radioassay (Fig. 4). Tetanus toxin did not inhibit A2B5 binding to cultured rat insulinoma cells. Furthermore, we have found that the rat islet cell line RINm5F, which synthesizes insulin, reacts with both antibody A2B5 and tetanus toxin while RINm14B cells, which synthesize somatostatin, react only with tetanus toxin (Fig. 5). Both tetanus toxin and antibody A2B5 also bind in an identical pattern to the plasma membrane of human insulinoma cells (Fig. 6).

The discovery that antibody A2B5 bound specifically to pancreatic islet cells suggested that other such APUD cells and their tumors would express receptors for antibody A2B5. As shown in Table 2, antibody A2B5 reacts with all such human APUD, neural tube, and neural crest tumors that we have studied (insulinoma, pheochromocytoma, medullary carcinoma of the thyroid, melanoma, glioblastoma, and neuroblastoma) while other tumors have been negative.

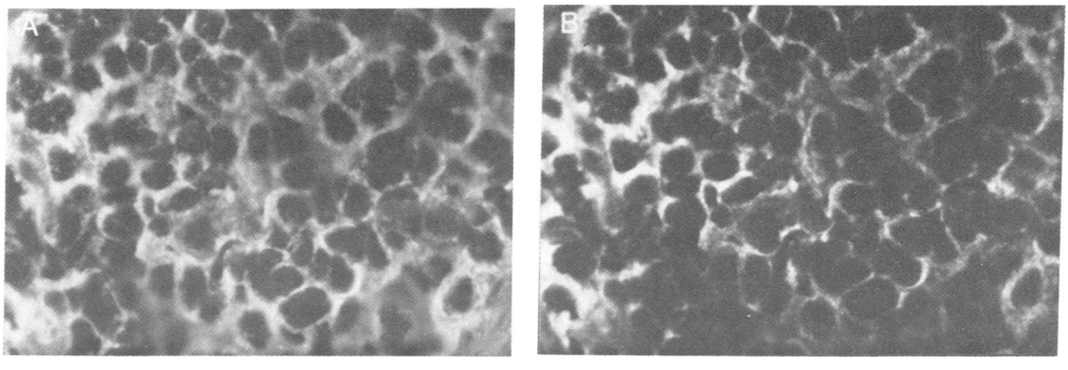

FIG. 6. Distribution of antibody A2B5 and tetanus toxin binding to a sectioned human insulinoma. Five-micrometer sections of human insulinoma were frozen in liquid nitrogen, fixed with acetone, and processed as described in Fig. 3. (*A*) Rhodamine staining of tetanus toxin. (*B*) Fluorescein staining of antibody A2B5. (×220.)

Table 2. Binding of antibody A2B5 to frozen sections, isolated cells, and cell lines of human tumors

	Frozen section	% positive isolated cells
Human tumors		
Benign insulinoma	++	80
Malignant insulinoma	++	NT
Benign pheochromocytoma	++	90
	++	85
		80
Medullary carcinoma	++++	85
(thyroid)	++++	80
	++++	90
Infiltrating ductal breast carcinoma	−	NT
Transitional cell carcinoma (kidney)	−	NT
Colorectal carcinoma	−	NT
Fibrosarcoma	−	NT
Large cell carcinoma, (lung)	−	NT
Rat cell lines		
RINm5F		90
RINm14B		<5
Human cell lines		
Melanomas (H99, H100)		40–50
Glioblastomas (H79, H80)		15–25
Neuroblastomas (H113, H134, H136)		15–20
Fibrosarcoma (H202)		0
Breast carcinoma (H185)		0
Colorectal carcinoma (H592)		0
Prostatic carcinoma (H112)		0
Pancreatic carcinomas (Colo 357, CAPAN1, CAPAN2, HPAF)		0

Binding was studied by indirect immunofluorescence using a 1:100 dilution of monoclonal antibody A2B5 in P_i/NaCl/1% albumin followed by fluorescein-conjugated rabbit anti-mouse antibody. No fluorescence was detected using a P3X63 mouse myeloma control with each cell type. −, Negative; ++++, strongly positive; ++, positive. NT, not tested.

DISCUSSION

Monoclonal antibody A2B5 was initially studied because of its reaction with chicken retina, brain, spinal cord, and dorsal root ganglion neurons and its lack of binding to heart, muscle, liver, kidney, fibroblasts, and erythrocytes (12). Islet cells and neurons share many biochemical and electrophysiological properties. To determine whether islet cells and neurons also expressed similar surface membrane molecules, we have studied the reaction of antibody A2B5 and tetanus toxin with islet cells. Antibody A2B5 reacted with the plasma membrane of viable islet cells. Specific binding to islet cells has been found with all vertebrate species studied, including chicken, human, rat, and mouse. This specificity of binding has allowed us to isolate islet cells from a suspension of pancreatic cells by using a fluorescent cell sorter.

Monoclonal antibody A2B5 reacts with a glycolipid of neurons with the solubility and chromatographic properties of a GQ ganglioside (12). Initial studies of the antigen expressed by RINm5F cells have given results identical to that with retina. Antigen A2B5 on RINm5F cells is resistant to trypsin but sensitive to neuraminidase digestion. The antigen can be pelleted from a water homogenate, dissolved in chloroform/methanol, and resuspended in water. Tetanus toxin, whose reaction with neurons has been studied extensively has high affinity for GD_{1b} and GT_1 gangliosides (15). Identical to the binding of antibody A2B5, tetanus toxin binds specifically to islet cells. Previous biochemical studies (12, 15), the fact that neither tetanus toxin nor antibody A2B5 block binding of the other molecule, and the differential expression of receptors for antibody A2B5 and tetanus toxin on RIN subclones, suggest that their respective receptors on the islet cell membrane are different. The expression of receptors for both of these neuronal markers indicates that islet cells and neurons share two similar cell surface differentiation antigens. It is probable that islet cells will express other neuronal cell surface differentiation antigens.

Both tetanus toxin and antibody A2B5 react with islet cell tumors, and other tumors derived from cells of the APUD series, including pheochromocytomas, medullary carcinomas of the thyroid (derived from thyroid C-cells), and melanomas also react with antibody A2B5 (Table 2). The variability in the percentage of cells reacting with antibody A2B5 and the marked difference between subclones RINm14B (<5% positive) and RINm5F (90% positive) is consonant with the variability of tumor expression of other APUD tumor markers such as histaminase, calcitonin, and L-DOPA decarboxylase (14). In addition to these tumors and the cell types previously discussed, rat adrenal medullary (not adrenal cortical) and anterior pituitary cells react with antibody A2B5 and tetanus toxin. Thus, despite the different embryonic origin, a number of APUD cells and neurons express on their surface receptors for tetanus toxin and antibody A2B5. It is likely that the expression of cell surface molecules, metabolic pathways (e.g., APUD), and enzymes (neuron-specific enolase) relate to shared functional and physiologic properties rather than to embryonic origin. A number of APUD tumors occur together in inherited syndromes of multiple endocrine neoplasia, including neuromas, pheochromocytomas, medullary carcinoma of the thyroid, islet cell tumors, and pituitary tumors. The localization of receptors for tetanus toxin and antibody A2B5 on the exterior surface of both neurons and APUD cells raises questions as to the role of common surface molecules in the pathogenesis of these syndromes. The physiologic functions of the receptors for antibody A2B5 and tetanus toxin, and for neuronal gangliosides in general, have not been defined. The large amounts of monoclonal antibody A2B5 and tetanus toxin available, as well as the discovery of expression of these molecules by APUD cells, should aid studies of their physiologic role.

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