PANCREATIC ISLET CELL SURFACE GLYCOPROTEINS CONTAINING Galβ1-4GlcNAc-R IDENTIFIED BY A CYTOTOXIC MONOCLONAL AUTOANTIBODY

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The insulin-dependent diabetic syndromes of the BB rat and man have several characteristics in common that implicate autoimmune processes in the etiology of pancreatic β cell destruction. The main arguments in favor of this are early infiltration of the islets by mononuclear cells (1, 2), and the presence of islet cell cytoplasmic (ICA)[1] and surface autoantibodies (ICSA) (3). ICSA are immunocytochemically detectable in the sera of patients with recent onset insulin-dependent diabetes mellitus (IDDM) and also in newly diagnosed diabetic BB rats. In vitro, ICSA can be cytotoxic for β cells (4–6), suggesting that these circulating autoantibodies may be direct effectors of β cell destruction in IDDM. However, what role they actually play in causing islet cell damage is still not completely clear. From this point of view it would be of interest to characterize β cell–specific autoantigens recognized by ICSA. Efforts have been made to isolate autoantigens using patient sera (7, 8) and mAbs against islets (9–11). In this study we describe the successful preparation by hybridoma technology of a complement-mediated cytotoxic monoclonal autoantibody from a diabetic BB rat that reacts with rat insulinoma cells (RINm5F) and primary rat islet cells. The functional properties of this antibody and the antigens to which it binds are defined.

Materials and Methods

Materials. Reagents were obtained from the following sources: trypsin/versene solution from Biofluids Inc. (Rockville, MD); *o*-phenylenediamine dihydrochloride (OPD) from Sigma Chemical Co. (St. Louis, MO); polyethylene glycol 1000 from J. T. Baker Chemical Co. (Phillipsburg, NJ). AntiIg reagents were obtained from CooperBiomedical, Inc., (Malvern, PA) and Kirkegaard & Perry Laboratories (Gaithersburg, MD); rabbit complement (Low-Tox-H) was from Accurate Chemical & Scientific Corp. (Westbury, NY); bovine testes hyaluronidase and *Vibrio cholerae* neuraminidase were from Behring

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[1] *Abbreviations used in this paper:* ICA, islet cell cytoplasmic autoantibodies; ICSA, islet cell surface autoantibodies; IDDM, insulin-dependent diabetes mellitus; OPD, *o*-phenylenediamine dihydrochloride.

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Diagnostics (San Diego, CA); *Clostridium perfringens* neuraminidase (Type VI) from Sigma Chemical Co.; collagenase from Wako Pure Chemical Industries (Osaka, Japan). BB rats were kindly supplied by Professor A. A. Like (University of Massachusetts Medical School, Department of Pathology, Worcester, MA). The HL-60 human promyelocytic leukemia cell line, rat fibroblast cell line, rat testicular Leydig cell line (LC540), rat pituitary tumor cell line (GH3), and rat basophilic leukemic cell line (2H3) were obtained from the American Type Culture Collection (Rockville, MD). The rat insulinoma cell line (RINm5F) was established by Gazdar et al. (12).

Carbohydrates were obtained as follows: α-lactose, lactulose, D-galactose, α-D-glucose, α-methyl-D-galactoside, β-methyl-D-galactoside, *p*-nitrophenyl-α-D-galactoside, *p*-nitrophenyl-β-D-galactoside, lactobionic acid were purchased from Sigma Chemical Co.; *N*-acetyl-lactosamine was from E-Y Laboratories, Inc. (San Mateo, CA); and ganglioside GM_1 from Supelco, Inc. (Bellefonte, PA). Insoluble immunoadsorbents (Synsorbs), coupled to monosaccharides and disaccharides, were a gift from D. Baker, Chembiomed Ltd. (Edmonton, Alberta, Canada). The glycolipids lactosyl ceramide, ceramide trihexoside, globoside, paragloboside, and (α2-3)sialosylparagloboside were isolated and purified from human red blood cells as previously described (13–15). The ganglioside (α2-6)-sialosylparagloboside was isolated from human meconium by the procedure described by Nilsson et al. (16). Asialo GM_1 was prepared from GM_1 by mild acid hydrolysis. Lacto-*N*-fucopentaosyl III ceramide and the H_1 blood group active glycolipid were gifts from D. Zopf (Bethesda, MD) and S. Hakomori (Seattle, WA), respectively. Glycolipids were also isolated from the HL-60 and RINm5F cell lines by extraction with chloroform/methanol/water 4:8:3 (17).

Isolation of Pancreatic Islets and Preparation of Single Cell Suspensions. Pancreatic islets of Langerhans were isolated by collagenase digestion (18) from 200–250 g male WF rats that were fed *ad libitum*. The islets were dissociated by gentle pipetting in a trypsin/versene solution (19). ~60% of the single cells were β cells by immunofluorescence staining with fluorescein-conjugated antiinsulin antibody.

Production of mAbs. Spleen cells of newly diagnosed BB rats were harvested within 20 d of onset of glycosuria and fused with the mouse myeloma cell line P3x63Ag8.653 by a modification of the procedure described by Köhler and Milstein (20). The fusion was carried out in the presence of 50% PEG 1000 at neutral pH at a ratio of spleen cell/myeloma cell of 4:1. After fusion, cells were resuspended in hypoxanthine/aminopterin/thymidine medium and seeded at 2×10^5 cells per well in 96-well tissue culture plates. When the hybridomas reached confluence, supernatants were tested for Ig production. Hybridomas producing autoantibodies were cloned twice by limiting dilution (21). Resident peritoneal macrophages from BALB/c mice, collected after injecting 5 ml of PBS, were used as feeder cells (5×10^3 cells/well).

Detection of Antibodies. Hybridoma supernatants were screened for rat Ig (IgG and IgM) production by an ELISA as follows: Immulon II plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 0.5 μg/100 μl/well of goat anti-rat IgG (H and L chain–specific) in 0.05 M carbonate/bicarbonate buffer (pH 9.6) overnight at 4°C. The remaining incubations were at room temperature. After five washes with PBS containing 0.05% Tween 20 (PBS/Tween), the wells were incubated for 1 h with 100 μl of hybridoma supernatants, washed five times with PBS/Tween, and then incubated with 100 μl of a 1:400 dilution of peroxidase-conjugated goat anti-rat IgG and anti-rat IgM for 1 h. After five washes with PBS/Tween, 100 μl of OPD solution was added to each well (22). The OD was read with a microtiter reader (Flow Laboratories, Inc., McLean, VA) after 30 minute incubation.

^{51}Cr-release Cytotoxicity Assay. The ^{51}Cr-release cytotoxicity assay was performed as follows (23). All incubations were conducted at 37°C. Cells at ~90% confluence in 96-well tissue culture plates were incubated with 100 μl of culture medium containing 1 μCi of ^{51}Cr–sodium chromate per well for 5 h. The cultures were gently washed and incubated with 100 μl of hybridoma supernatants for 1 h. The supernatants were removed and the cultures were washed and incubated for 45 min with 100 μl of a 1:5 dilution of rabbit complement in HBSS or buffer control. Aliquots (80 μl) from each reaction mixture were

removed and counted in a model 9000 gamma counter (Beckman Instruments, Inc., Fullerton, CA). A rabbit hyperimmune serum directed against rat islets served as a positive control. For cells in suspension, $5–10 \times 10^5$ cells/100 μl were incubated at 37°C for 5 h with 1 μCi of ^{51}Cr–sodium chromate in a microtube. For isolated rat islet cells, 2×10^4 cells were first cultured for 24 h and then incubated at 37°C for 3 h with 10 μCi of ^{51}Cr–sodium chromate (24). In some experiments, cells were treated with neuraminidase after ^{51}Cr labeling. For neuraminidase-treated cells, rabbit complement was preabsorbed with neuraminidase-treated RINm5F cells (5×10^7 cells/vial) for 2 h in ice and then was used at a 1:5 dilution. Results given are the average of duplicate determinations and are representative of two or more experiments. The percent ^{51}Cr release was determined by the following equation: % ^{51}Cr release = 100 × [(^{51}Cr release in presence of supernatant + complement) − (^{51}Cr release with complement alone)]/[(total ^{51}Cr release by Triton X-100) − (^{51}Cr release with complement alone)]. Release in the absence of antibody was <10% of total ^{51}Cr release.

Enzyme Treatment of Cells. To disperse cells from tissue culture flasks, the cultures were gently washed twice with PBS and incubated in PBS without Ca^{2+} and Mg^{2+}. After 30 min, cells were easily detached from tissue-culture flasks. Cells were washed twice with PBS, resuspended to $2–5 \times 10^7$/ml in the appropriate buffer with or without enzyme (*V. cholerae* neuraminidase, 5–200 μU/ml, in HBSS or trypsin, 0.1%–0.5%, or hyaluronidase, 0.2%, both in PBS) and incubated for 1 h at 37°C.

Indirect Immunofluorescence. Cells dispersed from tissue culture flasks were resuspended to $2–5 \times 10^6$/tube, and incubated with 100 μl of hybridoma culture supernatants. After washing, these cells were incubated with 5 μl of fluorescein-conjugated goat anti-rat IgM (μ chain–specific) for 1 h. All incubations were at room temperature.

Fresh-frozen sections of BB rat and blood group O human pancreatic tissues were also used for indirect immunofluorescence. Sialic acid was removed by incubations with 5 μU/ml *V. cholerae* neuraminidase in HBSS for 1 h at 37°C. After extensive washing in PBS, the sections were incubated with hybridoma culture supernatants for 1 h at 37°C, washed, and then incubated with an appropriate dilution of fluorescein-conjugated $F(ab')_2$ fragments of goat anti-rat IgM. Culture supernatant of the fusion partner and rat gammaglobulin (20 μg/ml) were used as controls.

Iodination. 100 μg of antibody in 100 μl PBS and 0.3 mCi Na ^{125}I were incubated for 5 min at room temperature in tubes coated with 20 μg of Iodogen (25). The mixture was applied to a PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.1% gelatin in PBS. The void volume was dialyzed against PBS.

Immunostaining of Glycolipids. The method was previously described (13, 26). All incubations were at room temperature. Briefly, glycolipids were chromatographed on high-performance thin-layer chromatography plates in chloroform/methanol/0.25% aqueous KCl, 5:4:1. After drying, the plates were soaked in 0.1% poly (isobutylmethacrylate) in hexane for 90 s. After drying, the plates were sprayed briefly with PBS and then incubated in Tris/BSA (0.05 M Tris, 0.15 M NaCl, pH 7.8, with 1% BSA, and 0.1% sodium azide) for 45 min to decrease nonspecific binding of antibody. The plates were overlaid with 60 μl/cm^2 of a 1:5 dilution in Tris/BSA of hybridoma culture supernatant for 1 h. After washing five times in PBS, the plates were overlaid with a dilution of ^{125}I-labeled goat anti-rat IgM in Tris/BSA (2×10^6 cpm/ml). After a 1-h incubation, the plates were washed in PBS, dried under a heat lamp, and exposed to x-ray film overnight.

In some experiments, the chromatography plates were treated with neuraminidase as follows. After the 45-min incubation in Tris/BSA to block nonspecific binding, the plates were washed three times in 0.05 M sodium acetate, 0.15 M NaCl, 0.009 M $CaCl_2$, pH 5.5. The plates were then overlaid with 0.2 U/ml of *C. perfringens* neuraminidase in this buffer or with buffer alone. After an overnight incubation at room temperature, the plates were washed five times in PBS and the assay was completed as above.

Radioimmunoassays. The method was similar to those previously described (13, 27). All assays were performed in triplicate at room temperature in 96-well flat-bottomed polyvinylchloride microtiter plates. We added 20 μl of a solution of glycolipid in methanol to each well. This was allowed to dry by evaporation. The wells were filled with Tris/BSA

and incubated for 45 min to decrease nonspecific antibody binding. The wells were emptied and 50 μl of a 1:5 dilution of hybridoma culture supernatant in Tris/BSA was added. After a 1 h incubation, the wells were washed three times with Tris/BSA, and 50 μl (100,000 cpm) of ^{125}I-labeled anti-rat IgM diluted in Tris/BSA was added to each well. After a 1-h incubation, the wells were washed five times with PBS, cut out, and counted in a model 1272 gamma counter (LKB Instruments, Inc., Gaithersburg, MD). The coefficient of variation for these assays is <10%.

Hapten Inhibition Studies. Several carbohydrates were tested for their ability to inhibit binding of mAb E5C2 to its target antigen. Each microtiter plate well was coated with crude glycolipids isolated from 2×10^6 HL-60 cells. A 1:10 dilution of antibody E5C2 in Tris/BSA was incubated at room temperature for 1 h with varying concentrations of these carbohydrates. The absorbed antibody solution was then tested by the RIA described above. Typically, when no inhibiting carbohydrate was used, ~3,000 cpm of ^{125}I bound to the well.

Adsorption studies with the insoluble immunoadsorbents were performed as described previously (28). A 1:16 dilution of E5C2 antibody in Tris/BSA was incubated with 50 mg/ml of Synsorbs. The mixture was gently rotated for one hour at room temperature, centrifuged briefly to sediment the Synsorbs, and the supernatant was tested by RIA exactly as above.

Western Blot Analysis. Cell cultures were washed twice with PBS and the cells were scraped from the flasks. The flasks were washed twice with PBS containing 1% NP-40 and 2 mM PMSF. The cell suspension was kept on ice for 30 min and then homogenized with a 10 Broeck tissue grinder. Aliquots of homogenate were either used immediately or stored at −20°C. Before use, the samples were centrifuged for 1 min in an Eppendorf microtube, and the protein content of the supernatant was determined (29). Aliquots of the supernatant were mixed with an equal volume of application buffer to yield final concentrations of 10% glycerol, 5% mercaptoethanol, 2.3% SDS, and 0.0625 M Tris (pH 6.8). The mixture was heated in a boiling water bath for 2 min. SDS-PAGE was performed using an 11% gel under reducing conditions (30). Aliquots of supernatant in application buffer were applied to each lane. Prestained molecular weight standards, 14,300 to 200,000 mol wt (Bethesda Research Laboratories, Bethesda, MD), were run in each gel. After electrophoresis, the gels were blotted onto nitrocellulose paper at 10°C for 90 min at 90 V (31) using a Biorad Transblot System (Bio-Rad Laboratories, Richmond, CA). The blots were cut into strips; some were stained with amido-black, while others were incubated overnight at room temperature in PBS containing 0.5% gelatin and 0.02% NaN_3 to block residual protein-binding sites. The blocked strips were incubated overnight in 0.05 M sodium acetate, 0.15 M NaCl, 0.009 M $CaCl_2$, pH 5.5, with or without 0.1 U/ml *V. cholerae* neuraminidase. They were then washed five times with PBS containing 0.1% gelatin, 0.05% Tween 20 and 0.02% NaN_3 (washing buffer). The blots were immunostained with culture supernatant containing mAb E5C2; the culture supernatant of the fusion partner was used as a control. Tris, Tween 20, and NaN_3 were added to those supernatants to yield final concentrations of 50 mM, 0.05%, and 0.02%, respectively, at pH 7.4. After overnight incubation at room temperature, the blots were incubated for 2 h at room temperature with 300,000 cpm/ml of ^{125}I-labeled goat anti-rat IgM in washing buffer. After five washes, the blots were exposed to x-ray film with an enhancing screen overnight at −70°C.

Results

Production of mAb E5C2. Spleen cells from newly diagnosed BB rats (killed within 20 d after onset of glycosuria) were fused with the myeloma cell line P3x63Ag8.653. Initial screening of culture supernatants from the resulting hybridomas was performed by solid-phase ELISA. ~90% of the 70,000 wells tested yielded hybridomas of which 10% produced rat IgG or IgM. Supernatants from >6,000 wells containing rat IgG or IgM were tested for cytotoxicity against rat insulinoma cells (RINm5F) using the ^{51}Cr-release cytotoxicity assay. 17 wells

TABLE I

^{51}Cr Release from Labeled Rat Cells by mAbs

Cell lines*	Percent ^{51}Cr release by mAbs:									
	E5C2	C3B2	D7G7	D9C4	G4D2	D2B3	D10D9	G4E10	C3F5	653‡
RINm5F	63	76	45	80	51	90	37	43	70	0
LC540	5	87	100	85	100	100	46	24	100	0
GH3	6	4	23	9	12	8	11	40	9	7
Fibroblast	0	53	51	33	23	32	94	58	74	0
2H3	0	ND	ND	ND	ND	ND	ND	ND	ND	0
Spleen cells	2	ND	ND	ND	ND	ND	ND	ND	ND	0

* RINm5F, rat insulinoma cells; LC540, rat Leydig cell tumor cells; GH3, rat pituitary tumor cells; 2H3, rat basophilic leukemic cells.

‡ 653 is the control supernatant from the P3 × 63 Ag8.653 fusion partner.

TABLE II

Binding of E5C2 to Viable RINm5F and Viable Primary Rat Islet Cells: Effect of Neuraminidase

Treatment of cells with neuraminidase	Antibody	Inhibitor	Percent positive cells by indirect immunofluorescence	
			RINm5F	Primary islet cells
−	E5C2	None	5	0
+	E5C2	None	75	45
+	E5C2	Lactose (200 mM)	0	0

were positive and 9 clones were established from these after two cloning steps (Table I). The antibody produced by one clone, E5C2, was only cytotoxic for RINm5F cells and not for four other rat cell lines (i.e., testicular Leydig cell line [LC540], pituitary tumor cell line [GH3], basophilic leukemia cell line [2H3], fibroblasts) or for normal spleen cells. The other eight clones were not specifically cytotoxic for RINm5F cells (Table I). The isotype of E5C2 was identified as IgMκ by solid-phase ELISA. E5C2 was used in all subsequent experiments.

Enzyme Treatment of RINm5F Cells, Primary Islet Cells, and Normal Pancreas

To investigate the nature of the antigen recognized by E5C2, viable RINm5F cells were treated with a variety of enzymes. Whereas only 5% of untreated RINm5F cells showed a strong pattern of surface immunofluorescence after exposure to E5C2, 75% of the neuraminidase treated cells (5 μU/ml) showed strong surface immunofluorescence after exposure to E5C2 (Table II). Hyaluronidase and trypsin did not increase antibody binding nor did fixation with paraformaldehyde (data not shown). This suggests that most of the antigen recognized by E5C2 is hidden on RINm5F cells by sialic acid residues. Viable primary rat pancreatic islet cells were also tested by indirect immunofluorescence with E5C2. No staining was evident without neuraminidase treatment (Fig. 1, *A* and *B*). After neuraminidase treatment, however, 45% of cells were stained by E5C2 (Table II). None of the insulin-negative cells were stained by E5C2 (data not shown), whereas 75% of insulin-positive cells were stained (Fig. 1, *B* and *C*).

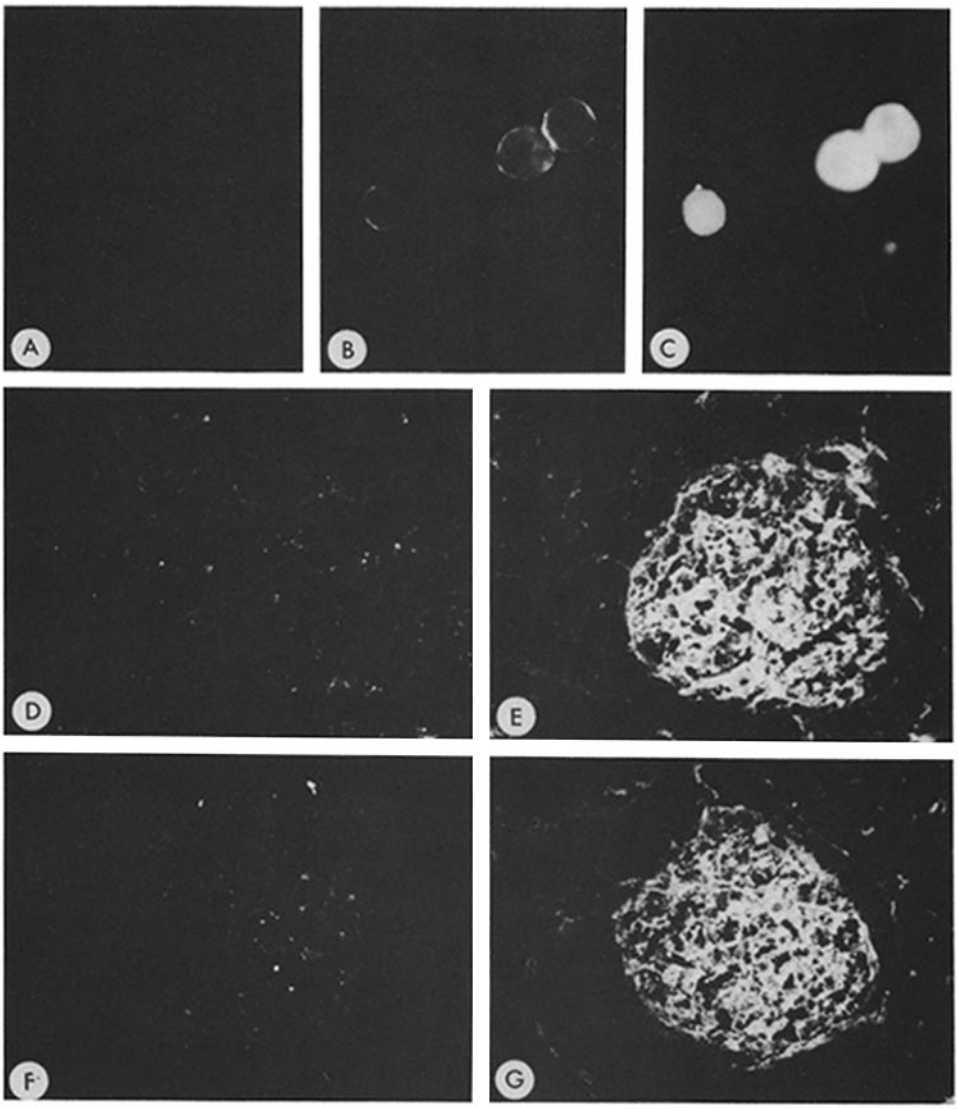

FIGURE 1. Binding of antibody E5C2 to rat and human islet cells by indirect immunofluorescence. Viable islet cells and frozen sections of pancreas were prepared and stained with E5C2 as described in Materials and Methods. (*A*) Viable primary rat islet cells, untreated, stained with E5C2 (× 254); (*B*) viable primary rat islet cells treated with neuraminidase and stained with E5C2 (× 254); (*C*) cells in *B* fixed in 1% paraformaldehyde and then stained with antiinsulin antibody (× 254). The same cells were examined in *B* and *C*. (*D*) Section of rat pancreas, untreated, stained with E5C2 (× 134); (*E*) section of rat pancreas, neuraminidase-treated, stained with E5C2 (× 134); (*F*) section of human pancreas, untreated, stained with E5C2 (× 134); (*G*) section of human pancreas, neuraminidase-treated, stained with E5C2 (× 134).

E5C2 did not stain frozen sections of untreated pancreatic tissue (Fig. 1, *D* and *F*), but stained both rat and human pancreatic islets after neuraminidase treatment (Fig. 1, *E* and *G*). Similar results were obtained with frozen sections of monkey pancreas (not shown). This strongly suggests that the E5C2 antigen is

TABLE III
RINm5F and Primary Rat Islet Cells Treated with Neuraminidase and Exposed to E5C2 and Complement

Treatment of cells with neuraminidase	Percent ^{51}Cr release	
	RINm5F	Primary islet cells
−	57	0
+	73	40

present in normal pancreatic islets, but is hidden by sialic acid residues. Rat kidney glomeruli were also stained by E5C2 after neuraminidase treatment, but other rat tissues (e.g., endocrine, nerve, liver, and muscle) were not stained with or without neuraminidase treatment.

Since neuraminidase treatment exposes the E5C2 antigen not only on RINm5F cells, but also on primary islet cells, the effect of neuraminidase treatment on E5C2-induced cytotoxicity was investigated. RINm5F cells and primary rat islet cells were labeled with ^{51}Cr, treated with neuraminidase, and subjected to the complement-mediated cytotoxicity assay described above. E5C2 was cytotoxic for RINm5F cells before and after neuraminidase treatment (Table III). In contrast, E5C2 was not cytotoxic for primary rat islet cells before neuraminidase treatment, but was cytotoxic after neuraminidase treatment. LC540, GH3, and rat fibroblasts were not rendered susceptible to E5C2-mediated cytotoxicity by neuraminidase treatment (data not shown).

Characterization of the Antigen Recognized by E5C2. Since neuraminidase treatment increased binding of E5C2 to both RINm5F cells and primary islet cells by indirect immunofluorescence, this suggested that antibody binding was blocked by the presence of sialic acid residues. This could be due to sialic acid covalently bound to a carbohydrate antigen or to the presence of sialic acid on neighboring nonantigenic molecules. The former possibility was tested using glycolipids isolated from the RINm5F cell line. Total glycolipids from RINm5F cells, HL-60 cells, and a ganglioside-enriched fraction from human RBCs were separated by thin-layer chromatography. The chromatograms were exposed to neuraminidase or to buffer and then immunostained with E5C2-containing culture supernatant (Fig. 2). No binding of E5C2 to untreated RINm5F glycolipids was found (Fig. 2 *A*). The faint staining near the top of lane *A* is artifactual. However, a band was identified at the origin after neuraminidase treatment (Fig. 2 *B*, *arrowhead*). Since a crude lipid extract was used, this band could represent a glycoprotein antigen(s) that is relatively soluble in organic solvents. It could also represent a glycolipid(s) with a large oligosaccharide moiety (15–20 sugars). In contrast, two distinct doublets were found with untreated HL-60 cells (Fig. 2*C*), and several additional bands were revealed by neuraminidase treatment (Fig. 2*D*). Glycolipids with identical sugar sequences often occur in doublets due to differences in the ceramide moiety. Similar findings were obtained with the human RBC extract (Fig. 2, *E* and *F*). Further experiments showed that the binding of E5C2 was directly proportional to the antibody concentration (Fig. 3). To confirm that these findings were due to antibody binding, purified E5C2 (10 μg/ml) was used. Identical results to those seen in Fig. 2 were obtained. In

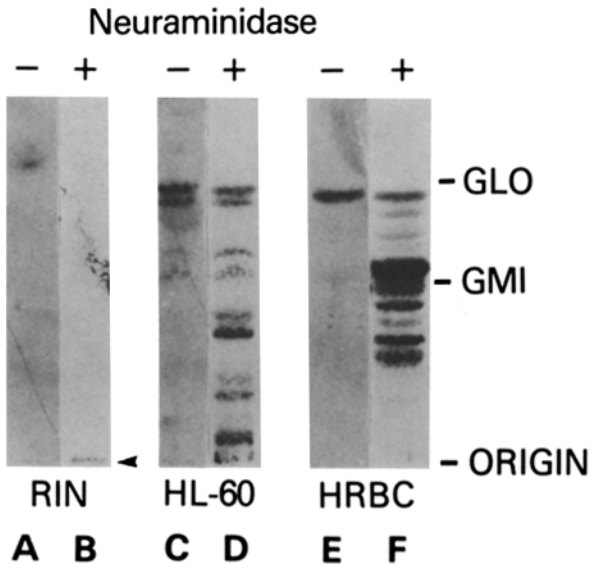

FIGURE 2. Binding of antibody E5C2 to untreated and neuraminidase-treated glycolipids separated by thin-layer chromatography. Glycolipid isolation, thin-layer chromatography, neuraminidase treatment, and immunostaining were performed as described in Materials and Methods. After thin-layer chromatography, the glycolipids were either not treated (*A*, *C*, and *E*) or treated with neuraminidase (*B*, *D*, and *F*) before immunostaining with E5C2. (*A* and *B*) Glycolipids isolated from 4×10^6 RINm5F cells; (*C* and *D*) glycolipids isolated from 2×10^6 HL-60 cells; (*E* and *F*) ganglioside-enriched preparation from human RBCs. Globoside (*GLO*) and ganglioside GM_1 (*GM1*) were used as chromatographic standards.

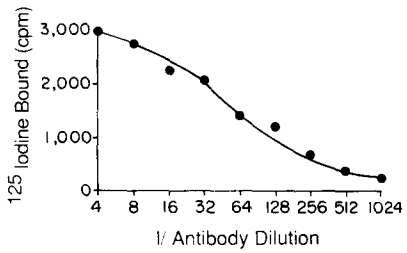

FIGURE 3. Binding of various dilutions of antibody E5C2 to HL-60 glycolipids. Glycolipids were extracted from HL-60 cells and the RIA was performed as described in Materials and Methods. Each well of the microtiter plate was coated with glycolipids isolated from 10,000 HL-60 cells.

contrast, culture supernatant from the myeloma fusion partner (No. 653) failed to bind. Taken together, these experiments argue that E5C2 recognizes a carbohydrate antigen on neutral glycolipids and that this antigen is hidden by covalently bound sialic acid residues.

The pattern of antibody binding in Fig. 2 was similar to that previously seen with another mAb, anti-My-28 (13). This suggested that E5C2 might recognize a sequence in the glycolipid paragloboside. Paragloboside was purified and tested by the immunostaining method (Fig. 4). By this technique, E5C2 could detect 5 ng of pure glycolipid (Fig. 4*B*). RIA was even more sensitive and E5C2 could detect as little as 1 ng of pure paragloboside (Fig. 5). Several other pure

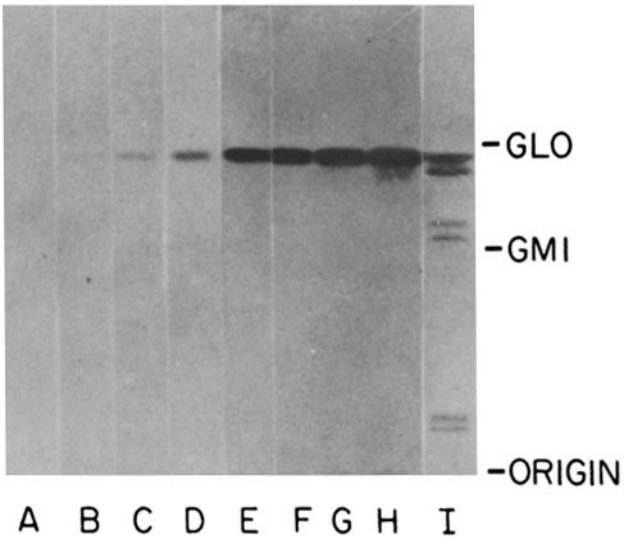

FIGURE 4. Binding of antibody E5C2 to glycolipids separated by thin-layer chromatography. Immunostaining of the thin-layer chromatogram was performed as described in Materials and Methods. (*A–H*) Pure paragloboside (*A*) 1 ng; (*B*) 5 ng; (*C*) 10 ng; (*D*) 20 ng: (*E*) 60 ng; (*F*) 120 ng; (*G*) 240 ng; (*H*) 600 ng; (*I*) glycolipids isolated from 2×10^6 HL-60 cells. The chromatographic standards were the same as those used in Fig. 2. (*GLO*) globoside; (*GM1*) ganglioside GM_1.

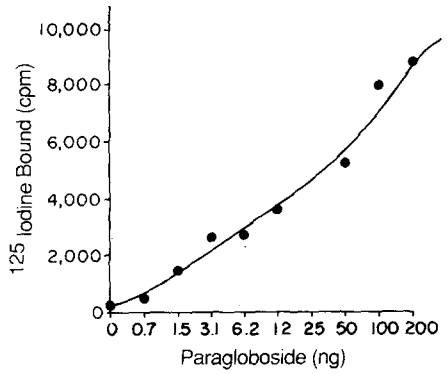

FIGURE 5. Binding of antibody E5C2 to purified paragloboside. The RIA was performed as described in Materials and Methods.

glycolipids were also tested by RIA including lactosyl ceramide, ceramide trihexoside, lacto-*N*-fucopentaosyl III ceramide, H_1 blood group active glycolipid, asialo GM_1, and (α2-3) and (α2-6) sialosylparagloboside. E5C2 did not bind to 200 ng of any of these glycolipids (Table IV). Interestingly, although E5C2 did not bind to (α2-3) or (α2-6) sialosylparagloboside, the E5C2 antigen was revealed after removal of the sialic acid from these glycolipids by neuraminidase (Table IV).

The specificity of antibody E5C2 for the sugar sequence found in paragloboside was further studied by hapten inhibition and adsorption studies. The best inhibitor tested was *N*-acetyllactosamine (Galβ1-4GlcNAc), which is the nonre-

TABLE IV

RIA of Binding of Antibody E5C2 to Untreated and Neuraminidase-treated Pure Glycolipids

Glycolipid	Structure	E5C2 binding	
		Untreated*	Neuraminidase treated
Lactosyl ceramide	Galβ1-4Glcβ1-1'cer	–	ND
Ceramide trihexoside	Galα1-4Galβ1-4Glcβ1-1'cer	–	ND
Paragloboside	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'cer	+	+
Lacto-*N*-fucopentaosyl III ceramide	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'cer Fucα1-3	–	ND
H_1	Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'cer	–	ND
Asialo GM_1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'cer	–	ND
(α2-3)sialosylparagloboside	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'cer	–	+
(α2-6)sialosylparagloboside	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'cer	–	+

* At least 200 ng of each glycolipid were tested

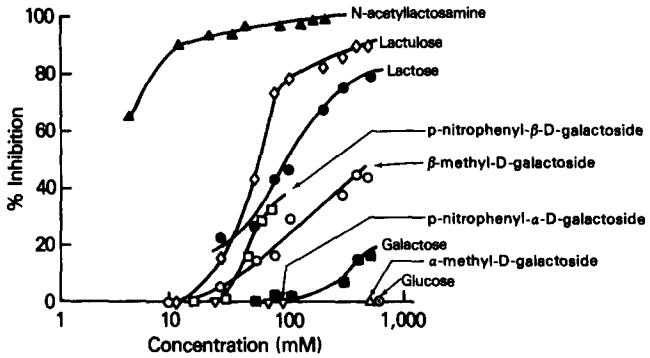

FIGURE 6. Inhibition of E5C2 binding to glycolipids by defined carbohydrates. The antibody was absorbed with various defined sugars and the RIAs were performed as described in Materials and Methods. Microtiter wells were coated with glycolipids isolated from 2×10^6 HL-60 cells. When no inhibiting sugar was present, ~3,000 cpm of ^{125}iodine-labeled second antibody bound to the well. The results with lactobionic acid are not shown, but it gave 50% inhibition at a concentration of ~200 mM.

ducing terminal disaccharide of paragloboside (Fig. 6). Lactose and lactulose were reasonable inhibitors but were ~20–30 times less effective. At the concentrations tested, the only carbohydrates that demonstrated any inhibition either had a terminal galactose in the β position or could assume a β configuration (free galactose). The fine specificity was also examined by adsorption studies with Synsorbs (Table V). These studies indicate that galactose-linked β1-4 to *N*-acetylglucosamine (as occurs in paragloboside) is an excellent inhibitor, while little or no inhibition is seen when the linkage is β1-3.

Western Blot Analysis. Extracts of RINm5F cells were subjected to SDS-PAGE and Western blotting. The results of immunostaining such blots are seen in Fig. 7. E5C2 specifically stained two bands, but only in neuraminidase-treated blots of the RINm5F cell extract (lane *A*). These bands had molecular weights of 60,000 and 68,000. Untreated blots of the RINm5F cell extract were not stained by E5C2 (lane *E*). Treated or untreated blots of GH3 cell extract were not stained (data not shown). The supernatant of the myeloma fusion partner (No. 653) also did not specifically stain any bands (lanes *D* and *F*). Lactose (200 mM) blocked E5C2 binding (lane *B*) while glucose at the same concentration did not

TABLE V

Galβ1-4GlcNAc, but not Galβ1-3GlcNAc, Inhibits E5C2 Binding: Immunoadsorption Studies with Solid-Phase Synthetic Carbohydrate Haptens

Hapten	^{125}I-Bound (cpm ± SD)	Percent inhibition
None	4107 ± 225	—
Glc	3961 ± 180	4
Galβ1-4GlcNAc	1182 ± 300	71
Galβ1-3GlcNAc	3958 ± 218	4

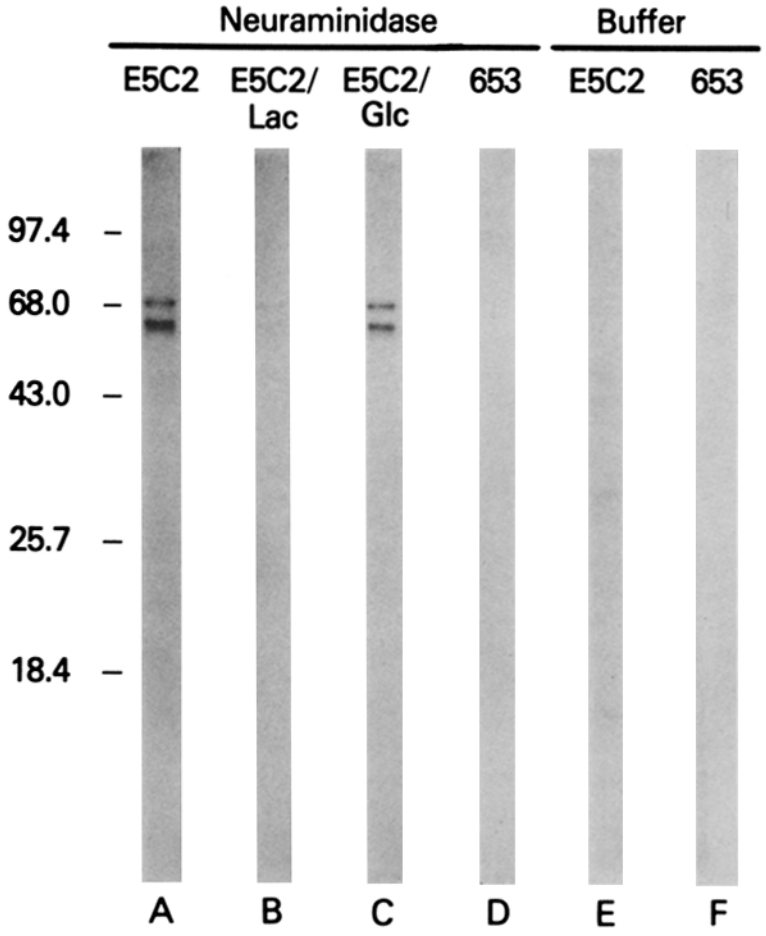

FIGURE 7. Western blot analysis of binding of antibody E5C2 to RINm5F cell extract. Immunoblots of RINm5F cell extract were prepared as described in Materials and Methods. Each lane contained 100 μg of protein. Blots were exposed to neuraminidase (*A–D*) or buffer (*E* and *F*) before incubation with antibody. Antibody E5C2 was preincubated with 200 mM lactose (*B*) or glucose (*C*). 653 is the supernatant of the myeloma fusion partner. Mol wt × 10^{-3} shown at left.

(Fig. 7 lane *C*). When intact viable RINm5F cells were treated with neuraminidase before extraction, blots of these extracts immunostained with E5C2 displayed a pattern similar to that seen with blots of RINm5F cell extract treated with neuraminidase after blotting (data not shown). The bands were of slightly lower molecular weight (~1,000) presumably due to removal of sialic acid residues before SDS-PAGE.

Inhibition of E5C2 Binding to Viable Cells by Lactose. Since lactose inhibited E5C2 binding to glycolipid antigens (Fig. 6) and to RINm5F cell glycoproteins (Fig. 7), its effect on antibody binding to intact cells was examined. By indirect immunofluorescence, 200 mM lactose blocked binding of E5C2 to neuraminidase-treated RINm5F cells and neuraminidase-treated primary islet cells (Table II). Lactose also blocked E5C2 binding to untreated RINm5F cells (data not shown). These results strongly indicate that lactose inhibits E5C2 binding to the intact cellular antigenic determinants. Taken together, these studies show that the E5C2 antigen is a carbohydrate moiety on cell surface glycoproteins.

Discussion

E5C2 is a rat IgMκ monoclonal anti–islet cell autoantibody that is specifically cytotoxic for RINm5F cells and for neuraminidase-treated isolated primary islet cells. By indirect immunofluorescence, neuraminidase increased E5C2 binding not only to RINm5F cells but also to primary islet cells. Since neuraminidase removes sialic acid residues from glycoconjugates, our studies indicate that the E5C2 antigenic determinants are partially exposed in RINm5F cells and are hidden by sialic acid residues in normal islet cells. This suggests that E5C2 may recognize a glycoconjugate antigen. In fact, E5C2 binds to glycolipids separated on thin-layer chromatography both before and after treatment with neuraminidase (Fig. 2). We elucidated the structure of the antigenic determinants as follows. E5C2 directly binds only to paragloboside (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-ceramide) and higher polylactosamine-containing structures, but not to related glycolipids (Table IV). Both lactose (Galβ1-4Glc) and *N*-acetyllactosamine (Galβ1-4GlcNAc) inhibit E5C2 binding to its antigen, although lactose is 20–30 times less effective. In addition, Galβ1-3GlcNAc is ineffective as an inhibitor (Table V); E5C2 did not bind to asialo GM_1 (Table IV), or to any carbohydrates that had a terminal galactose with an α linkage (Fig. 6). These results indicate that a β1-4-linked terminal galactose is a critical part of the antigenic determinant.

Our experiments argue that E5C2 antigen in RINm5F cells is not linked to a glycolipid (Fig. 2) but, as shown by Western blots, to a glycoprotein (Fig. 7). These antigens were revealed by treating blots with neuraminidase. E5C2 binding to the neuraminidase-treated glycoproteins was blocked by lactose but not by glucose, indicating that the carbohydrate determinant on the glycoprotein is similar to that found on the glycolipids. Based on these results, the probable carbohydrate structure on these glycoproteins is NeuAcα2-3/6Galβ1-4GlcNAc-R. After neuraminidase removes the sialic acid (NeuAc), E5C2 binds to the underlying structure. Since lactose inhibited binding of E5C2 to RINm5F cells and normal rat islets, as determined by immunofluorescence, this suggests that the antibody recognizes this carbohydrate antigen on intact cells. Thus, antibody E5C2 recognizes a carbohydrate moiety on cell surface glycoconjugates. With

RINm5F cells these antigenic determinants were found on glycoproteins with molecular weights of 60,000 and 68,000 but not on glycolipids. Baekkeskov et al. (7, 8) reported that sera from newly diagnosed IDDM patients and BB rats have autoantibodies to a 64,000 mol wt islet protein. It is not known whether these serum antibodies bind to the same proteins recognized by E5C2 or what roles the E5C2 glycoproteins play in the pathogenesis of IDDM. This is now under investigation.

Antibodies recognizing lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc) or its glycosphingolipid analogue, paragloboside, have been described previously (13, 32, 33). Recently, antilipid antibodies were measured in normal and pathologic human sera by means of a microplate method of complement-mediated immune lysis of fluorescent dye–trapped liposomes (34). No antibodies were observed against paragloboside in 11 healthy adult sera. High titers of these antibodies were found in some patients with idiopathic thrombocytopenic purpura, SLE, liver diseases, lymphoma, and leukemia. Cell surface sialic acids can occupy terminal positions on carbohydrate chains and neuraminidase may remove sialic acids, revealing carbohydrate structures not normally seen by the immune system of normal individuals. Neuraminidase is known to be present in many microorganisms and in mammalian cell lysosomes (35). It is possible that pathological conditions caused by certain bacterial and viral infections or by chemical inflammatory agents may remove sialic acid residues, exposing normally hidden autoantigens. Similarly, defective protein glycosylation, which may be genetically linked to the H-2 locus (36), could result in the expression of normally cryptic autoantigens. These observations raise the possibility that exposure of hidden antigenic determinants may trigger and be the target of autoimmune attack.

Summary

To investigate the autoimmune pathogenesis of spontaneously occurring diabetes mellitus in BB rats, spleen cells of newly diagnosed diabetic BB rats were fused with mouse myeloma cells. Hybridoma supernatants were screened for antibodies by indirect immunofluorescence and by ^{51}Cr-release assays using the RINm5F rat insulinoma cell line. One clone, E5C2, produced an IgMκ antibody that was cytotoxic for RINm5F cells, but not for other rat cell lines nor for primary rat islet cells. However, treatment of primary rat islet cells with neuraminidase exposed surface antigens and rendered the cells susceptible to complement-mediated lysis by antibody E5C2. Using immunostaining of glycolipids separated by thin-layer chromatography, hapten inhibition assays with defined carbohydrates, and Western blots, the antigens recognized by E5C2 on RINm5F cells were identified as glycoproteins with molecular weights of 60,000 and 68,000. The antibody recognizes a carbohydrate antigen containing the sequence Galβ1-4GlcNAc-R, which on RINm5F cells is predominantly hidden by covalently bound sialic acid. These studies raise the possibility that hidden antigenic determinants on islet cells exposed by a variety of means may be the target of autoimmune attack.

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