Expression of Galα(1,3)Gal on Neonatal Porcine Islet β-Cells and Susceptibility to Human Antibody/Complement Lysis

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Neonatal porcine pancreases may be a potential source of islets for transplantation into patients with type 1 diabetes; however, whether these cellular grafts will be susceptible to damage by human natural antibody-mediated rejection remains controversial. Although we and others have demonstrated that porcine islets bind human IgG and IgM, it remains unknown if they express the xenoreactive antigen Galα(1,3)Galβ(1,4)GlcNAc-R (Gal epitope). In this study, by using the Gal-specific lectin IB4 for immunohistochemistry and fluorescence-activated cell sorter (FACS) analysis, we determined which cell types present in porcine neonatal islet cell (NIC) aggregates express the Gal epitope and which ones are susceptible to lysis by activation of the human complement. After FACS analysis, 30.0 ± 3.0% of porcine NICs were shown to express Gal, whereas 70.0 ± 2.0% did not. Histological assessment of Gal-expressing cells revealed that 54.9 ± 8.8% stained positive for either insulin or glucagon. In contrast, 68.8 ± 8.4% of the Gal-negative population stained positive for the pancreatic hormones insulin and glucagon. Incubation of either the Gal-positive or -negative cells with human AB serum plus complement for 1.5 h resulted in the lysis of >90% of the cells. These results demonstrate that porcine NIC aggregates are composed of Gal-expressing cells and that expression of Gal is not restricted to nonendocrine cells. Furthermore, both Gal-positive and Gal-negative cells are susceptible to human antibody/complement-mediated cytolysis, suggesting that this form of immunological destruction is an obstacle that will need to be overcome before porcine NIC aggregates can be used clinically. *Diabetes* 47:1406–1411, 1998

Endocrine replacement by islet transplantation is an attractive alternative treatment for patients with type 1 diabetes. Transplantation of pancreatic islets in patients with diabetes has been shown to induce a state of normoglycemia (1–3), and long-term insulin independence can be achieved when a sufficient number of islets are transplanted. Widespread clinical application of this treatment, however, has been hampered by the shortage of cadaveric organs for transplantation. One possible solution to this problem is to use islet tissue from an abundant and accessible animal source. Pigs are a very appealing source of islets because they breed rapidly and share many biological features with humans, including a similarity in insulin structure (4). Despite many reports on the isolation of adult porcine islets, several factors, such as age, breed, and quality of organs, adversely affect the final yield (5,6), with the additional problem that, once isolated, adult porcine islets are fragile and difficult to maintain in tissue culture (7–9). A more attractive source of tissue that does not have the same problems associated with adult pigs is neonatal pigs. Recently we developed a method to isolate large numbers (50,000/pancreas) of porcine neonatal islet cell (NIC) aggregates from pigs ages 1–3 days (10). After 9 days of culture, porcine NIC aggregates were shown to correct diabetes in nude mice and exhibit growth both in vitro and in vivo (10).

Although porcine NIC aggregates constitute an attractive source of insulin-producing tissue for transplantation, the issue of humoral-mediated xenograft rejection (i.e., hyperacute rejection) of such implants needs to be addressed. This rejection process is initiated when naturally occurring xenoreactive antibodies in recipient sera bind to antigens present on the surface of endothelial cells of donor organs. Antibody binding in turn activates complement, which rapidly destroys the transplanted organ or tissue. The most important target for these antibodies has been identified as the carbohydrate Galα(1,3)Galβ(1,4)GlcNAc-R or the Gal epitope (11–13). This epitope is present in high concentrations on all porcine endothelial cells (13–15), and although it has been detected on fetal porcine islet cell clusters (16,17), the expression of Gal on porcine NIC aggregates has not been elucidated. Moreover, current literature suggests that Gal is not expressed on fully differentiated islet endocrine cells, but rather on intra-islet ductal and endothelial cells (16–19). However, it has been demonstrated that natural xenoreactive antibodies bind to fetal (18), neonatal (20), and adult (21,22) porcine islet cells, and exposure to human sera containing active complement in vitro results in rapid destruction of these tissues (20–22). In this study we examined the expression of Gal on cells present in porcine NIC aggregates and related this expression to the susceptibility of these cells to human antibody/complement-mediated lysis in vitro.

RESEARCH DESIGN AND METHODS

Preparation of neonatal porcine islets. The method used to prepare porcine NIC aggregates has been previously described (10). Briefly, Landrace-Yorkshire neonatal pigs ages 1–3 days (1.5–2.0 kg body wt) of either sex were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreases were removed, cut into small pieces, and digested with 2.5 mg/ml collagenase (Sigma, St. Louis, MO). After filtration through a nylon screen (500 µm), the tissue was cultured for 9 days in HAM's F10 medium (Gibco, Burlington, Canada)

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DAB, 3,3-diaminobenzidinetetrahydrochloride; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; NIC, neonatal islet cell; PAEC, porcine aortic endothelial cell.

containing 10 mmol/l glucose, 50 µmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 10 mmol/l nicotinamide (BDH Biochemical, Poole, U.K.), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Immunohistochemistry. The expression of the Gal epitope and the presence of insulin-positive cells within intact neonatal porcine pancreases and NIC aggregates were assessed by double immunohistochemistry staining. All samples were fixed in Bouin's solution for 2 h, washed three times with 70% ethanol, and embedded in paraffin. Next, 5-µm sections were stained with biotinylated BS-1 isolectin B4 from *Bandeiraea simplicifolia* BS-1 (1:25 dilution; Sigma) for 30 min at room temperature to detect the presence of the Gal epitope. The avidin-biotin complex/horseradish peroxidase (Vector Laboratories, Burlingame, CA) method was used and developed with 3,3-diaminobenzidinetetrahydrochloride (DAB) (BioGenex, San Ramon, CA) to produce a brown color. The same tissue sections were subsequently stained with guinea pig anti-porcine insulin antibody (1:1,000 dilution; Dako Laboratories, Mississauga, Canada) for 30 min followed by biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution; Vector Laboratories) for 20 min. The avidin-biotin complex/alkaline phosphatase method (Vector Laboratories) and Fast Red as chromogen (Vector Laboratories) were used to obtain a red reaction. Paraffin-embedded sections of porcine aorta and porcine aortic endothelial cells (PAECs) (Cell Systems, Kirkland, WA) placed on poly-L-lysine–coated slides were used as positive controls, since they are both known to express Gal. As expected, the vascular endothelium and isolated PAECs stained positive for the Gal antigen. Negative controls for the staining techniques consisted of omission of the primary antibodies as well as sections of paraffin-embedded human pancreases. These tests resulted in negative staining reactions.

Characterization of dissociated neonatal porcine islet cells. To further define the cellular specificity of Gal expression, 9-day culture porcine NIC aggregates were dissociated into single cells by gentle agitation in calcium-free media containing 15 µg/ml trypsin (Boehringer Mannheim, Laval, Canada) and 4 µg/ml DNase (Boehringer Mannheim). The cell suspension was filtered through a 63-µm nylon screen to remove cell clumps and then centrifuged through Percoll (Sigma) of 1.040 g/ml density to eliminate dead cells and debris. Single cell ($10–15 \times 10^6$) suspensions were subsequently incubated with fluorescein isothiocyanate (FITC)–conjugated BS-1 isolectin B4 (1:50; Sigma) for 1 h on ice, washed, and submitted to fluorescence-activated cell sorter (FACS) (EPICS Elite ESP flow cytometer; Coulter, Hialeah, FL) analysis for the purification of Gal-positive and -negative islet cells. The fluorescence emitted by FITC-labeled cells was selected as the sorting parameter. Positive controls for these experiments included PAECs stained with FITC-conjugated IB4 lectin using the same protocols as for NICs. The efficacy of the cell separation was evaluated by visually comparing the fluorescence of sorted Gal-positive and -negative cells, as well as by re-analyzing each population by flow cytometry to detect the relative percentage of Gal-positive cells in each population. The cellular composition of freshly dissociated and sorted cell populations was determined by immunohistochemical staining for insulin (as described above) and glucagon, after adherence of the cells on poly-L-lysine–coated slides and fixation in Bouin's fluid. Glucagon-positive cells were detected using rabbit anti-porcine glucagon antibody (1:1,000; Dako Diagnostics) followed by biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories) and DAB as chromogen (BioGenex).

To detect the binding of preformed human natural xenoreactive antibodies to porcine NICs, 1×10^6 cells were incubated in varying dilutions (1:2, 1:8, 1:16, and 1:32) of heat-inactivated pooled human AB serum (NABI, Miami, FL) for 1 h on ice. The cells were then washed, labeled with FITC-conjugated rabbit anti-human IgG or IgM (1:10; Dako Diagnostics) for 1 h on ice, and rewashed. The percentage of cells binding to human antibodies was determined by FACS analysis.

Cytotoxicity assay and morphology. To determine the susceptibility of porcine NICs to antibody/complement-mediated lysis, fractions of unsorted and FACS-purified Gal-positive and -negative cells were exposed to fresh human AB serum collected from one donor or heat-inactivated pooled human AB serum (NABI) with the addition of rabbit complement (Pel-Freeze, Brown Deer, WI) obtained from rabbits ages 3–4 weeks old. This complement was selected because rabbits at this age are believed not to exhibit xenoreactive antibodies to other species such as the pig. To confirm the absence of xenoreactive antibodies in this complement, we incubated dissociated porcine NICs in heat-inactivated rabbit complement (1:5 dilution; Pel-Freeze) for 1 h on ice. Positive controls for this experiment included porcine NICs incubated in heat-inactivated normal adult rabbit serum using the same protocol. Islet cells were then washed and stained with R-phycoerythrin–conjugated goat anti-rabbit IgG (1:100 dilution; Vector Laboratories) or mouse anti-rabbit IgM (0.44 mg/ml; Serotec, Mississauga, Canada) for 30 min on ice. Rabbit IgM antibody binding on porcine NICs was detected by further incubating the cells with Cy3-conjugated goat anti-mouse antibody (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min on ice. Stained cells were washed and analyzed by flow cytometry. Positive controls (i.e., adult rabbit serum) demonstrated that >90% of the islet cells bound both rabbit IgG and IgM. In contrast, porcine NICs incubated in rabbit complement exhibited <1% binding of rabbit IgG or IgM. These results confirmed that complement obtained from rabbits ages 3–4 weeks contained no xenoreactive antibodies to porcine NICs.

Before the cytotoxicity assay, single-islet cell suspensions were distributed over poly-L-lysine–coated 24-well plates (5×10^4 cells/well) and cultured overnight in 1.0 ml of HAM's F10 media (supplemented as previously described). Then 500 µl of medium was removed from each well, replaced with the same amount of human serum (final concentration 50%, vol/vol), and incubated for 1 h at 37°C. Next 200 µl of medium were removed, replaced with rabbit complement, and incubated for 30 min at 37°C. When fresh human serum was used (i.e., containing active complement), no rabbit complement was added to the well. Controls included cells incubated in medium or heat-inactivated serum only. After incubation, 60 µl of 2.5 mg/ml (final concentration 0.01%, wt/vol) stock neutral red solution (Fisher Scientific, Edmonton, Canada) were added to each well for 30 min and the number of neutral red-positive (live) and red-negative (dead) cells was determined. The percentage of total dead cells was calculated as follows:

$$\%\ \text{dead cells} = \frac{\text{cell viability (controls)} - \text{cell viability (serum + complement)}}{\text{cell viability (controls)}} \times 100$$

Statistical analysis. Data are expressed as means ± SE of n independent experiments. Statistical significance of differences was calculated with a one-way analysis of variance. $P < 0.05$ was considered significant.

RESULTS

Expression of Galα(1,3)Gal on neonatal porcine pancreases and islet cell aggregates. The expression of the Gal epitope within intact neonatal porcine pancreases was localized on the lumen of vascular endothelium and pancreatic ducts (Fig. 1*A*). Insulin-positive β-cells were scattered randomly in the pancreas as single cells or small clusters frequently arranged alongside or within the duct lining. The cell surface of a small proportion of these β-cells was stained positive for the Gal epitope. In freshly digested pancreatic fragments, Gal was detected on capillary/ductal fragments and on the majority of insulin-positive β-cells (Fig. 1*B*). After 9 days' culture, the cellular aggregates developed into spherical structures similar to adult pancreatic islets. Immunohistochemical analysis of these aggregates showed an increased staining for Gal localized along the islets' periphery as well as in the lumen of intra-islet ductal elements (Fig. 1*C*). Although the percentage of insulin-positive cells increased during culture, the proportion of β-cells expressing Gal appeared to decrease. To more accurately demonstrate whether a proportion of pancreatic β-cells expressed Gal, single-cell suspensions of 9-day culture islets were also examined. Immunohistochemical analysis of these preparations clearly demonstrated Gal expression on a proportion of the insulin-positive cells (Fig. 1*D*).

Characterization of porcine NICs. FACS analysis of the neonatal porcine single-islet cell suspensions stained with FITC-conjugated IB4 lectin demonstrated that 30.0 ± 3.0% (n = 15) of the cells expressed Gal (purity = 90.0 ± 2.1%), whereas 70.0 ± 2.0% (n = 15; purity = 98.3 ± 0.4%) did not (Fig. 2). A heterogeneity existed among the various pigs tested, since the mean percentage of islet cells staining positive for Gal in 4 of 15 piglets was lower (range, 8.7–17.1%) than those prepared from the remaining animals (range, 20.2–48.8%). Immunohistochemical analysis of unsorted porcine NIC preparations indicated the presence of 33.5 ± 2.7% (n = 12) insulin-positive cells and 39.1 ± 5.0% (n = 12) glucagon-positive cells (Table 1). The Gal-positive fractions contained 23.1 ± 5.1% (n = 10) insulin-positive cells and 31.8 ± 3.7% (n = 10) glucagon-positive cells. Similarly, the Gal-negative population contained 37.6 ± 4.4% (n = 12) insulin-positive cells and 31.2 ± 4.0% (n = 9) glucagon-positive cells.

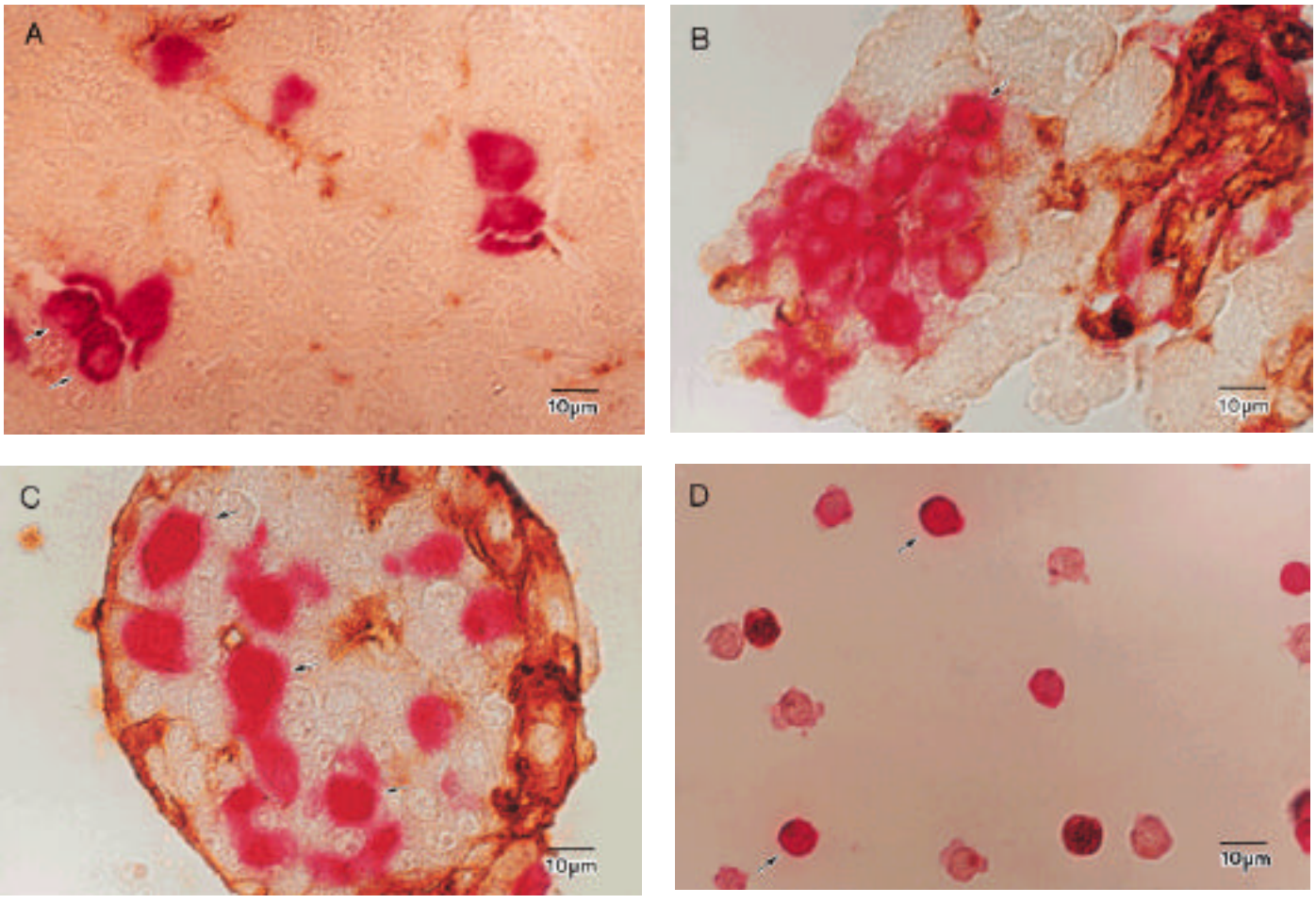

FIG. 1. Light micrographs of native neonatal porcine pancreas (*A*), freshly digested porcine NIC aggregates (*B*), 9-day culture islets (*C*), and 9-day culture dissociated islet cells (*D*). Sections were double immunohistochemically stained for Gal epitope (brown) and insulin (red). Arrows point to some double-positive cells.

The binding of preformed xenoreactive antibodies in normal human serum directed against porcine NICs was demonstrated by FACS analysis. At all tested dilutions of human sera (1:2 to 1:32), FACS analysis revealed >90% (range, 91–99%; n = 4) of the porcine NICs bound both human IgG and IgM antibodies (Fig. 3). When cells were incubated in the presence of FITC-labeled secondary antibodies, no significant fluorescence was detected.

Susceptibility to antibody/complement-mediated lysis. Incubation of porcine NICs in the presence of heat-inactivated pooled human AB serum containing rabbit complement resulted in the destruction of >90% of cells in unsorted as well as Gal-positive and -negative cell populations (Table 2). Similar results were obtained in all cell fractions when exposed to rabbit complement alone. In contrast, cell preparations that had been exposed to unmodified fresh human serum contained significantly (P < 0.05) fewer damaged cells (41–46% dead cells) (Table 2). When a 50% hemolytic activity assay was performed to determine complement activity, the condition in which rabbit complement was added exhibited values 1.5-fold higher (CH50, 300 U/ml) than that measured in fresh human serum (CH50, 193 ± 12.2 U/ml; n = 10). Incubation of islet cells in heat-inactivated serum containing no complement activity did not affect cell viability, as assessed by neutral red uptake (data not shown); these preparations demonstrated numerous intact cells. In contrast, the majority of cells exposed to heat-inactivated AB serum with rabbit complement or rabbit complement only exhibited ruptured plasma membranes.

DISCUSSION

Neonatal porcine islets constitute an attractive source of xenogeneic insulin-producing tissue for clinical transplantation; however, several aspects of the model need further investigation before this tissue can be considered for treating patients with type 1 diabetes. In particular, the susceptibility of porcine pancreatic endocrine cells to immunologic destruction via complement activation has not been fully elucidated. In the present study, neonatal porcine pancreatic islet cells were characterized for expression of the xenoreactive Gal epitope and sensitivity to human antibody/complement-mediated lysis in vitro. Our data indicate that Gal residues are expressed in neonatal porcine pancreases, and that this epitope is detected after collagenase digestion and 9-day culture of islet cell aggregates. In the native pancreas, Gal was detected on vascular endothelium within ductal lumen and on the surface of insulin-positive β-cells. Immunohistochemical staining of freshly digested pan-

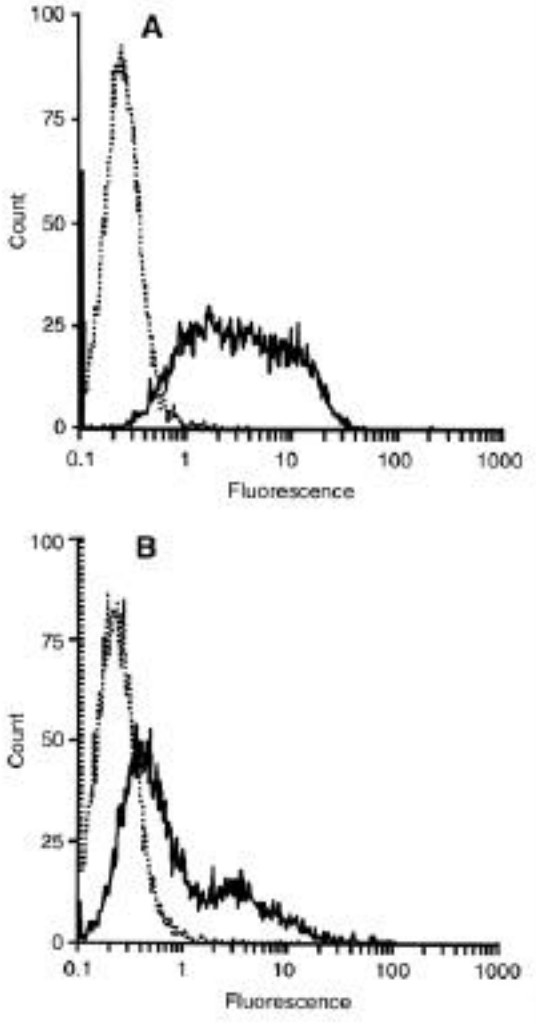

FIG. 2. FACS analysis of PAECs (*A*) and dissociated 9-day culture porcine NIC aggregates (*B*) stained with FITC-conjugated IB4 lectin. ---, negative controls not stained with IB4.

creatic fragments, 9-day cultured porcine NIC aggregates, and single-islet cell suspensions revealed that a proportion of insulin-containing β-cells clearly expressed Gal. To further identify which pancreatic islet cells express Gal, we used the lectin IB4 to FACS-purify cells with detectable levels of Gal. It was shown that ~30% of porcine NICs expressed Gal residues and that 55% of these cells stained positive for either insulin or glucagon. Thus ~17% of the total porcine NIC population could be characterized as insulin- or glucagon-positive cells that expressed Gal. On the other hand, the majority of porcine NICs (70%) were shown not to express Gal, and 69% of that population was composed of insulin- and glucagon-positive cells. Heterogeneity therefore exists among porcine NICs, in that a subpopulation of nonendocrine as well as endocrine cells express Gal.

This study also demonstrated that Gal is highly expressed in developing porcine NIC aggregates, and that the proportion of insulin-producing β-cells staining positive for Gal residues appear greater in freshly isolated preparations than in those allowed to mature further during the 9-day culture period. Furthermore, morphological assessment of porcine NIC grafts at >200 days posttransplantation in diabetic nude mice (i.e., after further maturation) revealed implants predominantly

TABLE 1
Cellular composition of neonatal porcine islet cells

Condition	Percentage of total cells	
	Insulin positive	Glucagon positive
Unsorted	33.5 ± 2.7 (12)	39.1 ± 5.0 (12)
Sorted		
Gal positive	23.1 ± 5.1 (10)	31.8 ± 3.7 (10)
Gal negative	37.6 ± 4.4 (12)	31.2 ± 4.0 (9)

Data are means ± SE (*n*) of independent experiments. Cell composition was determined by immunohistochemistry, as described in METHODS.

composed of insulin-positive cells, with Gal reactivity restricted to mouse endothelial cells originating from revascularization of the grafts (data not shown). It is therefore possible that Gal is expressed in a subpopulation of less mature porcine NICs that are precursors of hormone-secreting cells or transitional cytodifferentiated forms of insulin/glucagon-containing cells that eventually lose Gal expression upon becoming fully differentiated and/or functionally mature.

Our results differ from those obtained in other studies, suggesting that Gal is not present on fetal (17) or adult (17,19) pancreatic endocrine cells but limited to intra-islet ductal and endothelial cells (15,17,19). Because our data indicate that Gal is expressed on immature/developing β-cells, this possibly explains why Mirenda et al. (19) did not detect Gal on adult porcine β-cells, but it remains uncertain why McKenzie et al. (17) did not observe Gal residues on fetal porcine endocrine cells. It is possible that differences in experimental design as well as in age, strain, and even individual animals account for this variability in Gal expression. For example, we observed that islet cells isolated from Yorkshire-Landrace pigs exhibited variability in Gal expression. Similarly, Geller et al. (23) reported variation in porcine xenogeneic antigen expression among pigs within the same as well as different strains.

Using an in vitro cytotoxicity assay, we demonstrated that ~50% of porcine NICs are rapidly lysed by the activation of human complement. Exposure to heat-inactivated human serum containing rabbit complement led to the lysis of >90% of the islet cells. Because rabbit complement was tested for xenoreactive antibody content, the increased cytotoxicity seen in this condition was likely due to higher levels of complement activity than that measured in fresh human serum. Furthermore, results of the present study confirmed our previous data using intact porcine NICs (20) and agreed with those previously reported with fetal (18) and adult (21,22) porcine islet cells. In contrast, Mirenda et al. (19) recently showed no cytotoxic effect or alteration in adult porcine islet function, despite binding of human IgG or IgM antibodies and deposition of complement. It is possible that differences in age and strain of the pigs as well as selection of cytotoxicity protocols account for these differences. For example, previous reports that examined porcine islet viability after exposure to human sera provided no data regarding complement activity in the sera tested (18,19,22). It is therefore possible that an observed lack of islet cell toxicity is simply related to diminished complement activity during collection and/or the time required to pool sera from multiple donors. To exclude the possible loss of complement activity

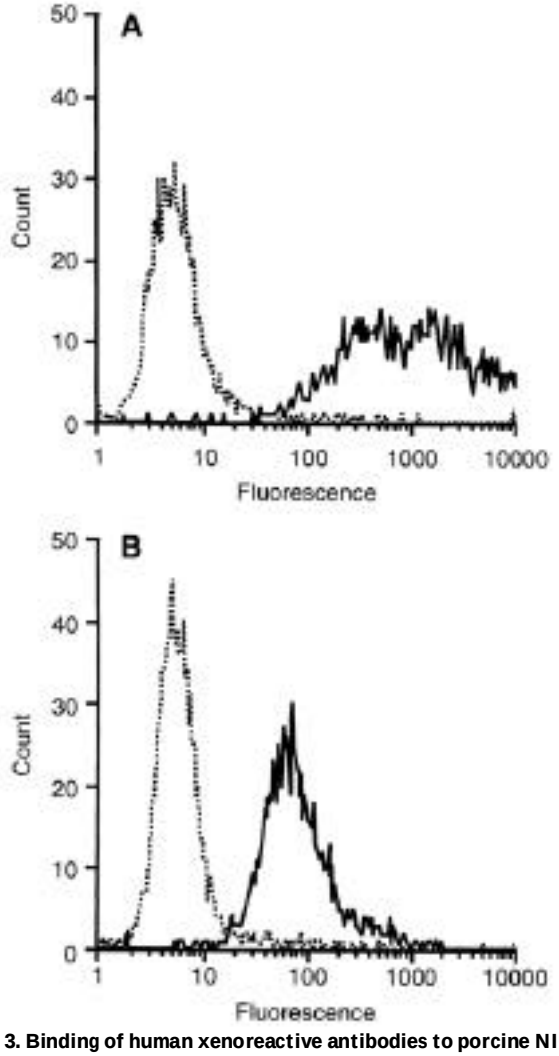

FIG. 3. Binding of human xenoreactive antibodies to porcine NICs. Single-cell suspensions of neonatal porcine islet cells were incubated with 1:32 dilution of heat-inactivated pooled human AB serum for 1 h, then incubated with 1:10 dilution of FITC-conjugated rabbit anti-human IgG (*A*) or IgM (*B*) for an additional 1 h. ---, negative control islet cells incubated in FITC-labeled secondary antibodies alone. Results are representative of three independent experiments.

during storage, we used freshly prepared serum from one individual or heat-inactivated serum (i.e., as a source of xenoreactive antibodies) supplemented with rabbit complement.

Our observation that porcine NICs were destroyed in the absence of xenoreactive antibodies with rabbit complement alone agrees with others from in vitro studies using porcine endothelial (24) or adult islet (22) cells. Although the role of classical and alternative pathways in complement activation within different species combinations remains controversial (24–29), several other xenotransplant models have also demonstrated hyperacute rejection of discordant xenografts mediated by complement activation via the alternative pathway (28,29). The present study suggests that Gal expression on porcine NICs might not be a critical factor in inducing complement-mediated lysis, since Gal-negative cells were also killed. It is possible that Gal-negative cells may be lysed by two different mechanisms: binding of xenoreactive antibodies to non-Gal epitopes (causing complement activation) or activation of the alternative complement pathway. Several

TABLE 2
Susceptibility of porcine NICs to human antibody/complement-mediated lysis

Condition	Percentage of dead cells		
	Unsorted	Gal positive	Gal negative
Heat-inactivated pooled AB serum + complement	97.4 ± 1.7 (9)	97.9 ± 1.3 (11)	97.9 ± 1.1 (15)
Complement	92.5 ± 3.1 (9)	91.4 ± 3.0 (10)	90.9 ± 2.9 (14)
Fresh AB serum	40.7 ± 4.9 (6)*	46.9 ± 7.7 (4)*	45.7 ± 3.2 (6)*

Data are means ± SE (n) of independent experiments. Cells were exposed to 50% human serum for 1 h and 20% rabbit complement for another 30 min. Fresh serum was from one individual with blood type AB. The percentage of dead cells was calculated as described in METHODS. *P < 0.05 vs. complement or heat-inactivated–pooled human AB serum + complement, one-way analysis of variance.

reports have suggested that human preformed anti-Gal antibodies are the major, if not exclusive, xenoreactive antibodies responsible for hyperacute rejection (11,30–32) and that adsorption of these antibodies may reduce the serum's cytotoxicity (33,34). However, it has also been shown that human anti-Gal antibodies are capable of recognizing alternative ligands, which still remain uncharacterized (35). In addition, Oriol et al. (14) reported that carbohydrate epitopes with terminal lactosamine (βGal1-4GlcNAc) or sialic acid (αNeuAc2-3βGal1-4GlcNAc) are also present on porcine vascular endothelium, suggesting the possibility that other xenoreactive antibodies are directed against different carbohydrate epitopes.

We conclude that porcine NIC aggregates are composed of Gal-expressing cells, and that expression of this epitope is not restricted to nonendocrine cells. Furthermore, both cells that express and cells that lack Gal are susceptible to complement-mediated lysis. This form of immunologic destruction takes place even in the absence of preformed natural antibodies, indicating a key role of the alternative pathway of the complement system in the destruction of porcine NICs in vitro. Taken together, these results suggest that porcine NIC grafts may be subjected to natural antibody/complement-mediated destruction if implanted into patients with type 1 diabetes; however, this must be confirmed in an in vivo model.

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