Growth-Promoting Effect of Rh(D) Antibody on Human Pancreatic Islet Cells

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Context/Objective: Hyperinsulinism with islet cell hyperplasia is a frequent complication, of unknown cause, in hemolytic disease of the newborn, occurring in Rh(D)-positive infants of Rh-isoimmunized Rh(D)-negative mothers, but not in infants with other hemolytic disorders. We investigated the possibility that trans-placentally acquired anti-D Ig is the cause of both conditions.

Design: Monolayer cultures of human islet cells were exposed to sera from Rh-isoimmunized mothers and newborns, where jaundice, hyperinsulinism, and hypoglycemia in the infant had ensued. Parallel cultures with anti-D, specific anti-D monoclonal antibodies, normal human Ig (15 μg/ml), and serum controls were also undertaken. Islet cell proliferation was determined by [^{3}H]thymidine incorporation. Insulin storage and chronic and acute insulin secretion to glucose were analyzed by RIA. Rh(D) surface antigen expression was determined on islet cells by flow cytometric analysis.

Results: Islet cell proliferation and insulin secretion were significantly greater in coculture with test sera ($P < 0.01$; n = 8) and with anti-D ($P < 0.001$; n = 8), compared with either controls or Ig. After 8 d of growth, the static incubation experiment showed a 3.5-fold response to glucose stimulus in all sera. Rh(D) antigen expression was detected on the islet cell surface by flow cytometry, and islet cell morphology was normal. Colocalization of the proliferation marker Ki67 with insulin by immunofluorescent staining further indicated that Rh(D) antibody promoted islet growth.

Conclusions: The anti-Rh(D) islet cell proliferative effect generates neonatal hyperinsulinism in Rh isoimmunization. Anti-Rh(D) may have application for islet cell proliferation in diabetes mellitus treatment for Rh(D)-positive subjects. Further analysis is required. (*J Clin Endocrinol Metab* 93: 3560–3567, 2008)

Hyperinsulinism, due to fetal islet cell hyperplasia, is a complication in the newborn of Rh(D)-isoimmunized mothers, but the basis for this is unknown. We suggest that this phenomenon may be a consequence of trans-placentally acquired anti-Rh(D) antibody, either alone or in combination with other trophic influences. Our confirmation of this specific, growth-promoting effect *in vitro* potentiates the identification and characterization of a factor that may ultimately have therapeutic application in the treatment of diabetes mellitus. This study investigated the *in vitro* growth-stimulating effect of commercially available anti-D monoclonal antibodies and maternal sera, or infant umbilical cord sera from subjects where maternal anti-D antibody has crossed the placenta, upon human islet cells.

Hyperinsulinemia and hypoglycemia are recognized complications of erythroblastosis fetalis in newborns of Rh-isoimmunized mothers (1, 2) and are associated with pancreatic islet cell

Abbreviations: DAT, Direct antibody test; HDN, hemolytic disease of the newborn; MCF, mean channel fluorescence intensity; PE, phycoerythrin; RT, room temperature.

hyperplasia, evident from postmortem findings from both fetuses and newborns (3, 4). This phenomenon in hemolytic disease of the newborn (HDN) seems to be specific to Rh isoimmunization and has been neither reported for other antibodies responsible for cases of HDN, such as in ABO incompatibility, nor observed with the nonimmune, hemolytic states, such as hereditary spherocytosis or α-thalassemia. Therefore, the mechanism of Rh-related induction of islet cell hyperplasia is unlikely to be due to excess breakdown of fetal red cells and their byproducts, such as glutathione, as previously suggested (5).

In normal pregnancy, there is an immunological tolerance between mother and fetus with progression of embryological and fetal development controlled by the trophic effects of numerous growth factors. These factors, in turn, interact with genetically predetermined fetal influences that, when disrupted, may be responsible for excessive cell proliferation in one or more organ systems, including the pancreatic islet cells with accompanying hyperinsulinism (6). There have been reports of islet cell proliferation after experimental pancreatic injury (7, 8), laying a foundation for possible strategies to promote pancreatic regeneration.

In this study, we have investigated the possibility that Rh(D) antibody might exert such islet growth-promoting actions. Samples of sera were collected from mother and infant umbilical cord blood at the time of delivery, when Rh(D) isoimmunization was suspected. After confirmation of the occurrence of HDN through transfer of maternal anti-D antibody across the placenta and evidence of both hypoglycemia and hyperinsulinemia, these sera samples were assessed, alone and in parallel with anti-D Ig or anti-D monoclonal antibodies (9, 10), for their ability to stimulate growth of human pancreatic islet cells. We have demonstrated an augmented proliferation of islet β-cells cultured in the presence of anti-D antibody.

Patients and Methods

Collection of sera

After approval from the Human Ethics Committee of the South Eastern Sydney Area Health Service, subjects were identified and recruited. Patients with medical conditions or on medications and those who had labor induced artificially were excluded. A 10-ml sample of maternal blood was collected in the week before onset of labor.

Serum 1 was collected from an Rh(D)-negative mother whose infant was confirmed to be Rh(D) positive and direct antibody test (DAT) positive was jaundiced and had both hypoglycemia and hyperinsulinemia. Serum 2 was collected from an Rh-negative mother whose infant was confirmed to be Rh(D) positive and strongly DAT positive and was jaundiced but had neither hypoglycemia nor hyperinsulinemia. Serum 3 was collected from an Rh(D)-negative, isoimmunized mother. Serum 4 was collected from the infant of serum 3 sample mother, and this infant had moderately severe hyperinsulinism but profoundly severe hemolytic disease. Normal human serum was used as a control throughout (Prince of Wales Hospital, Sydney, Australia). Sera were heat inactivated at 56 C for 30 min. Anti-D antibody was obtained from CSL Ltd. (Victoria, Australia) and intraglobin F (normal human Ig) from MDA Pharma (Sydney, Australia). Anti-D monoclonal antibodies BRAD-3, BRAD-5, and FOG-1, were gifts from Belinda Kumpel, National Blood Service (Bristol, UK).

Isolation and culture of islets

Ethics committee approval was obtained for the isolation of islets from pancreases received from 22 Rh(D)-positive and six Rh(D)-negative brain-dead, beating-heart organ donors sent to Westmead Hospital, New South Wales, and St. Vincent's Institute of Medical Research, Victoria. Islets were extracted from the whole pancreas by a modified Edmonton method, using an apheresis unit and density gradients (11). The islets were cultured in Ham's F10 media containing 6.1 mM glucose and 10% human serum for 24 h before transport at room temperature (RT) to the University of Technology Sydney. Because the islet preparation was 75–90% pure, the islets were centrifuged at $30 \times g$ for 5 min at RT and resuspended in RPMI 1640 (Thermo Electron Australia, Noble Park, Australia) medium containing 5.5 mM glucose plus normal human serum (10%). After dithizone staining (12), the islets were handpicked and cultured free-floating in the RPMI 1640 medium for another 24 h before monolayer culture. This procedure was performed on eight separate occasions, providing samples for eight individual experiments. Two or more samples from each batch of islets were used to assess viability, using an acridine orange (0.06 g/ml)/ethidium bromide (0.2 g/ml) vital stain. The sample was then examined by fluorescence microscopy using a band pass filter of wavelength 515–560 nm. Islets were used only if viability after preparation was more than 95%.

The islets were transferred to 12-well tissue culture plates coated with bovine corneal matrix (Bacto Laboratories Co., Liverpool, Australia) (20 islets per well) and cultured in RPMI 1640 medium plus normal human serum (10%). To prevent fibroblast overgrowth of the cultures, 0.46 μg/ml sodium ethylmercurithiosalicylate (thiomersal) (Sigma-Aldrich Co., Castle Hill, Australia) was added 24 h after plating for 48 h. This has been previously reported (13) and shown in our laboratory to prevent fibroblast contamination of primary islet cultures while not exerting detrimental effects upon islet growth or function. After thiomersal treatment, islets were washed three times and cultured in RPMI 1640 medium containing one of the sera listed above. The cultures were maintained for another 8 d, with the media changed every 2 d.

Proliferation assay

To the monolayer cultures treated with each of the sera and cultures supplemented with anti-D Ig (5–20 μg/ml), 15 μg intraglobin F, or monoclonal anti-D BRAD-3 (IgG3), BRAD-5 (IgG1), and FOG-1 (IgG1), 37 Mbq/ml of [^{3}H]thymidine was added to each well for 24 h. Subsequently, the cells were washed twice with ice-cold PBS and twice with ice-cold 10% trichloroacetic acid. The trichloroacetic acid-insoluble precipitate was solubilized with 2% SDS and analyzed (5 min/vial) using a β-counter (Wallac 1470 distributed by Pfizer Inc., New York, NY).

Acute insulin secretion

After 8 d of culture in the sera and/or antibody-supplemented media, monolayers were assessed for any acute response to a glucose stimulus (16.7 mM or for glucose dose-response curves, 1.25–20 mM), as previously described (14). Cellular DNA content was analyzed by fluorometry (15).

Expression of Rh(D) antigen on islet cells

The culture medium was removed from islet cells and the adherent cells rinsed with PBS before incubation at 37 C for 10 min with a standardized and stabilized bromelin solution (Diluent 1, DiaMed AG, Cressier-sur-Morat, Switzerland) to detach cells. This enzyme preparation does not destroy the Rh(D) antigen expressed on Rh-positive red cells. After two saline washes, a final suspension of cells was prepared in PBS with 0.3% BSA. Aliquots of this suspension (5×10^5 cells) were incubated at RT in the dark for 30 min with the appropriate concentration (as specified by the manufacturer) of the following fluorescently conjugated monoclonal antibodies of IgG isotype, anti-CD3-phycoerythrin (PE) (Becton Dickinson Co., Mountain View, CA), anti-CD16-PE (a low-affinity IgG receptor, expressed by granulocytes; Bec-

ton Dickinson), anti-CD14-PE (an endotoxin receptor on monocytes; Dako A/S, Glostrup, Denmark), and anti-Rh(D)-PE (Chemicon Australia, Boronia, Australia). After two washes in PBS, flow cytometric analysis was performed (Coulter Epics XL-MCL instrument; Beckman Coulter, Hialeah, FL).

Additional aliquots of islet cells were treated for 30 min at RT with either monoclonal anti-D biotin-conjugated antibody (in-house reagent) (16), monoclonal anti-CD38 biotin-conjugated (hemopoietic cell marker; Caltag Labs, South San Francisco, CA), or polyclonal antihuman Ig-biotin conjugated F(ab′)₂ fragment (custom-made by Silenus Ltd., Melbourne, Australia). The cells were washed twice with PBS and, together with a fourth test sample of cells not exposed to antibody, incubated for 30 min at RT and in the dark with the appropriate quantity of streptavidin-conjugated PE (Dako A/S, Denmark).

Immunohistochemical analysis

Immunohistochemistry was carried out on fixed islet monolayers, which had been trypsinized after 2 or 8 d growth in the various sera, using antibodies to human insulin, glucagon, somatostatin (Dako, Santa Barbara, CA), or pancreatic polypeptide (Miles, Naperville, IL), employing the alkaline phosphatase technique. For each preparation, four samples were stained for the different pancreatic hormones. One individual scored a total of 500 cells in every section, either positive or negative, in a blinded fashion. The possibility of a cell staining for two hormones (*e.g.* insulin and glucagon) was excluded by double staining the same sample of cells with different antibodies to distinguish the cell types (*e.g.* horseradish peroxidase for glucagon and alkaline phosphatase for insulin), as previously described (17). Dou-

ble immunofluorescence staining for insulin (Linco Research, St. Charles, MO) and Ki67 (GenTex, Carbondale, PA), a cell proliferation marker, was carried out on unfixed cultures as described (18). The secondary antibodies were biotinylated antirabbit IgG followed by fluorescein isothiocyanate-streptavidin (Vector Laboratories, Burlingame, CA) for Ki67 and Texas Red anti-guinea pig IgG (Vector) for insulin. Images were analyzed by microscopy with Image-Pro Plus (Media Cybernetics, Inc., Bethesda, MD).

Transmission electron microscopy

Pancreatic islet tissue from two donors was incubated in medium supplemented with serum 1 at 37 C for 4 d in tissue culture plates, processed as described (19), and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate followed by lead citrate.

Statistical analysis

Data are expressed as means ± SEM, and paired sample means were compared using Student's *t* test.

Results

Islet cell viability, morphology, and ultrastructure

Pancreatic β-cells used in this study had a normal appearance without damage and contained many secretory vacuoles (Fig. 1A, a). Crystalloids typical of human β-cells were present in the plane of section of many of the secretory granules. There were no necrotic cells or other abnormalities in any sample examined.

Epithelial cells grew from the attached islets, spreading as a monolayer in a typical cobblestone configuration. By d 8, the cells grown in the presence of serum 1 was confluent with no apparent fibroblasts (Fig. 1A, b). There were no apparent fibroblasts evident in any of the cultures examined.

Proliferation and insulin secretion

Monolayers from three separate experiments were stained for the pancreatic endocrine hormones insulin, glucagon, somatostatin, and pancreatic polypeptide. Over the 8-d culture period, the percentage of β-cells did not increase in cultures maintained in normal human serum but significantly ($P < 0.01$) increased in cultures maintained in serum 1 from 64.1 ± 4.1% at d 2 to 74.1 ± 3.2% at d 8 and, similarly, in the presence of anti-D ($P < 0.05$) from 62.3 ± 4.1% at d 2 to 68.2 ± 1.1% at d 8. There was also a significant increase in the percentage of β-cells ($P < 0.01$) within monolayers maintained in serum 3 and serum 4 ($P < 0.05$) but not in the presence of serum 2 or intraglobin F (results not shown). The numbers of cells staining for the other pancreatic hormones remained static over the culture period. The β-cell proliferation index, as measured by the percentage of Ki67-positive
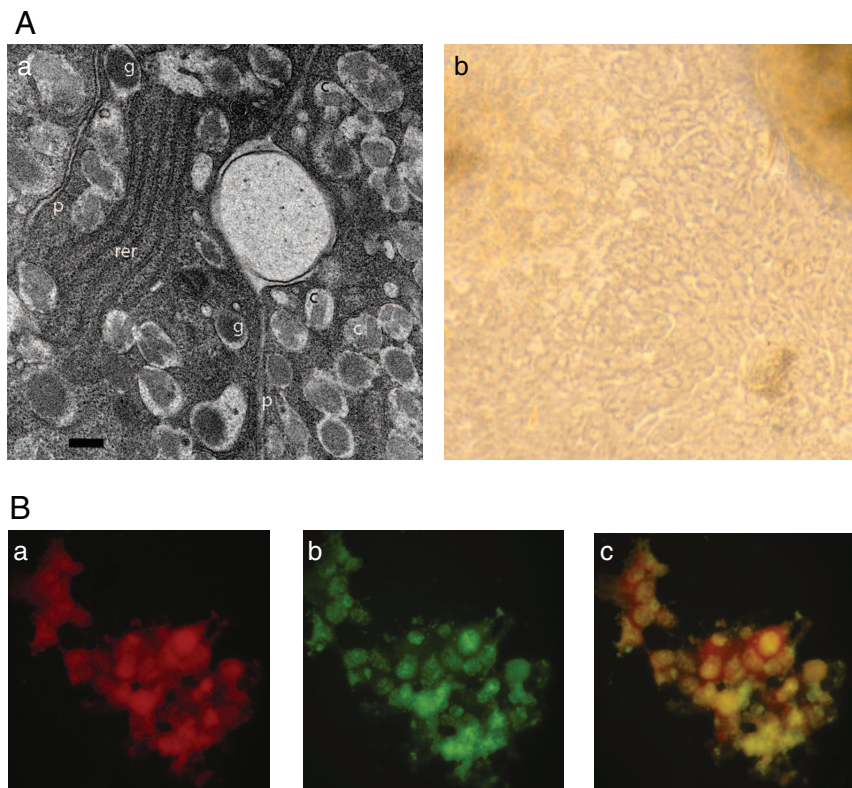

FIG. 1. A, Electron micrograph of adult human islet cells from Rh-positive donors cultured in serum 1 for 4 d (a) and light micrograph of monolayer cultures derived from human islets maintained in culture for 8 d (b). In a, three adjacent β-cells are present in the field of view, with the area where their plasma membranes abut labeled p. Rough endoplasmic reticulum (rer) was present. Some secretory vacuoles had granules without any crystalloids present in the field of view (g), whereas in others, the section plane contained crystalloids (c), typical of insulin-containing human β-cells (*bar*, 0.4 μm). In b, the cells have a regular cobblestone appearance (original magnification, ×40). B, Ki-67/insulin photomicrographs of human islet monolayers maintained in culture for 4 d in the presence of anti-D: a, insulin-positive cells; b, Ki-67-positive cells; c, Ki-67/insulin, double-positive cells (*bar*, 100 μm).

β-cells, for islet cells cultured in anti-D for 4 d was 87 ± 4.3% (Fig. 1B). Similar results were seen when the anti-D monoclonal antibodies were cocultured with islet cells.

An examination of the effect of increasing concentrations of anti-D (5–20 μg/ml), compared with normal sera, on the proliferation of adult pancreatic islets from Rh-positive donors, revealed that from d 4 of culture, all samples supplemented with anti-D antibody had shown significant ($P < 0.01$) cell proliferation (results not shown), and by d 8, concentrations of 15 and 20 μg/ml anti-D induced a 3-fold increase ($P < 0.001$) of 52.3 ± 0.25 and 54.0 ± 1.0 cpm × 10^3 (n = 4), respectively, above proliferation rates induced by normal serum (17.6 ± 0.5 cpm × 10^3; n = 4). There was no significant difference between the cell proliferation of cells grown in media supplemented with 15 μg/ml compared with 20 μg/ml anti-D. Similarly, an increased concentration of anti-D induced a corresponding increase in chronic insulin secretion by d 8, with the higher concentrations of anti-D (15 or 20 μg/ml) stimulating a 2-fold increase (5.2 ± 0.1 and 5.4 ± 0.1 pmol insulin/well·48 h; n = 4), respectively, compared with insulin secretion induced by normal serum (1.9 ± 0.1 pmol insulin/well·48 h; n = 4). Because there was no significant difference in the proliferation rates or levels of insulin secretion induced by 15 μg/ml compared with 20 μg/ml anti-D antibody, 15 μg/ml was used in subsequent experiments. Likewise, preliminary experiments indicated that 15 μg/ml was the appropriate concentration for the monoclonal antibodies employed in later experiments.

Figure 2A clearly shows that culture of adult pancreatic islets in the presence of serum 1 resulted in a significant ($P < 0.001$) increase in cell proliferation, compared with proliferation stimulated by both serum 2 and normal human serum. By d 8 of culture, the 4.5-fold increase in cell proliferation was mirrored by a significant ($P < 0.001$) 4-fold increase in chronic insulin secretion from the cells (Fig. 2B) compared with normal serum, indicating that the increase in cell proliferation was likely attributable to β-cell proliferation. This increase in the proliferation of cells, as measured by [³H]thymidine incorporation, was unlikely to be entirely a result of anti-D because by d 6 and 8 of culture, the increase in cell number resulting from addition of serum 1 was significantly ($P < 0.01$) higher than that recorded when the medium was supplemented with 15 μg/ml anti-D alone. However, supplementation of the culture medium with anti-D resulted in a significant ($P < 0.01$) increase in proliferation over that seen with normal serum alone or with serum 2. Likewise, whereas the addition of anti-D to the cultures resulted in a significant ($P < 0.001$) increase in insulin secretion, the insulin secretion by cells supplemented with serum 1 was again significantly ($P < 0.001$) higher than those grown in the presence of anti-D alone. After 8 d of growth in the four sera, static incubation experiments (Fig. 2C) showed a 3.5-fold response to a glucose stimulus from islets cultured in all four sera; however, because cultures supplemented with serum 1 or anti-D had a larger number of β-cells, absolute insulin levels were higher overall (Fig. 2C). Light microscopic examination indicated that the islet cultures remained healthy and viable throughout.

The proliferation of islet cultures grown in the presence of serum 3 was significantly ($P < 0.01$) greater from d 4 onwards,
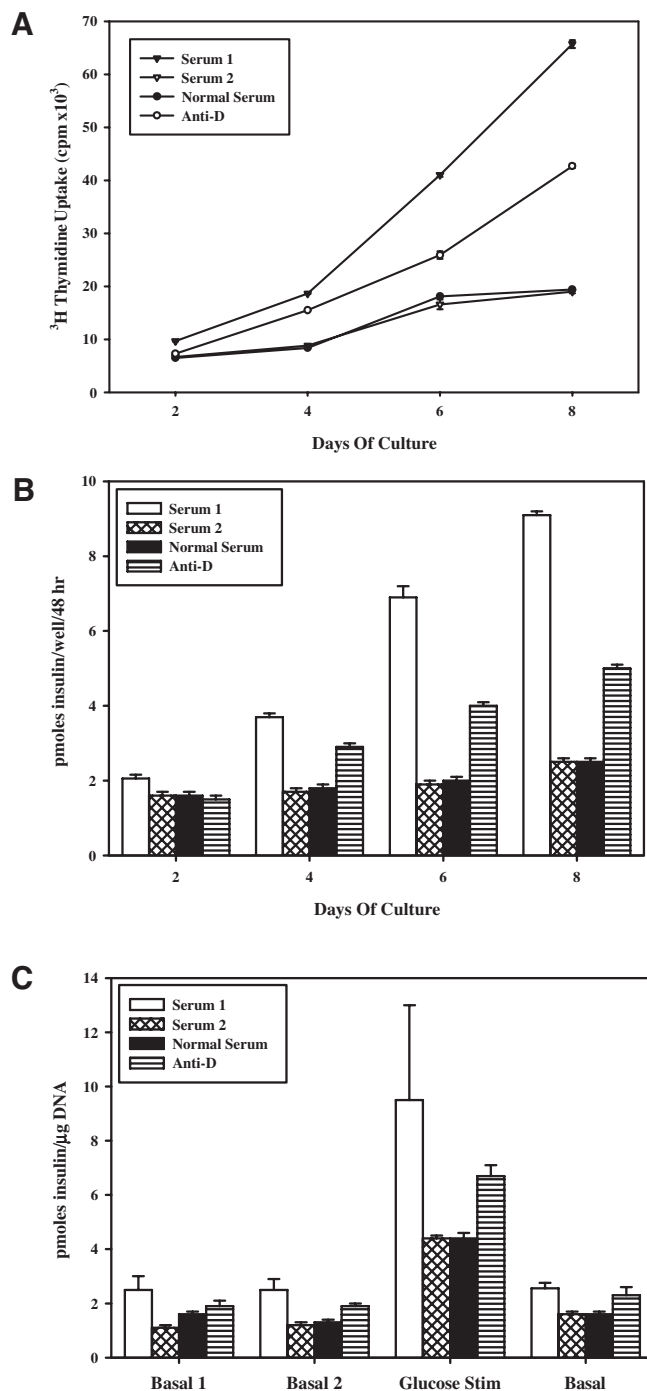

FIG. 2. Monolayer cultures of adult human islets from Rh-positive donors supplemented with patient sera 1 and 2, anti-D Ig (15 μg/ml), normal human Ig (intraglobin F) (15 μg/ml), and normal human serum. A, Cell proliferation as measured by [³H]thymidine incorporation; B, chronic insulin secretion; C, stimulation by 16.7 mM glucose on the acute regulated release of immunoreactive insulin. Cells were incubated in basal medium (PBS containing 1 mM CaCl₂ and supplemented with 20 mM HEPES and 2 mg/ml BSA, 2.8 mM glucose) for two consecutive 1-h periods before being exposed to the stimulus for 1 h; cells in the control group were treated throughout with basal medium. Results are expressed as the mean ± SEM (values represent eight replicates in each of four separate experiments; SE were very tight in cell proliferation studies and in some cases are contained within the data point).

as was cell proliferation in medium supplemented with anti-D ($P < 0.001$) when compared with normal serum and cells supplemented with intraglobin F (Fig. 3A). From d 6 onward, cell
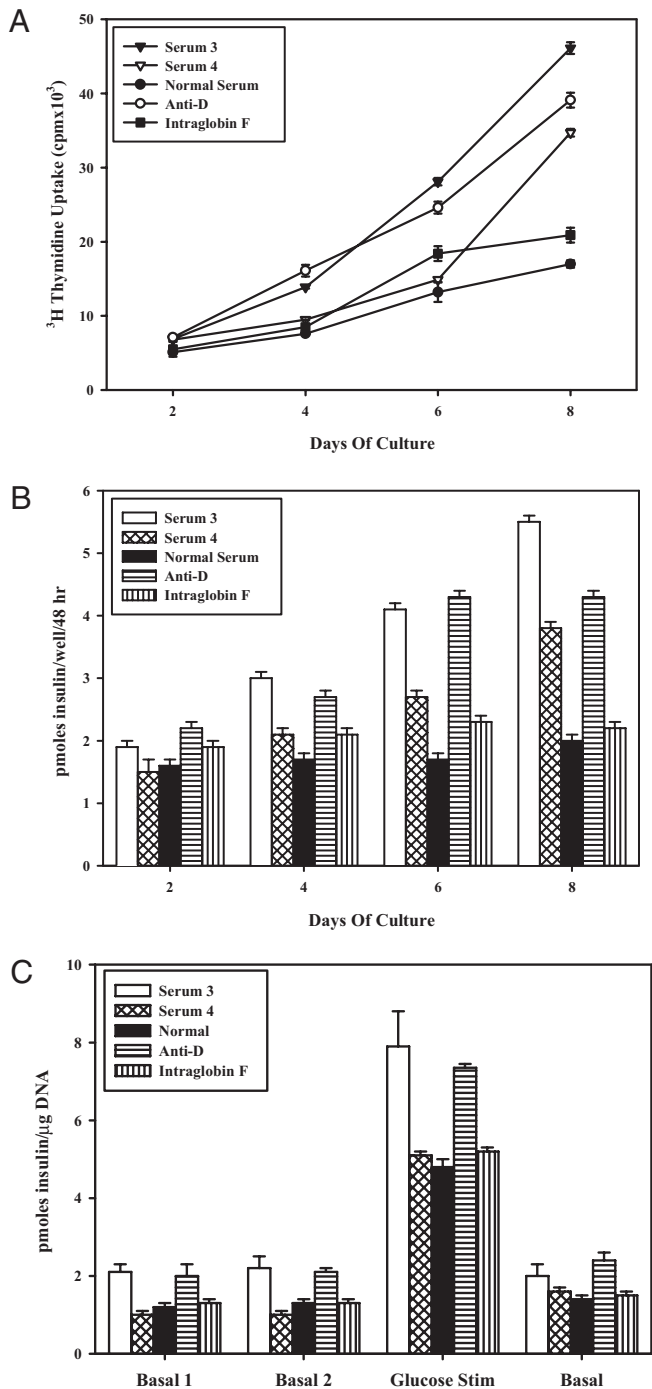
A

B

C

FIG. 3. Monolayer cultures of adult human islets from Rh-positive donors supplemented with sera 3 and 4, anti-D Ig (15 μg/ml), normal human Ig (intraglobin F) (15 μg/ml), and normal human serum. A, Cell proliferation as measured by [³H]thymidine incorporation; B, chronic insulin secretion; C, stimulation by 16.7 mM glucose on the acute regulated release of immunoreactive insulin. Cells were incubated in basal medium for two consecutive 1-h periods before being exposed to the stimulus for 1 h; cells in the control group were treated throughout with basal medium. Results are expressed as the mean $\pm$ SEM (values represent eight replicates in each of four separate experiments; SE were very tight in cell proliferation studies and in some cases are contained within the data point).

proliferation in cultures supplemented with serum 4 was significantly ($P < 0.01$) greater than coculture with normal serum and intraglobin F but remained significantly ($P < 0.01$) less than proliferation observed serum supplemented with serum 3 and
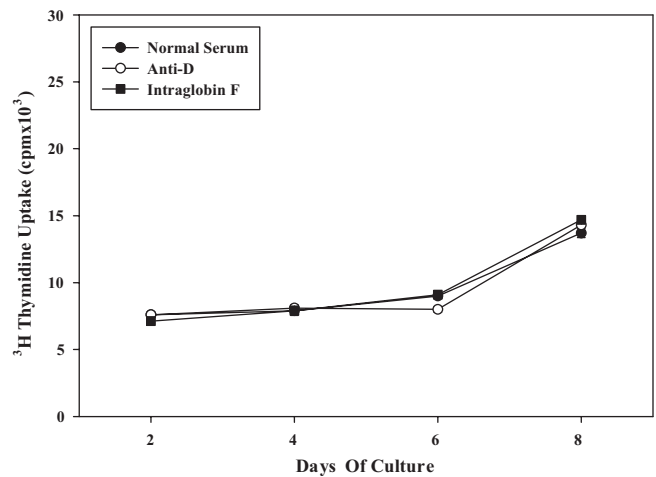

FIG. 4. Cell proliferation as measured by [³H]thymidine incorporation of monolayer cultures of adult human islets from Rh-negative donors supplemented with anti-D Ig (15 μg/ml), normal human Ig (intraglobin F) (15 μg/ml), and normal human serum. Results are expressed as the mean $\pm$ SEM (values represent eight replicates in each of four separate experiments; SE were very tight in cell proliferation studies and in some cases are contained within the data point).

anti-D ($P < 0.05$) throughout the experiment (Fig. 3A). However, with regard to chronic insulin secretion (Fig. 3B), by d 8 of culture, the level of insulin secreted from cultures supplemented with serum 3 was significantly ($P < 0.01$) higher than from cultures supplemented with either serum 4 or anti-D (Fig. 3B). From d 6 onward, insulin secretion from cells grown in normal serum or medium supplemented with intraglobin F was significantly ($P < 0.01$) less than other cultures, and by d 8, islets cocultured with serum 3 secreted three times the amount of insulin than islets cultured with normal serum. There was no significant difference in response to an acute glucose challenge (Fig. 3C). No growth potential was seen when serum either from normal and pregnant Rh(D)-positive women or from Rh(D)-negative, non-isoimmunized pregnant women were used to supplement the islet cell culture medium (results not shown). No increase in β-cell proliferation was seen when islets from Rh(D)-negative donors were supplemented with anti-D (Fig. 4).

The proliferation and insulin secretion of islet cultures in the presence of the anti-D IgG3 monoclonal antibody BRAD-3 was not significantly different from that seen in the presence of anti-D Ig (Fig. 5). However, both proliferation and insulin secretion in the presence of the anti-D IgG1 monoclonal antibodies BRAD-5 and FOG-1, although significantly ($P < 0.01$) higher than normal serum, were significantly ($P < 0.01$) lower than BRAD-3-supplemented cultures from d 6 onward (Fig. 5). As can be seen from Fig. 6, supplementation of the medium with anti-D Ig or BRAD-3 did not alter the kinetics of the response curve to increasing glucose concentrations.

Expression of Rh(D) on islet cells

Cultured islet cells did not express CD3, CD14, or CD16 antigens, showing low mean channel fluorescence intensity (MCF) of 2.8 $\pm$ 0.2, 2.8 $\pm$ 0.5, and 2.2 $\pm$ 0.6, respectively. In contrast, islet cells treated with anti-D showed significantly ($P < 0.001$) higher MCF of 30.2 $\pm$ 3.6. These results were confirmed
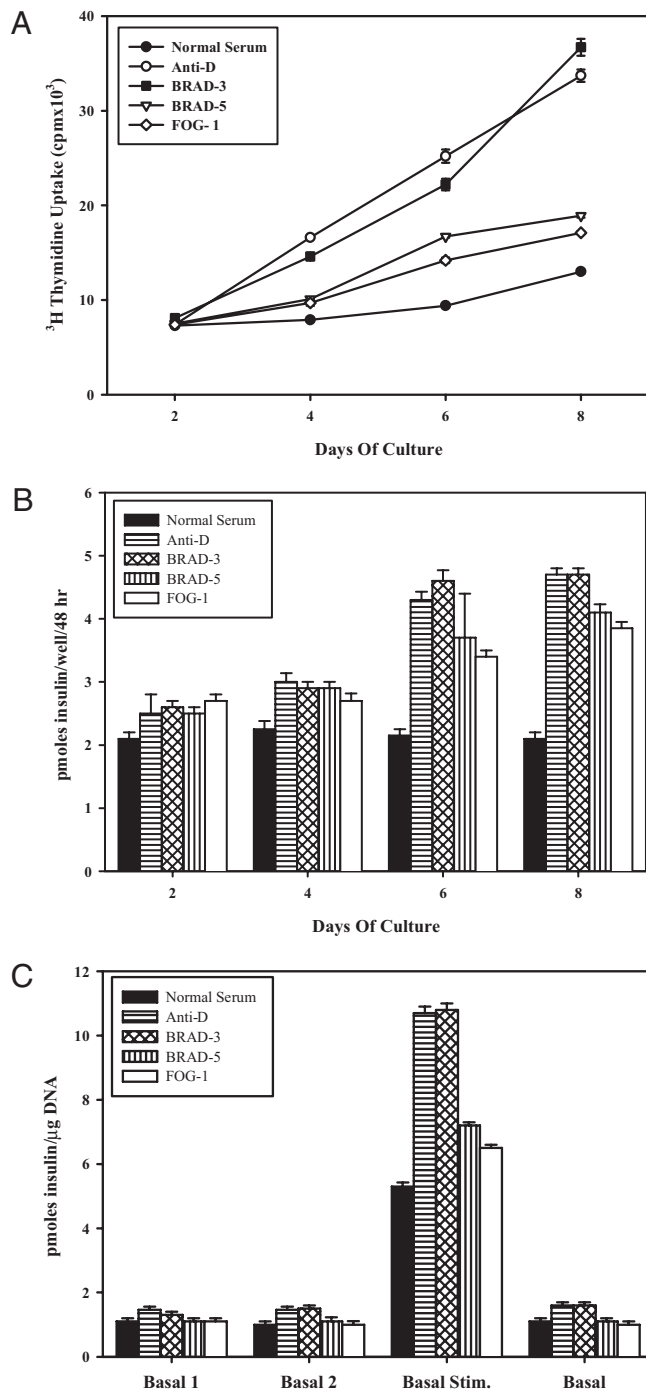
FIG. 6. Monolayer cultures of adult human islets from Rh-positive donors supplemented with anti-D Ig, anti-D monoclonal antibody BRAD-3 (15 μg/ml), and normal human serum. Dose-response curve to varying concentrations of glucose (0–20 mM). Results are expressed as the mean ± SEM (values represent eight replicates in each of four separate experiments; SE were very tight in cell proliferation studies and in some cases are contained within the data point).

[normal pooled human Ig, F(ab′)₂ fragment and mouse anti-CD38] gave results not significantly different from the MCF obtained with streptavidin-PE alone (4.0 ± 0.6).

Discussion

The hypothesis that fetal hyperinsulinism associated with Rh(D) isoimmunization might be a direct consequence of the induction of up-regulated islet cell activity by trans-placentally acquired maternal anti-D antibody was supported by the results of our *in vitro* study of human islet cells in the presence of sera from iso-immunized mothers. Similar up-regulation of both islet cell proliferation and insulin secretion by the cells were obtained after their exposure to anti-D Ig and specific Rh(D) monoclonal antibodies (9, 10) but not after exposure to control human Ig. However, serum 1 [collected from an Rh(D)-negative mother, whose infant was confirmed to be Rh(D) positive, DAT positive, and jaundiced and had both hypoglycemia and hyperinsulinemia], resulted in significantly ($P < 0.001$) higher cell proliferation and chronic insulin secretion when compared with cells grown in media supplemented with anti-D antibody alone. It is possible that some synergistic effect with other serum components, such as growth factors and cytokines, like TGF-β, which is induced by Rh-immune globulin (20), may have accounted for the observed results. It was also noted that the augmented islet cell activity behaved physiologically in that further enhancement of insulin secretion occurred upon exposure to an elevated glucose concentration, demonstrating that the increased cell division did not induce autonomous behavior in the treated islet cell population, with no sign of disordered glucose metabolism in glucose response data or dysmorphic changes in the islet cells, other than the exaggerated proliferation. The cultures remained viable and healthy throughout, and the number of β-cells increased significantly over the 8-d period in the presence of sera 1, 3, 4, and anti-D. The double Ki67 and insulin immunofluo-

FIG. 5. Monolayer cultures of adult human islets from Rh-positive donors supplemented with anti-D Ig, anti-D monoclonal antibodies BRAD-3, BRAD-5, and FOG-1 (15 μg/ml), and normal human serum. A, Cell proliferation as measured by [³H]thymidine incorporation; B, chronic insulin secretion; C, stimulation by 16.7 mM glucose on the acute regulated release of immunoreactive insulin. Results are expressed as the mean ± SEM (values represent eight replicates in each of four separate experiments; SE were very tight in cell proliferation studies and in some cases are contained within the data point).

via the indirect method of using a biotinylated antibody in the first step followed by a second incubation with streptavidin-PE to incorporate the fluorescent dye. Only the biotinylated monoclonal anti-D followed by streptavidin-PE showed strongly positive MCF (60.4 ± 9.7), whereas the two other biotinylated Igs
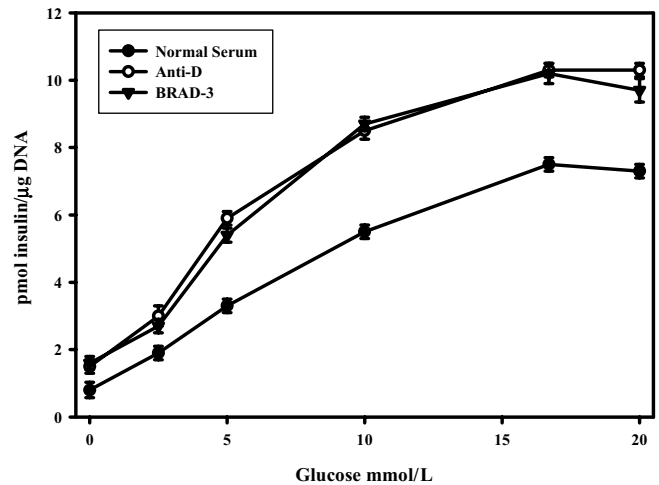
rescent staining confirmed that anti-D stimulated proliferation of β-cells and discounts the likelihood that the increase in insulin secretion was due to any extraneous factors. The fact that islet cell samples isolated from Rh(D)-negative human donors were unresponsive to exposure to anti-D Ig or maternal sera containing anti-D further supports our hypothesis that isoimmunization may induce islet cell proliferation.

The growth-stimulating effect of Rh(D) antibody *in vitro* was likely a consequence of the presence and/or binding of anti-D Ig at the cell surface. The Rh(D) antigen is not only expressed on erythrocytes but has also been described in other tissues from Rh(D)-positive individuals (21), although this is believed to be the first report of islet cells expressing the antigen. It is not surprising that the IgG3 monoclonal BRAD-3 was more effective in stimulating β-cell proliferation than the IgG1 monoclonals, because BRAD-3 has been shown in other studies to be more effective than IgG1 monoclonal antibodies in the clearing of Rh(D)-positive red cells (9, 10). The implication that the Rh glycoproteins are specific ammonium transporters may have some bearing on the observed proliferation of β-cells in the presence of anti-D, although RhD expression itself has not been implicated in such transport mechanisms in erythroid cells (22).

Aside from the possible associated problems of the widespread use of immunosuppressive drugs and other questions about impaired glucose tolerance (23, 24), the main obstacle for widespread islet transplantation for type I diabetes is scarcity of donors. Various growth factors have been reported to expand islet cell mass *in vitro* and *in vivo*, such as PTH-related protein (25), hepatocyte growth factor (26), prolactin (27), and IGF-I and -II (28, 29). In several studies, the proliferation of islet cells has been linked to the division of ductal cells (29, 30), which lose their specific duct phenotype with proliferation, resulting in multipotent cells that can subsequently differentiate into islet cells, in the presence of appropriate external stimuli, over a period of 3–4 wk (30). However, the proliferation of insulin-secreting, glucose-responsive tissue in this study occurred from 4 d onward and therefore may be indicative of proliferation of the original β-cell mass. Evidence from other studies has shown that terminally differentiated β-cells retain a proliferative capacity that is recognized as an important regulator of the adult β-cell mass (31–33).

Although preliminary, the results of this study suggest the presence of a factor in maternal serum containing a hemolytic anti-D antibody that exerts growth-stimulating effects on isolated primary human islet cells. Similar responses were observed after culture in medium containing the human anti-D Ig and specific anti-D monoclonal antibodies. Such monoclonal antibodies directed against a specific Rh(D) epitope may be ultimately developed for future therapeutic use.

Acknowledgments

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