Combination Therapy with Glucagon-Like Peptide-1 and Gastrin Restores Normoglycemia in Diabetic NOD Mice

Wilma L. Suarez-Pinzon,1 Robert F. Power,2 Yanhua Yan,3 Clive Wasserfall,4 Mark Atkinson,4 and Alex Rabinovitch1

From the 1Department of Medicine, University of Alberta, Edmonton, Alberta, Canada; 2Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, Canada; 3Waratah Pharmaceuticals, Woburn, Massachusetts, and 4Department of Pathology, University of Florida, Gainesville, Florida.

Address correspondence and reprint requests to:
Alex Rabinovitch, MD, 1-009 HRIF-East, University of Alberta, Edmonton, Alberta, Canada T6G 2E1
E-mail: alex.rabinovitch@ualberta.ca

Submitted 21 May 2008 and accepted 18 September 2008.
ABSTRACT

Objective. Glucagon-like peptide-1 (GLP-1) and gastrin promote pancreatic β-cell function, survival and growth. Here we investigated whether GLP-1 and gastrin can restore the β-cell mass and reverse hyperglycemia in NOD mice with autoimmune diabetes.

Research design and methods. Acutely diabetic NOD mice were treated with GLP-1 and gastrin, separately or together, twice daily for 3 weeks. Blood glucose was measured weekly and for a further 5 weeks following treatments, after which pancreatic insulin content and β-cell mass, proliferation, neogenesis, and apoptosis were measured. Insulin auto-antibodies were measured and adoptive transfer of diabetes as well as syngeneic islet transplant studies were done to evaluate the effects of GLP-1 and gastrin treatment on autoimmunity.

Results. Combination therapy with GLP-1 and gastrin, but not with GLP-1 or gastrin alone, restored normoglycemia in diabetic NOD mice. The GLP-1 and gastrin combination increased pancreatic insulin content, β-cell mass, and insulin-positive cells in pancreatic ducts, and β-cell apoptosis was decreased. Insulin auto-antibodies were reduced in GLP-1 and gastrin-treated NOD mice, and splenocytes from these mice delayed adoptive transfer of diabetes in NOD-scid mice. Syngeneic islet grafts in GLP-1 and gastrin-treated NOD mice were infiltrated by leukocytes with a shift in cytokine expression from interferon-γ (IFN-γ) to transforming growth factor-β1 (TGF-β1), and β-cells were protected from apoptosis.

Conclusions. Combination therapy with GLP-1 and gastrin restores normoglycemia in diabetic NOD mice by increasing the pancreatic β-cell mass and downregulating the autoimmune response.
Pancreatic β-cells can regenerate in response to experimental injury in adult animals (1-3), and can increase in humans in response to conditions such as pregnancy (4) and obesity (5). In addition, there is histological evidence of attempts at β-cell regeneration in humans with type 1 diabetes (6,7). Similarly, β-cell proliferation is increased before diabetes onset in NOD mice, an animal model for human type 1 diabetes, but not sufficiently to keep up with the ongoing autoimmune response that decreases the β-cell mass (8). Therefore, therapies directed at stimulating β-cell regeneration, in addition to arresting autoimmunity, may restore the β-cell mass and reverse type 1 diabetes.

Many putative β-cell growth factors have been identified, one of the most promising being glucagon-like peptide-1 (GLP-1), a peptide secreted from intestinal L-cells in response to nutrient ingestion (9). The actions of GLP-1 to stimulate glucose-dependent insulin secretion and inhibit glucagon release, gastric emptying and food intake (10) have led to its application as a therapy for type 2 diabetes (10). GLP-1 has additional actions that suggest a therapeutic role in conditions with a deficit in β-cell mass. GLP-1 and long-acting GLP-1 receptor agonists, such as exendin-4, increase the β-cell mass in rodents with surgically- or chemically-induced diabetes through stimulation of β-cell proliferation and islet neogenesis, and inhibition of β-cell apoptosis (12-15). Also, GLP-1 (16) and exendin-4 (17) reduce insulitis and protect β-cells in NOD mice when given before diabetes onset. Exendin-4 has also been reported to reverse diabetes in NOD mice; however, this required combination of exendin-4 with immunosuppressive therapy using anti-lymphocyte serum (18).

Gastrin is a gastrointestinal peptide reported to induce β-cell neogenesis from pancreatic exocrine duct cells in rodents (19,20). Combined gastrin and epidermal growth factor (EGF) treatment induces islet regeneration and restores normoglycemia in alloxan-treated mice (21) and ameliorates hyperglycemia after diabetes onset in NOD mice (22). Here, we report that addition of gastrin to GLP-1 treatment restored normoglycemia in acutely diabetic NOD mice, by increasing the pancreatic β-cell mass and downregulating the autoimmune response.

**RESEARCH DESIGN AND METHODS**

**Animals.** NOD female mice, age 6-8 weeks, were purchased from Taconic (Germantown, NY). NOD-scid female mice were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were housed and fed under specific pathogen-free conditions and were cared for according to the guidelines of the Canadian Council on Animal Care. The NOD mice were monitored daily by urine testing using Keto-Diastix reagent strips (Bayer, Etobicoke, ON, Canada). Diabetes onset was diagnosed by the presence of glucosuria (>6 mmol/l), ketonuria (>1.5 mmol/l), and a nonfasting blood glucose ≥10 mmol/l measured at 4-6 p.m. on 2 consecutive days, using a blood glucose meter and test strip (Ascencia Elite; Bayer). Treatments were started within 3-6 days after diabetes onset in NOD mice, age 12-16 weeks.

**Diabetes treatments.** Acutely diabetic NOD mice of similar ages were randomly allocated into seven groups: a pretreatment (baseline) group, and six groups treated for 3 weeks with twice-daily intraperitoneal injections of PBS vehicle (control), 10 µg/kg GLP-1, 100 µg/kg GLP-1, 1.5 µg/kg gastrin, 10 µg/kg GLP-1 plus 1.5 µg/kg gastrin, and 100 µg/kg GLP-1 plus 1.5 µg/kg gastrin. GLP-1 was synthetic human GLP-1 [7-36] amide (Bachem, Torrance, CA). Gastrin was human gastrin-17 synthesized and purified to >97% by
GLP-1 and gastrin cure diabetes in NOD mice

HPLC (Starr Biochemicals, Torrance, CA). This gastrin-17 has a leucine substitution for methionine at position 15 to prevent oxidation and is equipotent to native gastrin-17 (23). GLP-1 and gastrin endotoxin-free powders were dissolved in sterile 100 mmol/l NaCl and 50 mmol/l NaPO₄ (pH 7.4) at a stock concentration of 3 µg/ml, stored in aliquots at −70º C, then thawed, diluted in sterile PBS (pH 7.4), kept at 4º C, and used within 2 days. No insulin treatments were given. Blood glucose concentrations were measured in nonfasted mice at 4-6 p.m. once a week for 3 weeks during treatments and for another 5 weeks after treatments were stopped. Mice with blood glucose levels ≥27.5 mmol/l and losing weight were killed by sodium pentobarbital overdose before 8 weeks; all other mice were killed at 8 weeks (study end). Blood was collected for C-peptide and insulin auto-antibody assays. Pancreata were removed, kept on ice, cleaned of fat and lymph nodes, weighed, and divided longitudinally from head to tail into two equal portions to assay insulin content and for histological studies.

**Plasma C-peptide.** C-peptide levels in plasma were measured using a radioimmunoassay kit specific for rat or mouse C-peptide (Linco Research, St. Charles, MO).

**Insulin auto-antibodies.** Insulin auto-antibodies in serum were measured by a method previously described (24).

**Pancreatic insulin content.** One-half of each pancreas was weighed, minced with fine scissors in 1.0 ml acidified ethanol (75% ethanol, 1.5% 12 mmol/l HCl, and 23.5% H₂O₂), and incubated for 24 h at 4º C to extract insulin from tissue. The ethanolic extracts were diluted in assay buffer, and insulin was measured using a radioimmunoassay kit for mouse insulin (Linco).

**Pancreatic histology.** The other half of each pancreas was fixed in 10% buffered formalin and embedded in paraffin. Serial sections 4.5 µm thick were cut. Deparaffinized sections were stained for β-cells by an immunoperoxidase technique. The sections were incubated with a guinea pig anti-insulin antibody (Dako, Carpenteria, CA), then with a biotinylated goat anti-guinea pig antibody (Vector, Burlingame, CA) and a streptavidin peroxidase conjugate and chromogen (ISHC DAB kit; InnoGenex, San Ramon, CA). Sections were counterstained with hematoxylin.

**Pancreatic β-cell mass.** β-cell mass was determined by point counting morphometry on the insulin immunostained pancreatic sections using a Nikon E400 microscope connected to a video camera with a color monitor at 265x magnification. Each section was counted using a 192-point grid; at least 100 fields were counted for each tissue block. The β-cell relative volume was calculated by dividing the number of points over insulin-positive cells by the number of points over the total pancreatic tissue. β-cell mass was determined by multiplying the β-cell relative volume by the total weight of the pancreas.

**Pancreatic β-cell replication, apoptosis, and neogenesis.** Pancreatic sections were first treated with an enzymatic antigen-retrieval and blocker for mouse antigens (Ventana Medica Systems, Tucson, AZ). Replicating β-cells were identified by staining for insulin as described for the pancreatic histology studies, and then by incubation with a mouse anti-human monoclonal antibody (mAb) (clone PC-10, Dako) to the proliferating cell nuclear antigen (PCNA), followed by a universal secondary antibody and streptavidin alkaline phosphatase-blue kit (Ventana). To identify β-cells in apoptosis, pancreatic sections immunostained for β-cells were then stained using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method and a cell death detection kit (Roche Diagnostics, Laval, QC, Canada), followed by a streptavidin alkaline phosphatase-blue kit (Ventana). Replicating
duct cells were identified by staining for PCNA+ nuclei, followed by a mouse anti-human cytokeratin 20 (CK20) mAb (Dako) that identifies rodent ductal epithelial cells capable of islet cytodifferentiation (25), and then a biotinylated goat anti-human antibody and a streptavidin peroxidase-red kit (Ventana). Pancreatic duct cells that expressed insulin were identified by staining for insulin, then for duct cells using the CK20 mAb, followed by a biotinylated goat anti-human antibody and a streptavidin alkaline phosphatase-blue kit (Ventana). Sections were counterstained with 0.2% methyl green. Three sections (each, 15-25 mm²) cut 200 μm apart, were scanned for each set of stainings for each pancreas, using the Scanscope Imagescope system (Aperio Technologies, Vista, CA) at 40x magnification. The digital images were analyzed with Scanscope software (Aperio) and the number of positive signals as a percent of the total (positive + negative) signals representing the total pancreatic tissue area scanned was determined by the Positive Pixel Count Algorithm (Aperio).

**Adoptive transfer of diabetes.** Diabetic NOD mice treated with PBS vehicle for 3 weeks, and with a blood glucose of 20-25 mmol/l, provided diabetogenic splenic cells. Diabetic NOD mice that were treated with 100 μg/kg GLP-1 and 1.5 μg/kg gastrin for 3 weeks, and that became normoglycemic (3.5-6.5 mmol/l) for 5 further weeks without treatments, provided splenic cells to be tested for immunoregulatory activity. The mice were monitored for recurrence of hyperglycemia (>12 mmol/l) and islet grafts were examined histologically.

**Islet transplantation.** Islets were isolated from NOD female mice, age 6-8 weeks, by collagenase digestion of the pancreas and Ficoll density gradient centrifugation and then hand-picked. Islet transplant recipients were NOD female mice that had developed diabetes and been treated with 0.5 units of a 1:1 mix of regular pork and beef insulin (Eli Lilly, Indianapolis, IN) given by subcutaneous injection once daily for 3-5 weeks before transplantation. Fifty mice, pooled from 3-4 donor NOD mice, were transplanted under the left renal capsule in each diabetic NOD mouse 24 h after withdrawal of insulin, as previously described (26). The islet recipient mice were treated from the time of islet transplantation (day 0) with either PBS vehicle or 100 μg/kg GLP-1 and 1.5 μg/kg gastrin, given twice daily by intraperitoneal injection. Blood glucose returned to normal (3.5 – 6.5 mmol/l) for the first 5 days after islet transplantation in all mice. In the first study, the mice were monitored for recurrence of hyperglycemia (>12 mmol/l) and islet grafts were examined histologically. In the second study, islet grafts were removed at 10 days after transplantation and examined by immunocytochemistry for cell composition.

**Islet graft histology.** Islet grafts were removed with a portion of underlying kidney, fixed, embedded, sectioned, and stained for β-cells using an immunoperoxidase technique, as for the pancreatic histology studies.

**Immunocytochemistry of islet grafts.** Islet grafts were cut into small pieces with fine scissors, disrupted by syringe injection through progressively narrower gauge needles, and dissociated into single cells by incubation in Ca2+ Mg2+-free PBS with 0.2 mg/ml EDTA (Life Technologies, Burlington, ON, Canada). Total β-cells and β-cells in replication or apoptosis were identified by immunocytochemical methods, as previously described (27). Briefly, cells were fixed in 4% paraformaldehyde and placed on glass
glides coated with 3-aminopropyltriethoxysilane. To identify replicating β-cells, cells were stained with a guinea pig anti-insulin antibody, followed by staining with an antibody to PCNA, as for the pancreatic sections. To identify β-cells in apoptosis, cells were stained with a guinea pig anti-insulin antibody, then with a biotinylated goat anti-guinea pig antibody and a streptavidin alkaline phosphatase conjugate and chromogen (BCIP-NBT kit; Biomeda, Foster City, CA). This was followed by staining using the TUNEL method and a cell death detection kit (Roche), then a streptavidin peroxidase conjugate and a DAB nickel chromogen (Vector).

Leukocytes from islet grafts were stained with a rat mAb to mouse total leukocytes (CD45+ cells [Ly-5/T200]) or rat IgG control antibody, then biotinylated goat anti-rat mouse-absorbed IgG and streptavidin alkaline phosphatase conjugate and chromogen kit (Biomeda). Cytokine production by the CD45+ leukocytes was detected by methods previously described (27). Briefly, the CD45-stained cell preparations were permeabilized by incubation in 0.3% saponin in PBS, and then stained with either a rat anti-mouse IFN-γ mAb (XMG 1.2, rat IgG1; Cedarlane, Hornby, ON, Canada) or rat IgG1 control antibody. Other slides were stained with either a rabbit antibody specific for TGF-β1 (sc-146; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit IgG control antibody. Biotinylated rabbit anti-rat mouse absorbed IgG was the secondary antibody for the anti-IFN-γ primary antibody, and biotinylated goat anti-rabbit IgG was the secondary antibody for the anti-TGF-β1 primary antibody. All slides were incubated with a streptavidin peroxidase conjugate and DAB nickel chromogen (Vector). Cell preparations were stained in duplicate with each test or control antibody, and 3,000 cells were scored by two independent observers who each scanned 60 different microscopic fields (oil immersion, 100x).

**Statistical analyses.** Data are expressed as means ± SE. The Mann Whitney rank sum test was used to compare two groups, and a one-way ANOVA followed by Dunnett’s multiple comparison test was used when more than two groups were compared. Correlations were analyzed by non-linear regression and ANOVA. P<0.05 was considered significant. All analyses were done using InStat 3 Windows (GraphPad Software, San Diego, CA).

**RESULTS**

Effects of GLP-1 and gastrin on blood glucose and body weight. The diabetic mice treated with vehicle for 3 weeks were killed at 3-7 weeks because of weight loss (from 24.7 ± 0.7 g to 22.8 ± 0.5 g, n = 6) and severe hyperglycemia (≥27.5 mmol/l) (Fig. 1). Treatment with GLP-1 for 3 weeks restored normoglycemia (3.5-6.5 mmol/l) at 4-8 weeks in two of seven mice that received 10 µg/kg GLP-1 and also in two of seven mice treated with 100 µg/kg GLP-1. Treatment with gastrin slowed hyperglycemia progression in some mice; however, none of four mice that received gastrin alone were normoglycemic at 8 weeks. In contrast, 10 µg/kg GLP-1 plus gastrin restored normoglycemia in four of six mice at 4-8 weeks, and 100 µg/kg GLP-1 plus gastrin restored normoglycemia in seven of seven mice at 4-8 weeks (Fig. 1). The GLP-1 and gastrin-treated mice that became normoglycemic (11 of 13) gained weight, from 25.2 ± 0.5 g to 27.1 ± 0.6 g as they aged over 8 weeks similar to non-diabetic mouse strains.

**Pancreatic insulin content.** Figure 2A shows that the pancreatic insulin content in acutely diabetic mice before treatments (1.42 ± 0.40 µg) was greatly reduced after 3 weeks of vehicle treatment (0.04 ± 0.02 µg). Pancreatic insulin content was not significantly different from the pretreatment
GLP-1 and gastrin cure diabetes in NOD mice

level in mice that received gastrin, 10 µg/kg GLP-1, or 100 µg/kg GLP-1. In contrast, combined treatment with 10 µg/kg GLP-1 and gastrin increased pancreatic insulin content 3-fold over the pretreatment level (4.75 ± 2.00 µg vs. 1.42 ± 0.40 µg; P = 0.10, NS), and 100 µg/kg GLP-1 plus gastrin increased pancreatic insulin content 5-fold over the pretreatment level (7.61 ± 1.97 µg vs. 1.42 ± 0.40 µg; P <0.01). Pancreatic insulin content in acutely diabetic mice before treatment (1.42 ± 0.40 µg) was 14% of normal (10.25 ± 0.85 µg), whereas 100 µg/kg GLP-1 plus gastrin increased pancreatic insulin content to 74% of normal (7.61 ± 1.97 µg vs. 10.25 ± 0.85 µg).

**Plasma C-peptide.** Plasma C-peptide levels reflected pancreatic insulin contents. The plasma C-peptide level in the mice treated with 100 µg/kg GLP-1 and gastrin (0.95 ± 0.18 pmol/ml, n = 6) was ~ 50% of the C-peptide level in normoglycemic NOD-scid mice (1.96 ± 0.13 pmol/ml, n = 6), and barely detectable in vehicle-treated mice (0.03 ± 0.01 pmol/ml).

**Pancreatic β-cell mass.** Figure 2B shows that the pancreatic β-cell mass in acutely diabetic mice before treatments (0.25 ± 0.10 mg) was greatly reduced in vehicle-treated mice (0.01 ± 0.01 mg). Pancreatic β-cell mass was not significantly different from the pretreatment level in mice that received gastrin, 10 µg/kg GLP-1, or 100 µg/kg GLP-1. In contrast, combined treatment with 10 µg/kg GLP-1 and gastrin increased pancreatic β-cell mass 2-fold over the pretreatment level (0.57 ± 0.24 mg vs. 0.25 ± 0.10 mg; P = 0.15, NS), and 100 µg/kg GLP-1 plus gastrin treatment increased pancreatic β-cell mass 3-fold over the pretreatment level (0.74 ± 0.25 mg vs. 0.25 ± 0.10 mg; P <0.05). Pancreatic β-cell mass in acutely diabetic mice before treatment (0.25 ± 0.10 mg) was 18% of normal (1.40 ± 0.22 mg), whereas 100 µg/kg GLP-1 plus gastrin increased pancreatic β-cell mass to 53% of normal (0.74 ± 0.25 mg vs. 1.40 ± 0.22 mg).

**Correlations of blood glucose with pancreatic insulin and β-cell mass.** Reductions in hyperglycemia by gastrin, GLP-1, and GLP-1 plus gastrin correlated significantly with increases in pancreatic insulin content (Fig. 3A) and β-cell mass (Fig. 3B). Restoration of the pancreatic insulin content to ~30% of normal (~3 µg vs. ~10 µg in NOD-scid mice) was sufficient to bring the blood glucose level down to normal (5.4 ± 0.6 mmol/l in NOD-scid mice) (Fig. 3A). Similarly, restoration of the pancreatic β-cell mass to ~35% of normal (~0.5 mg vs. 1.4 mg in NOD-scid mice) was sufficient to correct hyperglycemia (Fig. 3B).

**Pancreatic histology.** Histological examination of NOD mice pancreata after diabetes onset revealed islets heavily infiltrated by leukocytes and few islets with remaining β-cells (Fig. 4A). Vehicle-treated mice were severely hyperglycemic and their islets were heavily infiltrated by leukocytes and almost devoid of β-cells (Fig. 4B). In contrast, normoglycemia was restored in GLP-1 and gastrin-treated mice, and islets with abundant insulin-containing β-cells were found; also, some islets were surrounded by leukocytes but most were not infiltrated (Fig. 4C).

**Pancreatic β-cell replication, apoptosis, and neogenesis.** The increase in pancreatic β-cells in GLP-1 and gastrin-treated NOD mice (Fig. 5A) was associated with a significant decrease in β-cell apoptosis (Fig. 5B). Pancreatic β-cell replication rates were low (~1% of total β-cells) and not changed by GLP-1 and gastrin therapy (Fig. 5C). In contrast, replication of pancreatic duct cells was significantly increased by GLP-1 and gastrin (Fig. 5D). Also, insulin-positive cells in ducts were found more frequently after GLP-1 and gastrin treatment (6 ± 1% of duct cells) than before treatment (2 ± 1%) or after vehicle treatment (1 ± 1%) (Figs. 5E and 5F).

**Insulin auto-antibodies.** Insulin auto-antibodies were present in acutely diabetic
NOD mice before treatment and these antibodies rose to very high levels in vehicle-treated mice, whereas insulin auto-antibodies were undetectable in GLP-1 and gastrin-treated mice (Fig. 6). This suggested that GLP-1 and gastrin therapy had altered the autoimmune response against β-cells.

**Adoptive transfer of diabetes.** Splenocytes from GLP-1 and gastrin-treated diabetic NOD mice in remission protected from diabetes development when mixed with diabetogenic splenocytes and transferred into immunodeficient NOD-scid mice (Fig. 7). This suggested activation of immunoregulatory cells in NOD mice treated with GLP-1 and gastrin.

**Islet transplantation.** Islet grafts were destroyed and diabetes recurred within 14 days after transplantation in all mice treated with vehicle, whereas 88% (7 of 8) islet grafts survived and maintained normoglycemia in GLP-1 and gastrin-treated mice (Fig. 8A). Histological examination of the islet grafts showed abundant insulin-stained cells interspersed with leukocytes in islet grafts of GLP-1 and gastrin-treated mice (Fig. 8B), whereas no insulin-stained cells remained in islet grafts of vehicle-treated mice (Fig. 8C). Recovery of β-cells in islet grafts of GLP-1 and gastrin-treated mice was associated with significantly reduced apoptosis of β-cells (Fig. 9D) and not with any change in replication of β-cells (Fig. 9C), similar to the findings in the pancreas (Figs. 5B and 5C).

**Cytokines expressed in islet grafts.** Fewer leukocytes produced IFN-γ in GLP-1 and gastrin-treated mice (Fig. 10B) than in vehicle-treated mice (Fig. 10A) and the difference was significant (Fig. 10C). In contrast, more leukocytes produced TGF-β1 in GLP-1 and gastrin-treated mice (Fig. 10E) than in vehicle-treated mice (Fig. 10D) and the difference was significant (Fig. 10F). Therefore, GLP-1 and gastrin appeared to have shifted the immune response from a cytotoxic (IFN-γ) to a regulatory (TGF-β1) one.

**DISCUSSION**

Previous reports of diabetes reversal in NOD mice following immunotherapy suggested β-cell regeneration (28-31). More recent studies, however, revealed that immunotherapy abrogated autoimmunity and allowed existent β-cells to recover their insulin secretory function, but β-cell mass did not increase (32,33). Also, addition of the GLP-1 analog, exendin-4, to anti-CD3 immunotherapy improved β-cell function but did not increase β-cell mass in diabetic NOD mice (34). Similarly, in the present study we found that GLP-1 therapy alone did not significantly increase either insulin content or β-cell mass in diabetic NOD mice; however, addition of gastrin to GLP-1 significantly increased both insulin content and β-cell mass in the diabetic mice. Gastrin induces β-cell neogenesis from pancreatic exocrine duct cells (19,20) and potentiates the growth-promoting effects of EGF on the pancreatic β-cell mass (21,22). We previously reported that this action of gastrin is via induction of the pancreatic and duodenal homeobox transcription factor, PDX-1, in pancreatic duct cells followed by insulin expression in these cells (35). Collectively, these findings suggest that gastrin can stimulate epithelial cells in the pancreatic ducts to differentiate along a β-cell pathway.

GLP-1 and exendin-4 can increase the β-cell mass in rodents with experimentally-induced deficits in β-cell mass through stimulation of β-cell proliferation and neogenesis, and inhibition of β-cell apoptosis (12-15). GLP-1 (16) and exendin-4 (17) reduced insulitis and protected β-cells in NOD mice when given before diabetes onset, and β-cell neogenesis from duct cells was reported (16). In the present study, we found that the increase in pancreatic β-cell mass induced by GLP-1 and gastrin combination
therapy was associated with an increase in duct-associated β-cells and a decrease in β-cell apoptosis. We do not know, however, to what extent the increase in duct-associated β-cells induced by GLP-1 and gastrin may have contributed to the increase in β-cell mass. We evaluated β-cell replication, neogenesis, and apoptosis at only one time point, three to five weeks after vehicle or GLP-1 and gastrin treatments, and compared these to the pre-treatment (baseline) values. Therefore, we cannot exclude an earlier contribution of β-cell proliferation during the GLP-1 and gastrin-induced restoration of β-cell mass and normoglycemia. Also, a time course study of PDX-1 and insulin staining of ductal cells would be required to provide more definitive evidence for β-cell neogenesis by GLP-1 and gastrin. Regarding the anti-apoptotic effect of GLP-1 and gastrin on β-cells, this may have been conferred on pre-existent as well as on newly generated β-cells.

Interestingly, partial (~35%) restoration of the pancreatic β-cell mass by GLP-1 and gastrin was sufficient to restore normoglycemia in the diabetic NOD mice. This is similar to findings in obese human subjects in whom hyperglycemia and type 2 diabetes was observed only after pancreatic β-cell mass was reduced below ~one-third of that in normoglycemic individuals (36).

The finding that normoglycemia persisted for at least 5 weeks after the 3-week course of GLP-1 and gastrin suggested that, in addition to restoration of β-cell mass, the expected autoimmune response had been arrested. Indeed, we found that insulin auto-antibodies rose to extremely high levels as diabetes progressed in vehicle-treated mice, whereas these auto-antibodies were undetectable in a few mice treated with either GLP-1 or gastrin, and in all the mice treated with GLP-1 and gastrin. This suggests that both GLP-1 and gastrin contributed to regulating the autoimmune response against islet β-cells. An adoptive transfer study suggested activation of immunoregulatory cell activity by GLP-1 and gastrin. In addition, the expected autoimmune response to syngeneic islet transplantation was largely abolished by GLP-1 and gastrin. Although leukocytic infiltration of the islet graft was extensive in GLP-1 and gastrin-treated mice, β-cells were not destroyed. Importantly, survival of β-cells in islet grafts of GLP-1 and gastrin-treated mice could be attributed to the anti-apoptotic effects of GLP-1 and gastrin on β-cells, and not to any stimulatory effects on β-cell replication, similar to our findings in the pancreas of GLP-1 and gastrin-treated mice.

Together with the anti-apoptotic effect of GLP-1 and gastrin on β-cells in the islet graft, there was a shift in the cytokine profile of the islet graft-infiltrating leukocytes from IFN-γ to TGF-β1 production. IFN-γ-producing leukocytes in islets are associated with β-cell destruction (37), whereas TGF-β1-producing leukocytes regulate (suppress) the autoimmune response (38). Therefore, the GLP-1 and gastrin-induced shift from an IFN-γ to a TGF-β1 type response may explain why the leukocytic infiltrate did not damage β-cells in the graft. Alternatively, or in addition, GLP-1 and gastrin may have had direct anti-apoptotic effects on β-cells, as reported for GLP-1 (15). Further studies are required to determine how GLP-1 and gastrin shifted the immune response from a cytotoxic to a regulatory one, and whether this was secondary to the anti-apoptotic effects of GLP-1 and gastrin on β-cells and/or a direct action of the gastrointestinal peptides on cells of the immune system. GLP-1 receptor mRNA transcripts have been detected in spleen, thymus, and lymph nodes of NOD mice (17). Also, a preliminary report demonstrated the expression of the GLP-1 receptor on human T lymphocytes and endogenous GLP-1 modulated CD8+ T cell migration in vitro (39). Further studies of the responses of leukocytes from GLP-1 and gastrin-treated NOD mice to NOD islet
antigens in vitro may help to clarify the mechanisms for the immunological effects we observed in vivo.

In summary, a short course of GLP-1 and gastrin partially restored pancreatic β-cell mass and arrested autoimmune destruction of β-cells in acutely diabetic NOD mice, thereby restoring normoglycemia. These findings suggest that GLP-1 and gastrin combination therapy may target both the cellular mechanisms that promote growth and survival of β-cells and the immunologic mechanisms that destroy β-cells, thereby offering a promising strategy for correction of the β-cell loss in type 1 diabetes.

ACKNOWLEDGEMENTS
This work was supported by grants from the Juvenile Diabetes Research Foundation International (17-2006-1024) and Transition Therapeutics, Inc., and by core support from the MacLachlan Fund of the University of Alberta Hospital Foundation, and the Muttart Diabetes Research and Training Centre at the University of Alberta.
REFERENCES

1. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE: A second pathway for regeneration of adult exocrine and endocrine pancreas: a possible recapitulation of embryonic development. *Diabetes* 42:1715-1720, 1993
2. Vinik A, Pittenger G, Rafaeloff R, Rosenberg L, Duguid WP: Determinants of pancreatic islet cell mass: a balance between neogenesis and senescence/apoptosis. *Diabet Rev* 4:235-263, 1996
3. Gu D, Sarvetnick N: Epithelial cell proliferation and islet neogenesis in IFN-γ transgenic mice. *Development* 118:33-46, 1993
4. Van Assche FA, Aerts L, DePrins F: A morphological study of the endocrine pancreas in human pregnancy. *Br J Obstet Gynaecol* 85:818-820, 1978
5. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102-110, 2003
6. Gepts W, deMay J: Islet cell survival determined by morphology. An immunocytochemical study of the islets of Langerhans in juvenile diabetes mellitus. *Diabetes* 27 (Suppl. 1):251-261, 1978
7. Meir JJ, Bhushan A, Butler AE, Rizza RA, Butler PC: Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration. *Diabetologia* 48:2221-2228, 2005
8. Sreenan S, Pick AJ, Levisetti M, Baldwin AC, Pugh W, Polonsky KS: Increased β-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse. *Diabetes* 48:989-996, 1999
9. Brubaker PL, Anini Y: Direct and indirect mechanisms regulating secretion of glucagon-like peptide-1 and glucagon-like peptide-2. *Can J Physiol Pharm* 81:1005-1012, 2003
10. Drucker DJ: Biological actions and therapeutic potential of the glucagon-like peptides. *Gastroenterology* 122:531-544, 2002
11. Holst JJ: Glucagon-like peptide-1: from extract to agent. The Claude Bernard Lecture, 2005. *Diabetologia* 49:253-260, 2006
12. Xu G, Stoffers DA, Habener JF, Bonnier-Weir S: Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 48:2270-2276, 1999
13. Tourrel C, Bailbé D, Meile MJ, Kergoat M, Portha B: Glucagon-like peptide-1 and exendin-4 stimulate β-cell neogenesis in streptozotocin-treated newborn rats resulting in persistently improved glucose homeostasis at adult age. *Diabetes* 50:1562-1570, 2001
14. Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ: Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. *J Biol Chem* 278:471-478, 2003
15. Brubaker PL, Drucker DJ: Minireview: glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. *Endocrinology* 145:2653-2659, 2004
16. Zhang J, Tokui Y, Yamagata K, Kozawa J, Sayama K, Iwahashi H, Okita K, Miuchi M, Konya H, Hamaguchi T, Namba M, Shimomura I, Miyagawa JI: Continuous stimulation of human glucagon-like peptide-1 [7-36] amide in a mouse model (NOD) delays onset of autoimmune type 1 diabetes. *Diabetologia* 50:1900-1909, 2007
17. Hadjiyanni I, Baggio LL, Poussier P, Drucker DJ: Exendin-4 modulates diabetes onset in nonobese diabetic mice. *Endocrinology* 149:1338-1349, 2008
18. Ogawa N, List JF, Habener JF, Maki T: Cure of overt diabetes in NOD mice by transient treatment with anti-lymphocyte serum and exendin-4. *Diabetes* 53:1700-1705, 2004
GLP-1 and gastrin cure diabetes in NOD mice

19. Wang TC, Bonner-Weir S, Oates PS, Chulak M, Simon B, Merlino GT, Schmidt EV, Brand SJ: Pancreatic gastrin stimulates islet differentiation of transforming growth factor alpha-induced ductular precursor cells. *J Clin Invest* 92:1349-1356, 1993

20. Rooman I, Lardon J, Bouwens L: Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreatic tissue. *Diabetes* 51:686-690, 2002

21. Rooman I, Bouwens L: Combined gastrin and epidermal growth factor treatment induces islet regeneration and restores normoglycemia in C57B16/J mice treated with alloxan. *Diabetologia* 47:259-265, 2004

22. Suarez-Pinzon WL, Yan Y, Power R, Brand SJ, Rabinovitch A: Combination therapy with epidermal growth factor and gastrin increases β-cell mass and reverses hyperglycemia in diabetic NOD mice. *Diabetes* 54:2596-2601, 2005

23. Petersen B, Christinsen J, Rehfeld JF: Acid potency and elimination of the 15-leucine gastrin-17 analogue in man. *Scand J Gastroenterol* 16:437-440, 1981

24. Yu L, Eisenbarth G, Bonifacio E, Thomas J, Atkinson M, Wasserfall C: The second murine autoantibody workshop: remarkable interlaboratory concordance for radiobinding assays to identify insulin autoantibodies in nonobese diabetic mice. *Ann NY Acad Sci* 1005:1-12, 2003.

25. Bouwens L, Wang R-N, DeBlay E, Pipeleers DG, Kloppel G: Cytokeratins as markers of ductal cell differentiation and islet neogenesis in the neonatal rat pancreas. *Diabetes* 43:1279-1283, 1994.

26. Wang T, Singh B, Warnock G, Rajotte RV: Prevention of recurrence of IDDM in islet-transplanted NOD mice by adjuvant immunotherapy. *Diabetes* 41:114-117, 1992

27. Rabinovitch A, Suarez-Pinzon WL, Shapiro AMJ, Rajotte RV, Power R: Combination therapy with sirolimus and interleukin-2 prevents spontaneous and recurrent autoimmune diabetes in NOD mice. *Diabetes* 51:638-645, 2002

28. Maki T, Ichikawa T, Blanco R, Porter J: Long-term abrogation of autoimmune diabetes in nonobese diabetic mice by immunotherapy with anti-lymphocyte serum. *Proc Natl Acad Sci U S A* 89:3434-3438, 1992

29. Chatenoud L, Thervet E, Primo J, Bach JF: Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 91:123-127, 1994

30. Zorina TD, Subbotin VM, Bertera S, Alexander AM, Haluszczak C, Gambrell B, Bottino R, Styche AJ, Trucco M: Recovery of the endogenous beta cell function in the NOD model of autoimmune diabetes. *Stem Cells* 21:377-388, 2003

31. Kodama S, Kuhfreiber W, Fujimura S, Dale EA, Faustman DL: Islet regeneration during the reversal of autoimmune diabetes in NOD mice. *Science* 302:1223-1227, 2003

32. Sherry NA, Kushner JA, Glandt M, Kitamura T, Brillantes A-MB, Herold KC: Effects of autoimmunity and immune therapy on β-cell turnover in type 1 diabetes. *Diabetes* 55:3238-3245, 2006

33. Phillips JM, O’Reilly L, Bland C, Foulis AK, Cooke A: Patients with chronic pancreatitis have islet progenitor cells in their ducts, but reversal of overt diabetes in NOD mice by anti-CD3 shows no evidence for islet regeneration. *Diabetes* 56:634-640, 2007

34. Sherry NA, Chen W, Kushner JA, Glandt M, Tang Q, Tsai S, Santamaria P, Bluestone JA, Brillantes A-MB, Herold KC: Exendin-4 improves reversal of diabetes in NOD mice treated with anti-CD3 monoclonal antibody by enhancing recovery of β-cells. *Endocrinology* 148:5136-5144, 2007
35. Suarez-Pinzon WL, Lakey JRT, Brand SJ, Rabinovitch A: Combination therapy with epidermal growth factor and gastrin induces neogenesis of human islet β-cells from pancreatic duct cells and an increase in functional β-cell mass. *The Journal of Clinical Endocrinology & Metabolism* 90:3401-3409, 2005
36. Ritzel BA, Butler AE, Rizza RA, Veldhuis JD, Butler PC: Relationship between β-cell mass and fasting blood glucose concentration in humans. *Diabetes Care* 29:717-718, 2006
37. Rabinovitch A, Suarez-Pinzon WL: Role of cytokines in the pathogenesis of autoimmune diabetes mellitus. *Reviews in Endocrine and Metabolic Disorders* 4:291-299, 2003
38. You S, Thieblemont N, Alyanakian M-A, Bach J-F, Chatenoud L: Transforming growth factor-β and T-cell-mediated immunoregulation in the control of autoimmune diabetes. *Immunological Reviews* 212:185-202, 2006
39. Masur K, Beinborn M, Zaenker KS: Glucagon-like peptide-1 (GLP-1) is not only an important incretin hormone but also a modulator of the cellular immune system. *Diabetes* 53:A49-LB, 2004
FIG. 1. GLP-1 and gastrin combination therapy restores normoglycemia in NOD mice. Beginning 3-6 days after diabetes onset (0 weeks) and for the next 3 weeks, NOD mice were treated with twice-daily intraperitoneal injections of vehicle (n = 6); 10 µg/kg GLP-1 (n = 7); 100 µg/kg GLP-1 (n = 7); 1.5 µg/kg gastrin (n = 4); 10 µg/kg GLP-1 plus 1.5 µg/kg gastrin (n = 6); and 100 µg/kg GLP-1 plus 1.5 µg/kg gastrin (n = 7). Blood glucose concentrations in individual mice are shown during the 3 weeks of treatments and for the additional 5 weeks after treatments were stopped. Mice with blood glucose levels ≥ 27.5 mmol/l and losing weight were killed (†). Shaded bars show the normal range for blood glucose (3.5 – 6.5 mmol/l).
FIG. 2. GLP-1 and gastrin combination therapy increases pancreatic insulin content (A) and β-cell mass (B) in NOD mice. Pancreata were removed from acutely diabetic NOD mice before and after 3 weeks of the treatments indicated plus an additional 5 weeks after treatments were stopped, or sooner if the mice were killed because of severe diabetes as shown in Fig. 1. NOD-scid mice provide normal values for pancreatic insulin content (A) and β-cell mass (B). Values are means ± SE. *P<0.05, **P<0.01 vs. pretreatment values for the numbers of mice shown in parentheses.
FIG. 3. Partial restoration of the pancreatic insulin content (A) and β-cell mass (B) corrects hyperglycemia in NOD mice. The mice were treated for 3 weeks with vehicle (○, n = 4); 1.5 µg/kg gastrin (▲, n = 4); 10 or 100 µg/kg GLP-1 (●, n = 14); and 1.5 µg gastrin plus 10 or 100 µg/kg GLP-1 (□, n = 12). Blood glucose values at 5 weeks after treatments were stopped (or earlier if blood glucose was ≥27.5 mmol/l; Fig. 1) are plotted against the corresponding values for pancreatic insulin content (A) and β-cell mass (B) in each mouse. The curved lines were derived using nonlinear regression analysis (r = 0.985; P<0.001 in panel A, and r = 0.874; P<0.001 in panel B). Shaded bars show the mean ± SD range for normal values, as determined in NOD-scid mice (n = 6).
FIG. 4. Photomicrographs of pancreatic tissue sections. A: An NOD mouse pancreas 5 days after diabetes onset and before the start of treatments. Islets are infiltrated by leukocytes (dark blue-stained) and the few islets with remaining β-cells (brown-stained with insulin antibody) are surrounded and infiltrated by abundant leukocytes (inset a). B: An NOD mouse pancreas after treatment with vehicle for 3 weeks and an additional 3 weeks without treatment. Islets are heavily infiltrated by leukocytes and almost devoid of β-cells (inset b). C: An NOD mouse pancreas after treatment with 100 µg/kg GLP-1 and 1.5 µg/kg gastrin for 3 weeks and an additional 5 weeks without treatment. Islets with abundant insulin-stained β-cells are found and some islets are surrounded by leukocytes but most are not infiltrated and destroyed (inset c).
FIG. 5. GLP-1 and gastrin combination therapy decreases β-cell apoptosis and increases pancreatic duct-associated β-cells. β-cells (insulin⁺) were more abundant (A) and β-cell apoptosis (TUNEL⁺ insulin⁺) was decreased (B) in the pancreas of NOD mice treated with 100 µg/kg GLP-1 and 1.5 µg/kg gastrin compared with diabetic NOD mice before treatment (baseline). Replication of β-cells (PCNA⁺ insulin⁺) was similar in the pancreas of NOD mice before (baseline) and after treatments with vehicle or GLP-1 and gastrin (C), whereas replication of pancreatic duct epithelial cells (PCNA⁺ CK20⁺) was increased in GLP1 and gastrin-treated mice (D). Insulin stained cells in ducts (insulin⁺ CK20⁺) were increased in GLP-1 and gastrin-treated mice (E). Insulin-positive cells (brown-stained) in and budding from pancreatic ducts (blue-stained) are indicated by arrows (F). Values are means ± SE for 5-6 mice. *P<0.05, **P<0.01 vs. baseline.
FIG. 6. GLP-1 and gastrin combination therapy eliminates insulin auto-antibodies (IAA) in NOD mice. IAA levels were elevated (>4 IAA index, dashed line) in serum of acutely diabetic mice (GLP-1 −, gastrin −) and IAA rose to very high levels when diabetes had progressed in vehicle-treated mice (GLP-1 0, gastrin 0). Mean IAA levels (open bars) in NOD mice treated with 100 µg/kg GLP-1 only or 1.5 µg/kg gastrin only remained at approximately pretreatment levels, whereas IAA were undetectable in all the mice treated with GLP-1 and gastrin.

FIG. 7. GLP-1 and gastrin combination therapy induces immunoregulatory cell activity in NOD mice. The median time (50% diabetes incidence) for diabetes onset (blood glucose >12 mmol/l) in NOD-scid mice was 18 days when the mice were injected intravenously with 1.5 x 10⁷ splenic cells from diabetic NOD mice (△, n = 5), and 39 days when injected with 0.5 x 10⁷ splenic cells from diabetic NOD mice (○, n = 8). Diabetes onset was delayed to a median of 69 days when NOD-scid mice were injected with 0.5 x 10⁷ splenic cells from diabetic NOD mice mixed with 1.0 x 10⁷ splenic cells from GLP-1 plus gastrin-treated NOD mice (●, n = 8), and to 107 days when NOD-scid mice were injected with 0.5 x 10⁷ splenic cells from diabetic NOD mice mixed with 3.0 x 10⁷ splenic cells from GLP-1 plus gastrin-treated NOD mice (■, n = 8).
FIG. 8. GLP-1 and gastrin combination therapy prevents autoimmune destruction of syngeneic islet grafts in diabetic NOD mice. After 30 days of treatment with 100 µg/kg GLP-1 and 1.5 µg/kg gastrin, NOD islet grafts survived and maintained normoglycemia in 88% (7 of 8) mice, whereas no islet grafts (0 of 6) survived beyond 14 days and diabetes recurred in vehicle-treated mice (A). Abundant insulin-stained β-cells (in brown) interspersed with abundant leukocytes (dark blue) are seen in an islet graft under the renal capsule of an NOD mouse treated with GLP-1 and gastrin for 30 days (B), whereas no insulin-stained cells remain in an islet graft of an NOD mouse treated with vehicle for 10 days (C).

FIG. 9. GLP-1 and gastrin combination therapy prevents β-cell apoptosis. Leukocytes (CD45+) were decreased (A) and β-cells (insulin+) were more abundant (B) in syngeneic islet grafts in NOD mice treated with 100 µg/kg GLP-1 and 1.5 µg/kg gastrin than in islet grafts of NOD mice treated with vehicle for 10 days. Replication of β-cells (PCNA+ insulin+) was low and not different in islet grafts of NOD mice treated with vehicle or GLP-1 and gastrin (C). Apoptosis of β-cells (TUNEL+ insulin+) was less in islet grafts of NOD mice treated with GLP-1 and gastrin than in islet grafts of vehicle-treated NOD mice (D). Values are means ± SE for 8 mice. **P<0.01 vs. vehicle.
FIG. 10. GLP-1 and gastrin combination therapy shifts the cytokine expression of leukocytes infiltrating syngeneic islet grafts from IFN-γ to TGF-β1. At 10 days after syngeneic islet transplantation in diabetic NOD mice, leukocytes (blue-stained) expressing IFN-γ (black-stained) were more abundant in vehicle-treated mice (A) than in mice treated with 100 µg/kg GLP-1 and 1.5 µg/kg gastrin (B), whereas leukocytes (blue-stained) expressing TGF-β1 (black-stained) were more abundant in GLP-1 and gastrin-treated mice (E) than in vehicle-treated mice (D). The percent of leukocytes that produced IFN-γ (IFN-γ⁺CD45⁺) was significantly greater in vehicle-treated mice than GLP-1 and gastrin-treated mice (C), whereas the percent of leukocytes that produced TGF-β1 (TGF-β1⁺CD45⁺) was greater in GLP-1 and gastrin-treated mice than in vehicle-treated mice (F). Values are means ± SE for 8 mice. *P<0.05, **P<0.01 vs. vehicle.