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

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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 after treatments, after which pancreatic insulin content and β-cell mass, proliferation, neogenesis, and apoptosis were measured. Insulin autoantibodies were measured, and adoptive transfer of diabetes and 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 autoantibodies 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 to transforming growth factor-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. ***Diabetes*** **57:3281–3288, 2008**

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 (11). 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 antilymphocyte 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

NOD female mice, 6–8 weeks of age, were purchased from Taconic (Germantown, NY). NOD-scid female mice were purchased from The Jackson Laboratories (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 4:00–6:00 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 12- to 16-week-old NOD mice.

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 high-performance liquid chromatography (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

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Received 21 May 2008 and accepted 18 September 2008.

Published ahead of print at http://diabetes.diabetesjournals.org on 3 October 2008. DOI: 10.2337/db08-0688.

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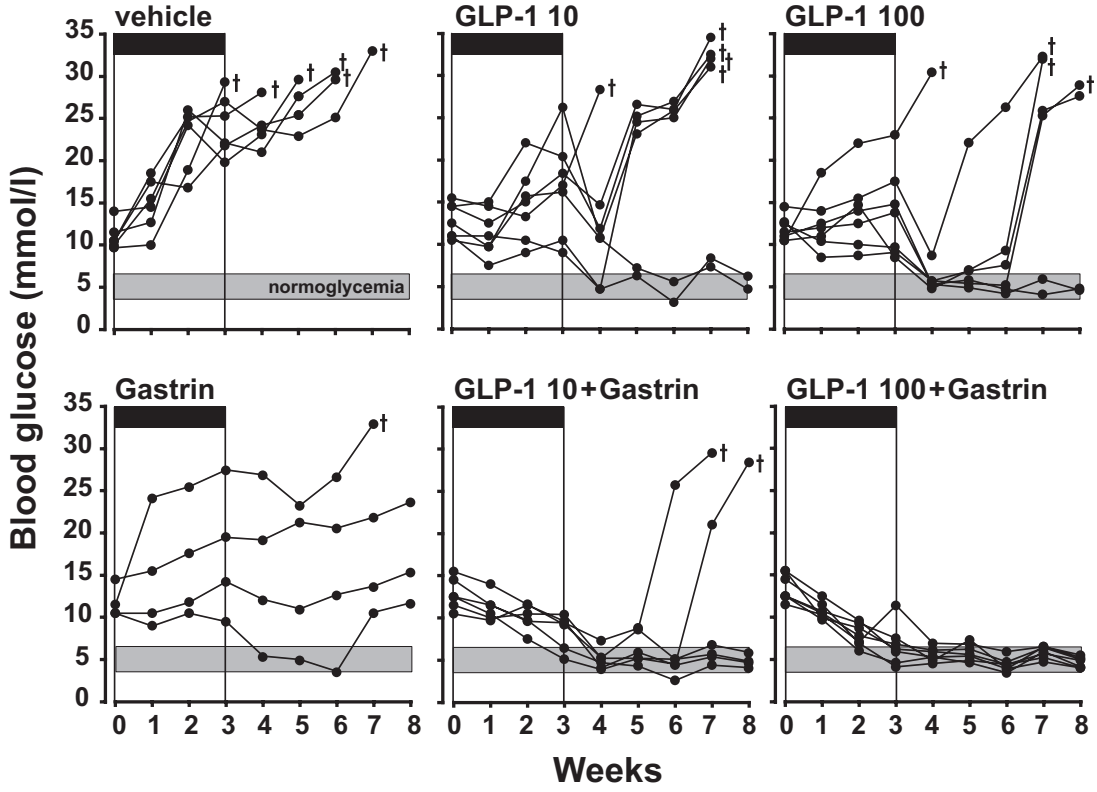

FIG. 1. GLP-1 and gastrin combination therapy restores normoglycemia in NOD mice. Beginning 3–6 days after diabetes onset (0 weeks) and for the next 3 weeks, NOD mice were treated with twice-daily intraperitoneal injections of vehicle (n = 6), 10 μg/kg GLP-1 (n = 7), 100 μg/kg GLP-1 (n = 7), 1.5 μg/kg 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).

endotoxin-free powders were dissolved in sterile 100 mmol/l NaCl and 50 mmol/l $NaPO_4$ (pH 7.4) at a stock concentration of 3 μg/ml, stored in aliquots at −70°C, then thawed, diluted in sterile PBS (pH 7.4), kept at 4°C, and used within 2 days. No insulin treatments were given. Blood glucose concentrations were measured in nonfasted mice at 4:00–6:00 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 autoantibody 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 autoantibodies. Insulin autoantibodies 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% H_2O), and incubated for 24 h at 4°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), and then with a biotinylated goat anti–guinea pig antibody (Vector, Burlingame, CA) and a streptavidin peroxidase conjugate and chromogen (iso-IHC 3,3′-diaminobenzidine (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 magnification ×265. 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 a blocking reagent for mouse antigens (Ventana Medical 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 by 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 and 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 mm^2) cut 200 μm apart were scanned for each set of stainings for each pancreas using the Scanscope Imagescope system (Aperio Technologies, Vista, CA) at magnification ×40. The digital images were analyzed with Scanscope software (Aperio), and the number of positive signals as a percentage of the total (positive plus 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. Splenic cells were pooled from six diabetic NOD mice and separately from six NOD mice treated with GLP-1 and

gastrin. The former cells, either alone or mixed together with the latter cells, were injected intravenously into five to eight NOD-scid mice. Diabetes development in the NOD-scid mice was determined by daily monitoring for glucosuria and then by detecting a blood glucose >12 mmol/l.

Islet transplantation. Islets were isolated from 6- to 8-week-old NOD female mice 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 unit 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. Five hundred islets, pooled from three to four 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 was done 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 Ca^{2+} Mg^{2+}-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 and then with a biotinylated goat anti–guinea pig antibody and a streptavidin alkaline phosphatase conjugate and chromogen (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium kit; Biomeda, Foster City, CA). This was followed by staining using the TUNEL method and a cell death detection kit (Roche) and 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 and 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 interferon-γ (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 transforming growth factor-β1 (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, ×100).

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 nonlinear 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 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 was 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 to 27.1 ± 0.6 g, as they aged over 8 weeks, similar to nondiabetic mouse strains.

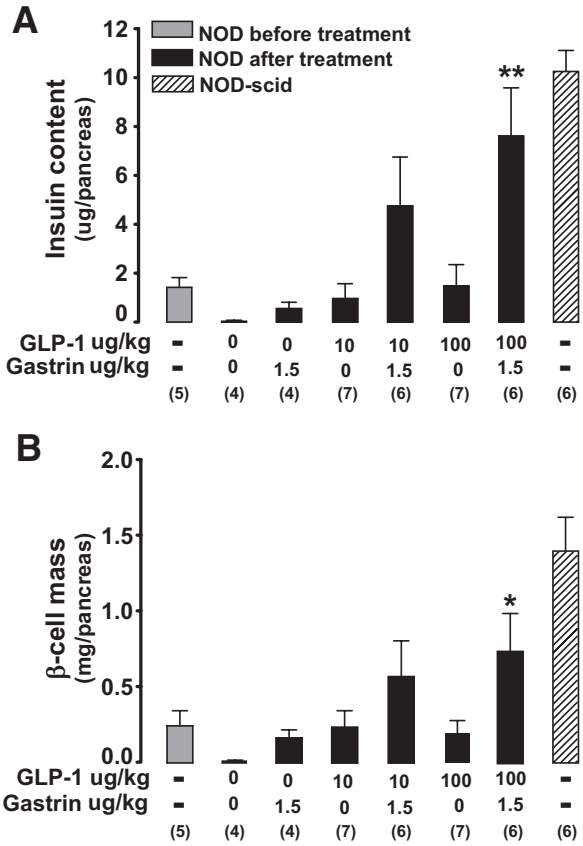

FIG. 2. GLP-1 and gastrin combination therapy increases pancreatic insulin content (*A*) and β-cell mass (*B*) in NOD mice. Pancreata were removed from acutely diabetic NOD mice before and after 3 weeks of the treatments indicated plus an additional 5 weeks after treatments were stopped or sooner if 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.

Pancreatic insulin content. Fig. 2*A* 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 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 threefold over the pretreatment level (4.75 ± 2.00 vs. 1.42 ± 0.40 μg; $P = 0.10$, NS), and 100 μg/kg GLP-1 plus gastrin increased pancreatic insulin content fivefold over the pretreatment level (7.61 ± 1.97 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 vs. 10.25 ± 0.85 μg).

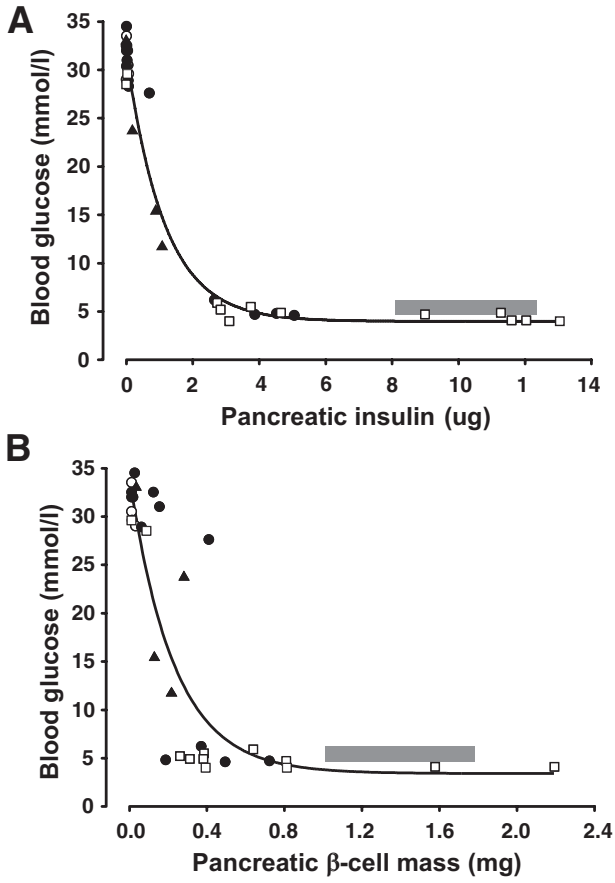

FIG. 3. Partial restoration of the pancreatic insulin content (*A*) and β-cell mass (*B*) corrects hyperglycemia in NOD mice. The mice were treated for 3 weeks with vehicle (○, $n = 4$), 1.5 μg/kg gastrin (▲, $n = 4$), 10 or 100 μg/kg GLP-1 (●, $n = 14$), and 1.5 μ*g* gastrin plus 10 or 100 μg/kg GLP-1 (□, $n = 12$). 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 *A*, and $r = 0.874$; $P < 0.001$ in *B*). Shaded bars show the mean ± SD range for normal values, as determined in NOD-scid mice ($n = 6$).

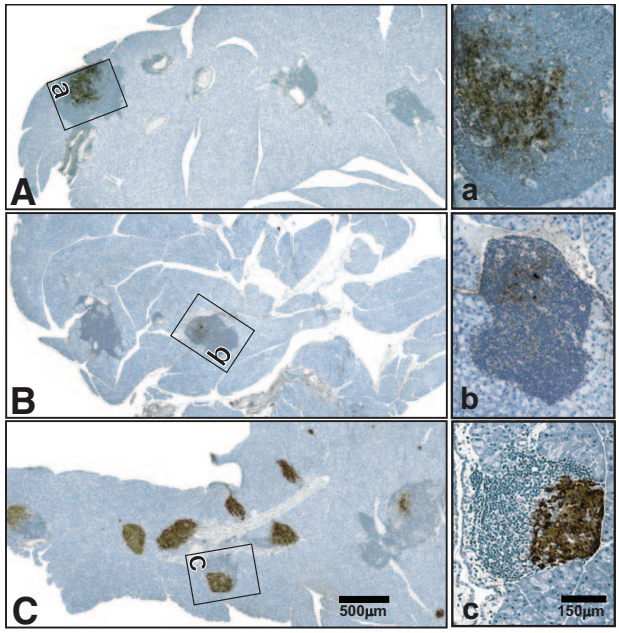

FIG. 4. Photomicrographs of pancreatic tissue sections. *A*: An NOD mouse pancreas 5 days after diabetes onset and before the start of treatments. Islets are infiltrated by leukocytes (dark blue-stained), and the few islets with remaining β-cells (brown-stained with insulin antibody) are surrounded and 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*). (Please see http://dx.doi.org/10.2337/db08-0688 for a high-quality digital representation of this figure.)

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. Fig. 2*B* 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 twofold over the pretreatment level (0.57 ± 0.24 vs. 0.25 ± 0.10 mg; $P = 0.15$, NS), and 100 μg/kg GLP-1 plus gastrin treatment increased pancreatic β-cell mass threefold over the pretreatment level (0.74 ± 0.25 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 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. 3*A*) and β-cell mass (Fig. 3*B*). Restoration of the pancreatic insulin content to ~30% of normal (~3 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. 3*A*). Similarly, restoration of the pancreatic β-cell mass to ~35% of normal (~0.5 vs. 1.4 mg in NOD-scid mice) was sufficient to correct hyperglycemia (Fig. 3*B*).

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. 4*A*). Vehicle-treated mice were severely hyperglycemic, and their islets were heavily infiltrated by leukocytes and almost devoid of β-cells (Fig. 4*B*). 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. 4*C*).

Pancreatic β-cell replication, apoptosis, and neogenesis. The increase in pancreatic β-cells in GLP-1– and gastrin-treated NOD mice (Fig. 5*A*) was associated with a significant decrease in β-cell apoptosis (Fig. 5*B*). Pancreatic β-cell replication rates were low (~1% of total β-cells) and not changed by GLP-1 and gastrin therapy (Fig. 5*C*). In contrast, replication of pancreatic duct cells was significantly increased by GLP-1 and gastrin (Fig. 5*D*). Also,

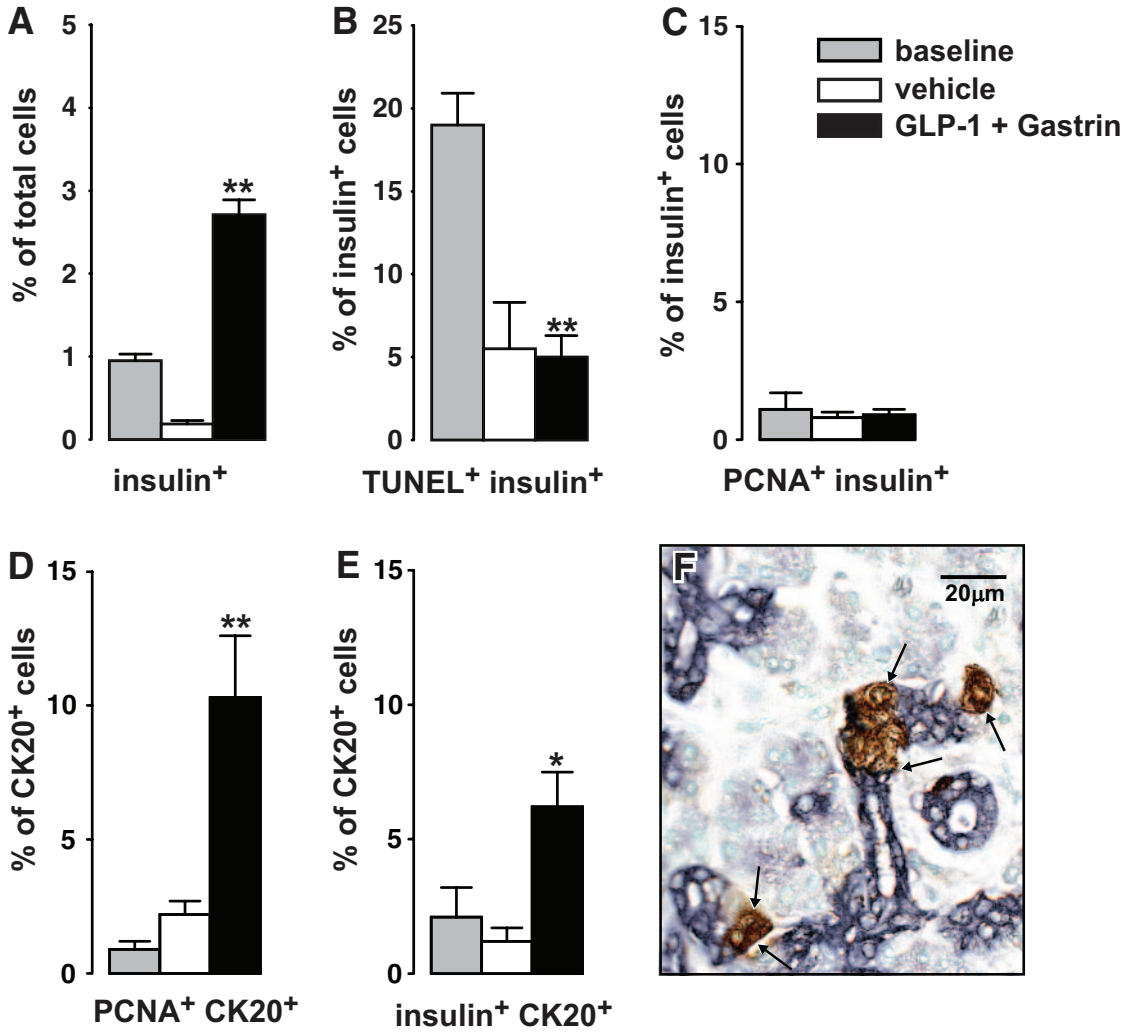

FIG. 5. GLP-1 and gastrin combination therapy decreases β-cell apoptosis and increases pancreatic duct-associated β-cells. β-Cells (insulin$^+$) were more abundant (*A*) and β-cell apoptosis (TUNEL$^+$ insulin$^+$) was decreased (*B*) in the pancreas of NOD mice treated with 100 μg/kg GLP-1 and 1.5 μg/kg 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*). *E*: Insulin-stained cells in ducts (insulin$^+$ CK20$^+$) were increased in GLP-1– and gastrin-treated mice. *F*: Insulin-positive cells (brown-stained) in and budding from pancreatic ducts (blue stained) are indicated by arrows. Values are means ± SE for five to six mice. *P<0.05, **P < 0.01 vs. baseline. (Please see http://dx.doi.org/10.2337/db08-0688 for a high-quality digital representation of this figure.)

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%) (Fig. 5*E* and *F*).

Insulin autoantibodies. Insulin autoantibodies were present in acutely diabetic NOD mice before treatment, and these antibodies rose to very high levels in vehicle-treated mice, whereas insulin autoantibodies 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 against 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% (seven of eight) of islet grafts survived and maintained normoglycemia in GLP-1– and gastrin-treated mice (Fig. 8*A*). 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. 8*B*), whereas no insulin-stained cells remained in islet grafts of vehicle-treated mice (Fig. 8*C*). Recovery of β-cells in islet grafts of GLP-1– and gastrin-treated mice was associated with significantly reduced apoptosis of β-cells (Fig. 9*D*) and not with any change in replication of β-cells (Fig. 9*C*), similar to the findings in the pancreas (Fig. 5*B* and *C*).

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

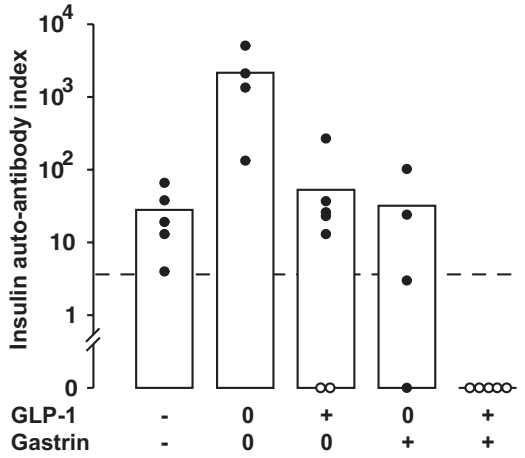

FIG. 6. GLP-1 and gastrin combination therapy eliminates insulin autoantibodies in NOD mice. Insulin autoantibody levels were elevated (>4 insulin autoantibody index, dashed line) in serum of acutely diabetic mice (GLP-1 −, gastrin −), and insulin autoantibodies rose to very high levels when diabetes had progressed in vehicle-treated mice (GLP-1 0, gastrin 0). Mean insulin autoantibody levels (□) in NOD mice treated with 100 μg/kg GLP-1 only or 1.5 μg/kg gastrin only remained at approximately pretreatment levels, whereas insulin autoantibodies were undetectable in all the mice treated with GLP-1 and gastrin. ○, normoglycemic; ●, diabetic.

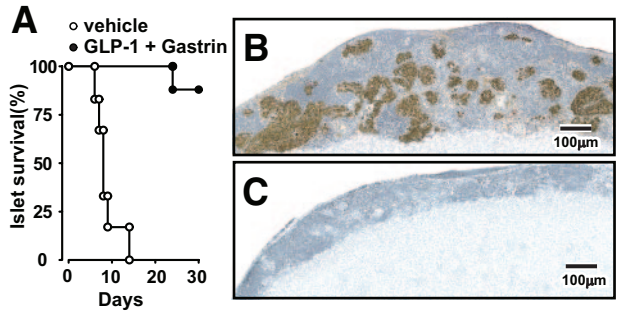

FIG. 8. GLP-1 and gastrin combination therapy prevents autoimmune destruction of syngeneic islet grafts in diabetic NOD mice. *A*: After 30 days of treatment with 100 μg/kg GLP-1 and 1.5 μg/kg gastrin, NOD islet grafts survived and maintained normoglycemia in 88% (seven of eight) of mice, whereas no islet grafts (zero of six) survived beyond 14 days and diabetes recurred in vehicle-treated mice. 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*). (Please see http://dx.doi.org/10.2337/db08-0688 for a high-quality digital representation of this figure.)

DISCUSSION

Previous reports of diabetes reversal in NOD mice after 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).

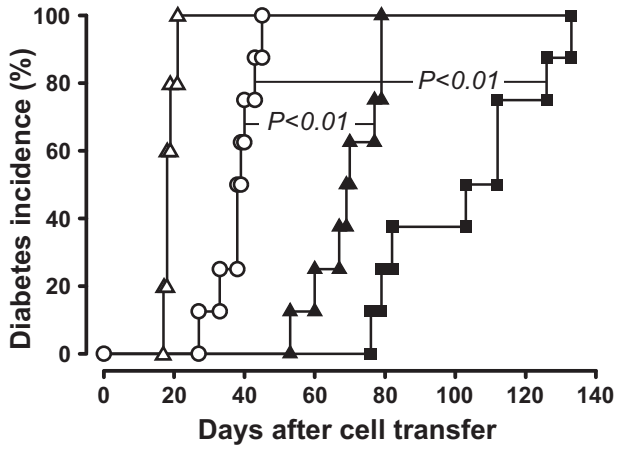

FIG. 7. GLP-1 and gastrin combination therapy induces immunoregulatory cell activity in NOD mice. The median time (50% diabetes incidence) for diabetes onset (blood glucose >12 mmol/l) in NOD-scid mice was 18 days when the mice were injected intravenously with 1.5×10^7 splenic cells from diabetic NOD mice (△, $n = 5$) and 39 days when injected with 0.5×10^7 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 0.5×10^7 splenic cells from diabetic NOD mice mixed with 1.0×10^7 splenic cells from GLP-1–plus gastrin-treated NOD mice (●, $n = 8$) and to 107 days when NOD-scid mice were injected with 0.5×10^7 splenic cells from diabetic NOD mice mixed with 3.0×10^7 splenic cells from GLP-1–plus gastrin-treated NOD mice (■, $n = 8$).

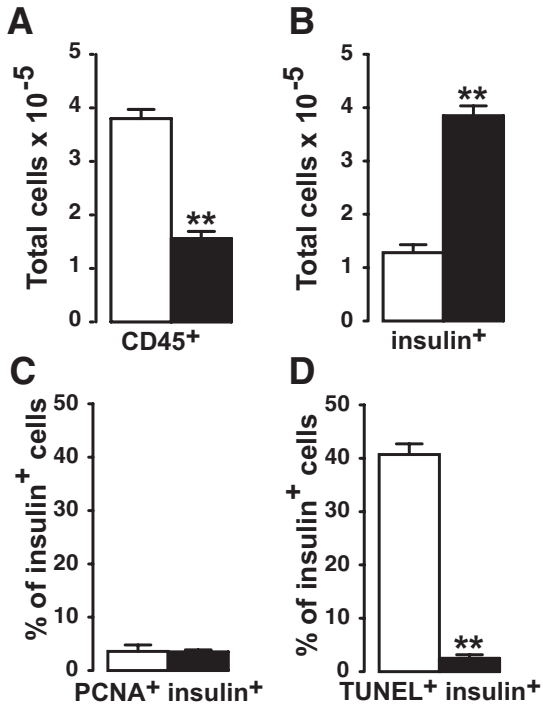

FIG. 9. GLP-1 and gastrin combination therapy prevents β-cell apoptosis. Leukocytes ($CD45^+$) were decreased (*A*) and β-cells ($insulin^+$) were more abundant (*B*) in syngeneic islet grafts in NOD mice treated with 100 μg/kg GLP-1 and 1.5 μg/kg gastrin than in islet grafts of NOD mice treated with vehicle for 10 days. *C*: 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. *D*: 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. Values are means ± SE for eight mice. □, vehicle; ■, GLP-1 + gastrin. **$P < 0.01$ vs. vehicle.

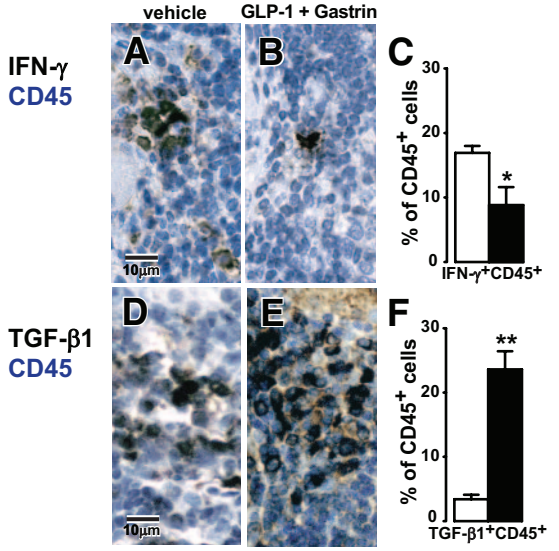

FIG. 10. GLP-1 and gastrin combination therapy shifts the cytokine expression of leukocytes infiltrating syngeneic islet grafts from IFN-γ to TGF-β1. At 10 days after syngeneic islet transplantation in diabetic NOD mice, leukocytes (blue stained) expressing IFN-γ (black stained) were more abundant in 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 percentage of leukocytes that produced IFN-γ (IFN-γ$^+$ CD45$^+$) was significantly greater in vehicle-treated mice than in 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*). □, vehicle; ■, GLP-1 + gastrin. Values are means ± SE for eight mice. *P < 0.05, **P < 0.01 vs. vehicle. (Please see http://dx.doi.org/10.2337/db08-0688 for a high-quality digital representation of this figure.)

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, 3–5 weeks after vehicle or GLP-1 and gastrin treatments, and compared these values to the pretreatment (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 antiapoptotic effect of GLP-1 and gastrin on β-cells, this may have been conferred on preexistent and 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 approximately 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. We found that insulin autoantibodies rose to extremely high levels as diabetes progressed in vehicle-treated mice, whereas these autoantibodies 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 antiapoptotic 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 antiapoptotic 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 antiapoptotic 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 to determine whether this was secondary to the antiapoptotic 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-cells and exogenous 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 that 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 immunological mechanisms that destroy β-cells, thereby offering a promising strategy for correction of the β-cell loss in type 1 diabetes.

ACKNOWLEDGMENTS

This work was supported by grants from the Juvenile Diabetes Research Foundation International (17-2006-1024) and Transition Therapeutics 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.

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