Original Article

Combination Therapy With Epidermal Growth Factor and Gastrin Increases β-Cell Mass and Reverses Hyperglycemia in Diabetic NOD Mice

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Combination therapy with epidermal growth factor (EGF) and gastrin induces β-cell regeneration in rodents with chemically induced diabetes. We investigated whether EGF plus gastrin could correct hyperglycemia in NOD mice with autoimmune diabetes. Combined treatment with EGF (1 μg/kg) and gastrin (3 μg/kg) for 2 weeks restored normoglycemia after diabetes onset in NOD mice, whereas EGF or gastrin alone did not. Fasting blood glucose remained normal (3.5–6.5 mmol/l) or mildly elevated (<11 mmol/l) in five of six mice (83%) for 10 weeks after EGF plus gastrin treatment was stopped, whereas all mice treated with vehicle or EGF or gastrin alone became severely hyperglycemic (12–35 mmol/l). Pancreatic β-cell mass was increased threefold and insulin content was increased eightfold in mice treated with EGF plus gastrin compared with pretreatment values. The correction of hyperglycemia correlated significantly with increases in pancreatic β-cell mass and insulin content. In addition, splenic cells from mice treated with EGF plus gastrin delayed diabetes induction by adoptive transfer of diabetogenic cells into immunodeficient NOD-scid mice, suggesting the induction of immunoregulatory cells in NOD mice treated with EGF plus gastrin. We conclude that a short course of combined EGF and gastrin therapy increases pancreatic β-cell mass and reverses hyperglycemia in acutely diabetic NOD mice; the impact of this combined therapy may result from the effects of EGF and gastrin on β-cells, immune cells, or both. *Diabetes* 54:2596–2601, 2005

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EGF, epidermal growth factor; FBG, fasting blood glucose.

Successful transplantation of human pancreatic islets provides the prospect of a cure for type 1 diabetes (1); however, the lack of sufficient donor pancreata greatly limits the widespread use of this approach (2). Recent discoveries showing the regenerative capabilities of the endocrine pancreas suggest that β-cell regeneration may be a nonmutually exclusive alternative to allogeneic islet transplantation (3,4).

Pancreatic β-cell mass can increase in adult life in response to physiological stimuli such as pregnancy (5) and obesity (6). In addition, β-cells can regenerate under conditions of tissue injury and repair, such as partial pancreatectomy, pancreatic duct ligation, cellophane wrapping of the gland, administration of alloxan or streptozotocin, and transgenic overexpression of γ-interferon in β-cells (7–9). Although β-cell proliferation is increased before diabetes onset in NOD mice, this is not sufficient to keep up with the ongoing autoimmune response that decreases the β-cell mass (10). Normoglycemia can be restored in diabetic NOD mice, however, when the β-cell–directed autoimmune response is abrogated by treatments with anti–T-cell antibodies (11,12) or by replacing NOD immunocompetent cells with bone marrow or splenic cells from diabetes-resistant donors (13,14). These findings suggest that β-cell regenerative processes can occur after diabetes onset; therefore, therapies directed at stimulating β-cell regeneration may restore the β-cell mass in type 1 diabetes.

Many putative β-cell growth factors have been identified, including epidermal growth factor (EGF) and other EGF family members, such as transforming growth factor-α and betacellulin (15–19). In addition, gastrointestinal peptides such as glucagon-like peptide-1 (20,21) and gastrin (22,23) can stimulate β-cell neogenesis. In the rat pancreatic duct-ligated model of pancreas regeneration, gastrin enhances β-cell neogenesis from pancreatic duct cells (22); further, endogenous gastrin may be necessary for β-cell neogenesis in this model (23). Combined EGF and gastrin treatment has been found to increase β-cell mass and reduce hyperglycemia in streptozotocin-induced diabetic rats (24) as well as induce islet regeneration from pancreatic duct cells and restore normoglycemia in alloxan-induced diabetic mice (25). The choice of EGF and gastrin combination therapy in the current studies (24,25)

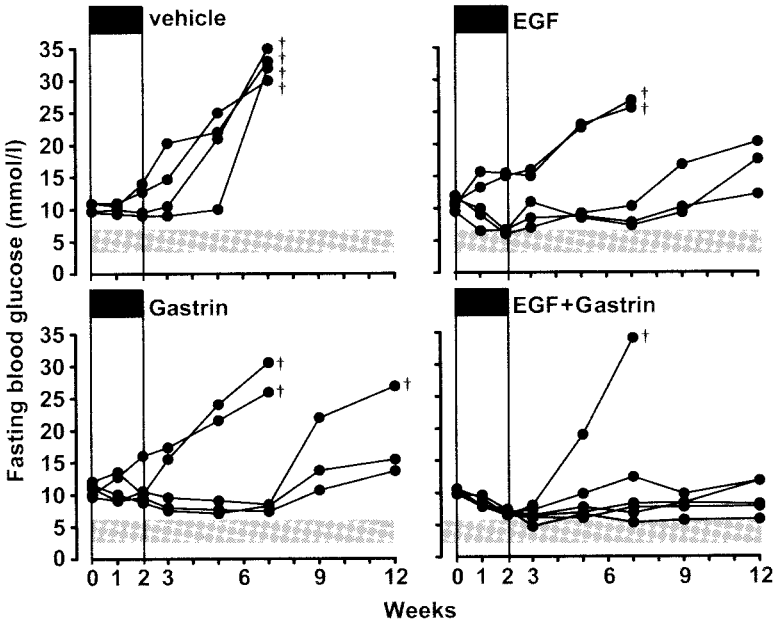

FIG. 1. Effects of EGF and gastrin on blood glucose. Beginning 3–6 days after diabetes onset (0 weeks) and continuing for 2 weeks, NOD mice were treated with twice-daily intraperitoneal injections of vehicle (n = 4); 1 μg · kg^{-1} · day^{-1} EGF (n = 5); 3 μg · kg^{-1} · day^{-1} gastrin (n = 5); or EGF plus gastrin (n = 6). FBG concentrations are shown in individual mice during the 2 weeks of treatments and for the 10 additional weeks after treatments were stopped. Mice with FBG ≥25 mmol/l and losing weight were killed (†). Shaded bars show the normal range for FBG (3.5–6.5 mmol/l).

was based on a previous study in which an increase in islet mass was observed in double transgenic mice that expressed transforming growth factor-α, an EGF receptor ligand, and gastrin locally in the pancreas (26).

In the present study, we investigated whether combination therapy with EGF and gastrin could restore the pancreatic β-cell mass and reverse hyperglycemia in NOD mice with autoimmune diabetes. We report that a short course of EGF and gastrin combination therapy increases β-cell mass and reverses hyperglycemia in acutely diabetic NOD mice concurrently with the induction of immunoregulatory cells.

RESEARCH DESIGN AND METHODS

NOD female mice, age 6–8 weeks, were purchased from Taconic (Germantown, NY). 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 mice were monitored daily for diabetes onset by urine testing using Keto-Diastix (Bayer, Etobicoke, Canada). Diabetes onset was diagnosed by the presence of glucosuria (>6 mmol/l), ketonuria (>1.5 mmol/l), and a 10- to 12-h fasting blood glucose ≥9 mmol/l on 2 consecutive days, measured on a glucose meter (Glucometer Elite; Bayer). Treatments were started within 3–6 days after diabetes onset in NOD mice age 10–15 weeks.

Acutely diabetic NOD mice of similar ages were randomly allocated into four groups and treated for 2 weeks with twice-daily intraperitoneal injections of PBS vehicle (control), 1 μg · kg^{-1} · day^{-1} EGF, 3 μg · kg^{-1} · day^{-1} gastrin, or 1 μg · kg^{-1} · day^{-1} EGF plus 3 μg · kg^{-1} · day^{-1} gastrin. Both EGF and gastrin were lipopolysaccharide- and endotoxin-free preparations. The EGF used in this study was recombinant human EGF_{1-51} expressed in yeast *Pichia pastoris* and purified to >95% by high-performance liquid chromatography and mass spectroscopy. Recombinant human EGF_{1-51} has an asparagine substitution for glutamate at position 51 and has equal biological potency to native human EGF_{1-53} (27). The gastrin used in this study was human gastrin-17 synthesized and purified to >97% by high-performance liquid chromatography (Starr Biochemicals, Torrance, CA). Gastrin-17 has a leucine substitution for methionine at position 15 to prevent oxidation and is equipotent to native gastrin-17 (28). EGF and gastrin preparations 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 and were stored in aliquots at −70°C. EGF and gastrin stocks were thawed, diluted in sterile PBS (pH 7.4), kept at 4°C, and used within 2 days. Fasting blood glucose (FBG) concentrations in mice were measured once a week for 2 weeks while the mice were receiving treatments and for an additional 10 weeks after treatments were stopped (total of 12 weeks). Mice with FBG >25 mmol/l and losing weight were killed by sodium pentobarbital overdose before the end of the study; all other mice were killed at 12 weeks. 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 the insulin content and for histological studies.

Pancreatic insulin content. For insulin assays, half of each pancreas was weighed, minced with fine scissors in a small beaker with 1.0 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 insulin assay buffer, and insulin was measured using a radioimmunoassay kit for rat and mouse insulin (Linco, St. Charles, MO).

Histological studies. 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 (insulin-positive) by an immunoperoxidase technique. The sections were first incubated with a polyclonal 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 substrate kit (InnoGenex iso-IHC DAB kit; San Ramon, CA) that stained insulin-positive cells a golden brown. The sections were then counterstained with hematoxylin. Coded slides were examined by light microscopy.

Insulitis scoring. Pancreatic islet inflammation (insulitis) was graded on a 0–3 scale, according to the extent of intra-islet infiltration by leukocytes, with 0 = none, 1 = <20% infiltration, 2 = 20–50% infiltration, and 3 = >50% infiltration. A mean insulitis score was calculated for each pancreas by dividing the sum of the insulitis scores for individual islets by the number of islets examined.

Analysis of β-cell mass. β-Cell mass was determined by point counting morphometry on the β-cell (insulin-positive) immunostained pancreatic sections by means of a Nikon E400 microscope connected to a video camera with a color monitor at a final magnification of ×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 β-cells by the number of points over the total pancreatic tissue. β-Cell mass was determined by multiplying the relative volume by the total weight of the pancreas.

Adoptive cell transfer. Adult NOD-scid female mice (Jackson Laboratory, Bar Harbor, ME) were used as recipients of splenic cells from NOD mice. Splenic cells were prepared from acutely diabetic NOD mice treated with PBS vehicle for 2 weeks (FBG 12–16 mmol/l; diabetogenic cells) and from acutely diabetic NOD mice that had been treated with EGF plus gastrin for 2 weeks and had become normoglycemic or near normoglycemic (FBG 6.0–7.5 mmol/l; putative immunoregulatory cells) (Fig. 1). Splenic cells were pooled from six diabetic NOD mice or six NOD mice treated with EGF plus gastrin and adoptively transferred into four to eight NOD-scid mice. NOD-scid mice were injected intravenously with 1.5×10^7 diabetogenic cells alone or with a mixture of 1.5×10^7 diabetogenic cells and 0.5×10^7 putative immunoregulatory cells. Other NOD-scid mice received 0.5×10^7 diabetogenic cells alone or with 0.5×10^7 or 3.0×10^7 putative immunoregulatory cells. Diabetes development in the NOD-scid mice was determined by daily monitoring for

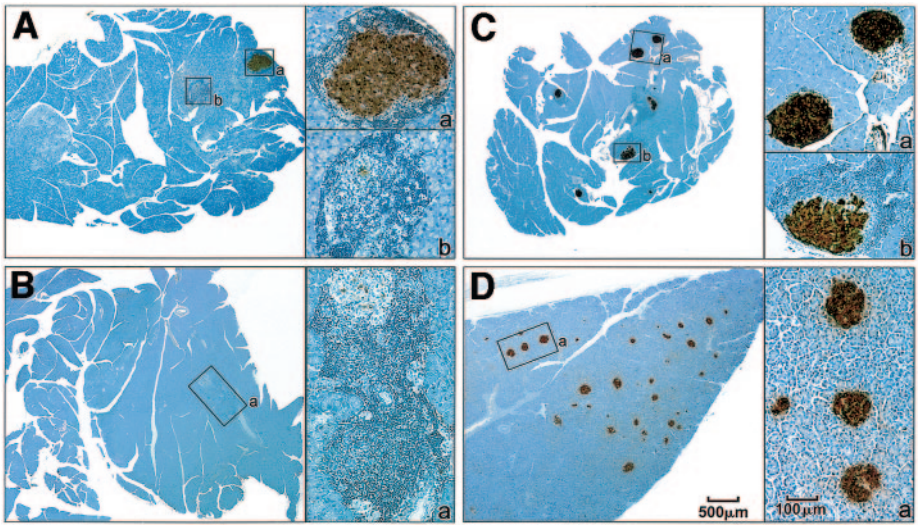

FIG. 2. Photomicrographs of the pancreata of NOD mice. *A*: An NOD mouse 4 days after diabetes onset and before the start of treatments (FBG = 13.2 mmol/l). Some islets still contain β-cells (brown-stained with insulin antibody) surrounded by leukocytes (small cells with blue-stained nuclei) (*inset a*) and some islets are almost devoid of β-cells and are infiltrated by leukocytes (*inset b*). *B*: An NOD mouse after treatment with vehicle for 2 weeks and an additional 5 weeks without treatment (FBG = 33.0 mmol/l). Islets are devoid of β-cells and heavily infiltrated by leukocytes (*inset a*). *C*: An NOD mouse after treatment with EGF plus gastrin for 2 weeks and an additional 10 weeks without treatment (FBG = 7.2 mmol/l). Islets with abundant β-cells are found without leukocytes (*inset a*) and with surrounding leukocytes (*inset b*). *D*: A normoglycemic NOD-scid mouse (FBG = 4.7 mmol/l). Islets contain β-cells without associated leukocytes (*inset a*).

glucosuria, followed by blood glucose measurement; a blood glucose ≥12 mmol/l marked the onset of diabetes after splenic cell transfer.

Statistical analyses. Data are expressed as means ± SE. Differences between groups were analyzed by one-way ANOVA followed by the Bonferroni multiple comparisons test (for pancreatic insulin content and β-cell mass) and the unpaired Student's *t* test (for insulitis scores). Correlations were analyzed by linear regression analysis using Spearman's rank correlation test. $P < 0.05$ was considered significant.

RESULTS

Effects of EGF and gastrin on blood glucose. Female NOD mice had FBG concentrations of 10.0–13.2 mmol/l (normal FBG <6.5 mmol/l) 3–6 days after diabetes onset. The mice treated with vehicle for 2 weeks (controls) became progressively more hyperglycemic and had to be killed at 7 weeks because of severe hyperglycemia (FBG >25 mmol/l) and weight loss (Fig. 1). Treatment with EGF alone reduced FBG to normal levels in three of five mice, but hyperglycemia recurred in these mice when EGF treatment was stopped. Treatment with gastrin alone reduced FBG to near normoglycemic levels in three of five mice, but this effect did not last beyond 5 weeks after stopping gastrin treatment. In contrast, treatment with EGF plus gastrin reduced FBG in five of five mice (from 10.2 ± 0.2 to 6.9 ± 0.2 mmol/l; $P < 0.01$) after 2 weeks, and FBG remained normal (<6.5 mmol/l) or mildly elevated (<11 mmol/l) in five of six mice (83%) for 10 weeks after EGF and gastrin combination therapy was stopped (Fig. 1).

Pancreatic histology. Histological examination of NOD mice pancreata 3–6 days after diabetes onset (just before treatments were started) revealed some islets that still contained abundant insulin-stained β-cells with a halo of leukocytes and other islets that were almost devoid of β-cells and heavily infiltrated by leukocytes (Fig. 2*A*). After 2 weeks of vehicle (control) treatment and an additional 5 weeks without treatment, the mice were severely hyperglycemic (FBG >30 mmol/l) and their islets were either devoid of β-cells or heavily infiltrated by leukocytes (Fig. 2*B*). In contrast, after 2 weeks of treatment with EGF plus gastrin and an additional 10 weeks without treatment, many islets with abundant β-cells were found, some with and some without surrounding leukocytes (Fig. 2*C*). Leukocytic infiltration of islets (insulitis score) was significantly less in pancreata of mice treated with EGF plus gastrin (0.72 ± 0.14; $n = 5$) than in mice before treatments were started (1.41 ± 0.15; $n = 4$; $P < 0.02$).

Pancreatic insulin content. Figure 3 shows that the pancreatic insulin content in diabetic NOD mice before treatments (basal, 0.21 ± 0.04 μg) was reduced even further after 2 weeks of vehicle (control) treatment plus 5 weeks off treatment (0.08 ± 0.01 μg). In contrast, pancreatic insulin content was significantly increased above the pretreatment level (0.21 ± 0.04 μg) in mice that survived for 10 weeks after being treated for 2 weeks with EGF (1.13 ± 0.07 μg; $P < 0.05$) or EGF plus gastrin (1.71 ± 0.18 μg; $P < 0.01$). Pancreatic insulin content in NOD mice treated with EGF plus gastrin (1.71 ± 0.18 μg) was 28% of that in normoglycemic NOD-scid mice (6.02 ± 0.33 μg), whereas the pancreatic insulin content in NOD mice before treatment (0.21 ± 0.04 μg) was only 3% of that in NOD-scid mice.

Pancreatic β-cell mass. Figure 4 shows that pancreatic

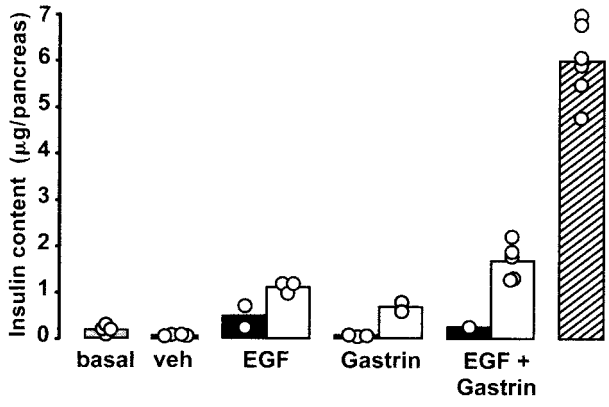

FIG. 3. Effects of EGF and gastrin on pancreatic insulin content in mice treated as described in Fig. 1. Values for individual mice (○) and mean values for groups (bars) are shown for diabetic NOD mice before treatments (basal), after treatment with vehicle for 2 weeks and then no treatment for 5 weeks (veh), after treatment with EGF for 2 weeks and then no treatment for 5–10 weeks (EGF), after treatment with gastrin for 2 weeks and then no treatment for 5–10 weeks (Gastrin), and after treatment with EGF plus gastrin for 2 weeks and then no treatment for 5–10 weeks (EGF + Gastrin). NOD mice were either severely hyperglycemic (FBG >25 mmol/l) and killed at 5 weeks after treatments were stopped (■) or they survived for 10 weeks after treatments were stopped (□). Pancreatic insulin content is also shown for normoglycemic NOD-scid mice (▨).

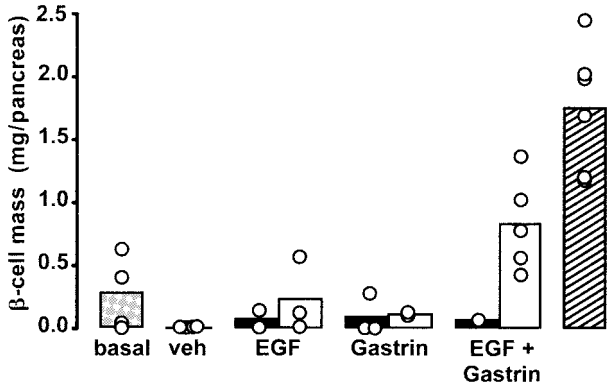

FIG. 4. Effects of EGF and gastrin on pancreatic β-cell mass in mice treated as described in Fig. 1. Values for individual mice (○) and mean values for groups (bars) are shown for diabetic NOD mice before treatments (basal), after treatment with vehicle for 2 weeks and then no treatment for 5 weeks (veh), after treatment with EGF for 2 weeks and then no treatment for 5–10 weeks (EGF), after treatment with gastrin for 2 weeks and then no treatment for 5–10 weeks (Gastrin), and after treatment with EGF plus gastrin for 2 weeks and then no treatment for 5–10 weeks (EGF + Gastrin). NOD mice were either severely hyperglycemic (FBG >25 mmol/l) and killed 5 weeks after treatments were stopped (■), or they survived for 10 weeks after treatments were stopped (□). Pancreatic β-cell mass is also shown for normoglycemic NOD-scid mice (▨).

β-cell mass in diabetic NOD mice before treatment (basal, 0.27 ± 0.15 mg) was reduced even further after 2 weeks of vehicle (control) treatment plus 5 weeks off treatment (0.01 ± 0.01 mg). In contrast, pancreatic β-cell mass was significantly increased above the pretreatment level (0.27 ± 0.15 mg) in mice that survived for 10 weeks after being treated for 2 weeks with EGF plus gastrin (0.83 ± 0.17 mg; $P < 0.05$). Pancreatic β-cell mass in NOD mice treated with EGF plus gastrin (0.83 ± 0.17 mg) was 47% of that in normoglycemic NOD-scid mice (1.76 ± 0.20 mg), whereas the β-cell mass in NOD mice before treatment (0.27 ± 0.15 mg) was only 15% of that in NOD-scid mice.

Correlations of blood glucose with pancreatic insulin and β-cell mass. Reductions in hyperglycemia by EGF, gastrin, and EGF plus gastrin correlated significantly with increases in pancreatic insulin content and β-cell mass induced by the respective treatments (Fig. 5).

Immunologic effects of EGF and gastrin. Our finding that a short course of combined EGF and gastrin therapy led to long-lasting control of hyperglycemia (Fig. 1) and reduced insulitis (Fig. 2) suggested that EGF and gastrin had an effect against the autoimmune response in diabetic NOD mice. Therefore, we determined whether mice treated with EGF plus gastrin harbored cells capable of suppressing autoimmunity. Using a standard adoptive transfer model, we injected splenic cells prepared from acutely diabetic NOD female mice (diabetogenic cells) into NOD-scid mice together with or without splenic cells from NOD mice that underwent remission after treatment with EGF plus gastrin (putative immunoregulatory cells.) As shown in Fig. 6, cotransfer of cells from mice treated with EGF plus gastrin significantly delayed diabetes induction by two different doses of diabetogenic cells (Fig. 6*A* and *B*) in a dose-dependent fashion (Fig. 6*B*). These findings suggest that combination therapy with EGF and gastrin induces immunoregulatory cells that may inhibit autoimmunity, thereby contributing to the long-lasting survival of the increased β-cell mass induced by combined EGF plus gastrin therapy.

DISCUSSION

Recent studies have shown that the endocrine pancreas is capable of regeneration after injury in adult life (3,4). Furthermore, β-cell regenerative processes may be active even after diabetes onset in NOD mice with autoimmune diabetes (11–14). This report, to our knowledge, is the first to demonstrate an increase in pancreatic β-cell mass after diabetes onset in NOD mice where the increase was quantified. This increase was achieved by combination therapy with EGF and gastrin. Pancreatic β-cell mass was increased from 15 to 47% of normal, an increase that was sufficient to normalize blood glucose levels. We ascribe the glucose-lowering effects of EGF and gastrin to the abilities of these peptides to increase pancreatic β-cell mass and insulin content, because blood glucose levels were significantly correlated with both pancreatic β-cell mass and insulin content in the mice.

In another recent study, the glucagon-like peptide-1 analog, exendin-4, was reported to reverse hyperglycemia in diabetic NOD mice; however, this required concurrent immunosuppressive treatment with antilymphocyte serum (29). In the present study, combination therapy with EGF and gastrin did not require additional immunotherapy to induce remission in diabetic NOD mice. Also, we achieved a long-lasting remission with EGF plus gastrin (blood glucose <11 mmol/l in 83% of mice) similar to that

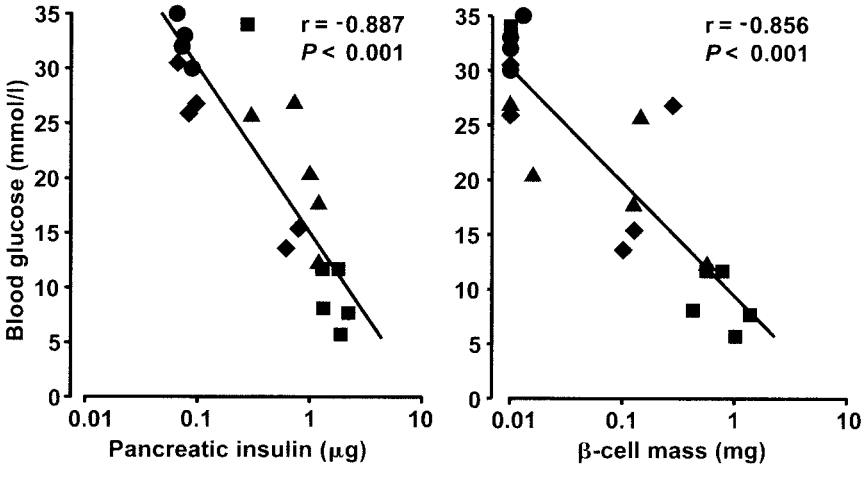

FIG. 5. FBG is inversely correlated with pancreatic insulin content and β-cell mass in NOD mice treated with vehicle (●; $n = 4$), EGF (▲; $n = 5$), gastrin (◆; $n = 5$), or EGF plus gastrin (■; $n = 6$). FBG values 5-10 weeks after treatments were stopped (Fig. 1) are plotted against the corresponding values for pancreatic insulin content (Fig. 3) and β-cell mass (Fig. 4).

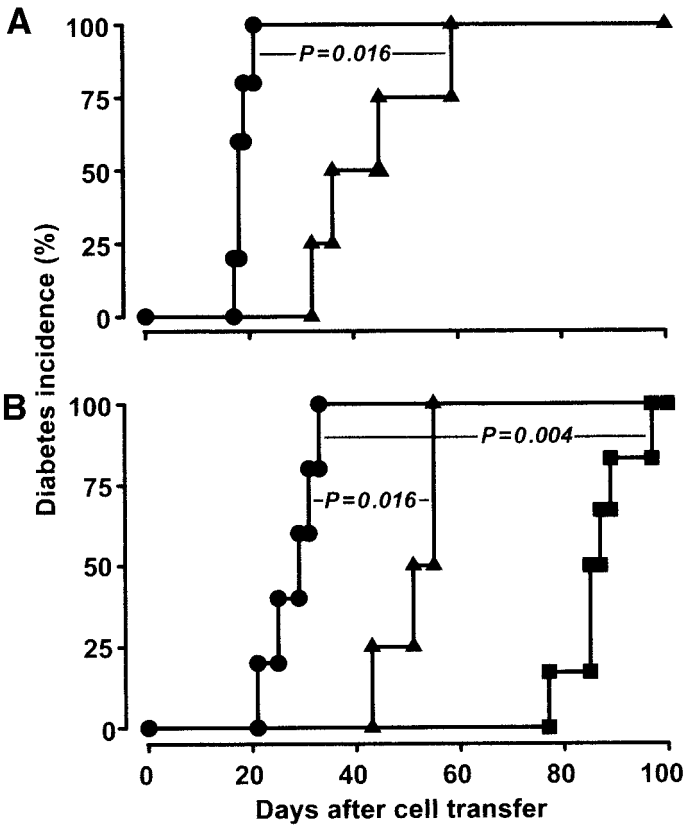

FIG. 6. Development of diabetes after adoptive cell transfer. *A:* Splenic cells (1.5×10^7) from diabetic NOD mice either alone (●; $n = 5$) or together with splenic cells (0.5×10^7) from NOD mice treated with EGF plus gastrin (▲; $n = 4$) were injected intravenously into NOD-scid mice. *B*: Splenic cells (0.5×10^7) from diabetic NOD mice, either alone (●; $n = 5$) or together with splenic cells (▲, 0.5×10^7, $n = 4$; or ■, 3.0×10^7, $n = 6$) from NOD mice treated with EGF plus gastrin were injected intravenously into NOD-scid mice.

achieved with exendin-4 plus antilymphocyte serum (blood glucose <11 mmol/l in 88% of mice) (29). Our finding that a short course (2 weeks) of EGF plus gastrin treatment induced a long-lasting (at least 10 weeks) remission from hyperglycemia in diabetic NOD mice without immunotherapy was unexpected. The finding of reduced islet inflammation (insulitis) in mice treated with EGF plus gastrin suggests that EGF and gastrin therapy may have interfered with the autoimmune response against β-cells. This interpretation is supported by the finding of immunoregulatory cell activity in mice treated with EGF plus gastrin; however, there may be other explanations for the decreased insulitis. For example, there could be nonimmunoregulatory cell−dependent effects of EGF and gastrin on the balance of proinflammatory (Th1) and anti-inflammatory (Th2 or Th3) cytokines. In fact, EGF receptor ligands have been reported to either increase or suppress inflammatory mediators (30).

Regarding possible mechanisms by which EGF plus gastrin therapy induced increases in β-cell mass in diabetic NOD mice in the present study, this therapy was recently reported to induce neogenesis of β-cells from pancreatic exocrine duct cells in mice with alloxan-induced diabetes (25). These findings are consonant with the different roles that EGF and gastrin have in islet development. EGF receptor ligands are expressed in the developing pancreas, and EGF receptor signaling stimulates proliferation and branching morphogenesis of fetal pancreatic ducts (31). This process is impaired and islet cell differentiation is delayed in mice lacking EGF receptors (32). Endogenous gastrin expression is activated in the developing pancreas during the secondary transition phase when proto-differentiated ducts develop into the fully differentiated exocrine and endocrine pancreas (33). Further study is needed to determine whether or not the increase in pancreatic β-cell mass in NOD mice induced by EGF plus gastrin in the present study resulted from β-cell neogenesis from duct cells, as reported when the combination of EGF and gastrin was used to treat mice with alloxan-induced diabetes (25). Similarly, we recently reported that combination therapy with EGF and gastrin significantly increased β-cell mass in adult human pancreatic islets in vitro and in vivo, an increase that appeared to result from the induction of β-cell neogenesis from pancreatic exocrine duct cells (34).

In summary, a short course of combined EGF and gastrin treatment of NOD mice after diabetes onset increased pancreatic β-cell mass and insulin content, decreased insulitis, and reversed hyperglycemia. These changes were accompanied by the induction of immunoregulatory cells. We conclude, therefore, that EGF plus gastrin combination therapy induces β-cell regeneration while inhibiting autoimmune β-cell destruction, and that this may dispense with the need for conventional immunosuppressive drug therapy for type 1 diabetes.

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