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Decrease in β-Cell Proliferation Precedes Apoptosis during Diabetes Development in Bio-Breeding/Worcester Rat: Beneficial Role of Exendin-4

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In autoimmune type 1 diabetes mellitus, proinflammatory cytokine-mediated apoptosis of β-cells has been considered to be the first event directly responsible for β-cell mass reduction. In the Bio-Breeding (BB) rat, an *in vivo* model used in the study of autoimmune diabetes, β-cell apoptosis is observed from 9 wk of age and takes place after an insulitis period that begins at an earlier age. Previous studies by our group have shown an antiproliferative effect of proinflammatory cytokines on cultured β-cells in Wistar rats, an effect that was partially reversed by Exendin-4, an analogue of glucagon-like peptide-1. In the current study, the changes in β-cell apoptosis and proliferation during insulitis stage were also determined in pancreatic tissue sections in normal and thymectomized BB rats, as well as in Wistar rats of 5, 7, 9, and 11 wk of age. Although stable β-cell proliferation in Wistar and thymectomized BB rats was observed along the course of the study, a decrease in β-cell proliferation and β-cell mass from the age of 5 wk, and prior to the commencement of apoptosis, was noted in BB rats. Exendin-4, in combination with anti-interferon-γ antibody, induced a near-total recovery of β-cell proliferation during the initial stages of insulitis. This highlights the importance of early intervention and, as well, the possibilities of new therapeutic approaches in preventing autoimmune diabetes by acting, initially, in the insulitis stage and, subsequently, on β-cell regeneration and on β-cell apoptosis. (***Endocrinology*** **151: 2538–2546, 2010)**

Autoimmune type 1 diabetes mellitus (T1DM) is characterized by a loss of β-cell mass due to an autoimmune process (insulitis). In a preonset phase, immune cells infiltrate the pancreatic islets creating an inflammatory microenvironment responsible for the β-cell-specific toxicity. Proinflammatory cytokines, such as IL-1β, TNF-α, and interferon-γ (IFN-γ) secreted by activated lymphocytes, macrophages, and β-cells (1) have been implicated in the pathophysiology of T1DM. The effects on β-cells are wide-ranging and include inhibition of insulin secretion (2, 3) and apoptotic death of islet β-cells (4). Many mechanisms underlying cytokine-mediated apoptosis of β-cells have been proposed. Increase in nitric oxide production (5) and Fas/CD95 death receptor up-regulation (6) have been described in the response to proinflammatory cytokines, together with activation of signaling pathways such as nuclear factor-κB (7), p38 (8), or c-Jun N-terminal kinase (JNK) (9), which are connected to the apoptotic process. IL-1β alone is capable of inducing apoptosis in β-cells, but TNF-α and IFN-γ act synergistically in promoting the effects of IL-1β.

Islet ß-cell regenerative response is crucial in maintaining glucose homeostasis when islet integrity is adversely affected. In conditions of substrate oversupply, and in 60% pancreatectomy models, an increase in β-cell mass has been found to be able to restore a normal plasma

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Abbreviations: BB, Bio-Breeding; BrdU, 5-bromo-2-deoxyuridine; GLP-1, glucagon-like peptide-1; IFN-γ, interferon-γ; IPGTT, ip glucose tolerance test; JNK, c-Jun N-terminal kinase; T1DM, type 1 diabetes mellitus; TUNEL, terminal deoxynucleotidyltransferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling.

glucose level (10, 11). Although remaining β-cell self-replication has been proposed as the main form for β-cell mass recovery after islet injury (12), recent reports propose neogenesis as the key to the regenerative response (13). We have previously demonstrated that proinflammatory cytokines exert an important antiproliferative effect on cultured β-cells, an effect that is mediated, at least in part, by an inhibition of MAPK-belonging ERK1/2 pathway activation (14). We reported, as well, that a glucagon-like peptide-1 (GLP-1) analog, Exendin-4, is able to induce partial recovery.

In the case of T1DM, there is a paucity of information on the regenerative response of β-cells. In animal models, studies performed in Bio-Breeding (BB) rats demonstrated that insulitis was preceded by a reduction in the β-cell volume (15). However, in humans, evidence of proliferating β-cells in islets of recent-onset-diabetes patients has only recently been reported (16–18).

The BB rat is one of the best models of spontaneous autoimmune diabetes. This Wistar-derived colony, generated in 1974 at Bio-Breeding Laboratories (Ottawa, Canada), develops an absolute insulin deficiency similar to human T1DM. The animals are not obese, and the disease occurs equally in both sexes. Diabetes onset in BB rats is generally between 60 and 120 d of age (median age, 96 d). The cumulative incidence of diabetes over 120 d of age is 60–80% (19). As in human T1DM, BB diabetes onset is preceded by an insulitis period characterized by a monolymphocytic infiltration of islets (20, 21) and, subsequently, a more evident infiltration by T CD4+, T CD8+, and B lymphocytes. Contrary to humans, natural killer cells are present in BB rat infiltrates (22). Proinflammatory cytokines secreted by infiltrating cells can be detected in pancreatic tissue from the first stages of lymphocytic infiltration (23, 24), whereas β-cell death by apoptosis has been observed before the clinical onset of diabetes (25). Because of its autoimmune origin, immunosuppression therapies such as neonatal thymectomy have been reported to be effective in generating diabetes-free BB rats (26).

In the present study, we describe, in an *in vivo* system, a new stage in the insulitis process that occurs before the increase in ß-cell apoptosis. This stage is characterized by a halting of ß-cell proliferation, which is mediated, at least in part, by the effect of IFN-γ and which can be partially modified by the action of Exendin-4, an analog of GLP-1.

Materials and Methods

Animals

All animal procedures were performed with the approval of the Cádiz University School of Medicine (Cádiz, Spain) Committee for the Ethical Use and Care of Experimental Animals.

BB and Wistar rats were kept under conventional conditions in an environment-controlled room (20–21 C; 12-h light, 12-h dark cycle) with water and standard laboratory rat chow available *ad libitum*. Blood extracted from the tail vein was used for random glucose measurements every 4 d using an automatic glucose monitor (Accucheck Optimun; Roche Diagnostic, Basel, Switzerland). The rats were killed at 5, 7, 9, and 11 wk of age. The BB rats (55–85 g in weight) were thymectomized as previously described (27). Briefly, animals were anesthetized, and a midline incision was made in the skin from the base of the neck to the breastbone. The first rib was cut at the insertion point with the breastbone, and both structures were separated to widen the surgical field. The thymus was removed by gentle suction.

Detection of proliferation and apoptosis

To determine β-cell proliferation, animals were treated with ip 5-bromo-2-deoxyuridine (BrdU) (100 mg/kg body weight) 6 h before killing. Samples from pancreas were snap frozen, embedded in OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands), and cryostat 10-μm sections were generated and fixed in 4% methanol-free formaldehyde. Proliferation was assessed by double immunostaining using monoclonal mouse antibromodeoxyuridine (Dako Cytomation, Glostrup, Denmark) and polyclonal guinea pig antiinsulin (Sigma-Aldrich, St. Louis, MO) antibodies according to the manufacturer's instructions. Sections were incubated for 30 min with 0.1% Triton X-100 (vol/vol) in PBS for tissue permeabilization and washed twice with PBS. Then, sections were treated with HCl (2 N) in PBS for 30 min, neutralized with borax/borate buffer (0.1 M, pH 8.9) for 30 min, washed, and incubated overnight at 4 C with antibromodeoxyuridine and antiinsulin antibodies. Stained sections were revealed using antimouse IgG antibody (Alexa-546 conjugated) and antiguinea pig IgG (Alexa-488 conjugated) antibodies (Molecular Probes, Inc., Eugene, OR). To determine the proliferating fraction, insulin-positive/BrdU-positive cells and islet areas were quantified in a total of 50 islets per condition, using a confocal scanning microscope (Leica Microsystems, Wetzlar, Germany). Results were noted under randomized conditions by a single investigator (G.P.-A.) and expressed as number of insulin+/BrdU+ cells/mm^2 of islet.

β-Cell apoptosis was determined using the DeadEnd Fluorometric terminal deoxynucleotidyltransferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling (TUNEL) System (Promega, Madison, WI) according to the manufacturer's instructions. Insulin was simultaneously stained using polyclonal guinea pig antiinsulin (Sigma-Aldrich). Quantification was as above, and the results were expressed as number of insulin+/TUNEL+ cells/mm^2 of islet.

Histological examination of β-cells

Histological examination of pancreatic islets was performed in Harris' H&E-stained pancreas sections using ×20 objective lens. The severity of insulitis was graded as a function of the mononuclear cell infiltration of the pancreatic islets: 0, no infiltrate; 1, peri-ductular infiltrate; 2, peri-islet infiltrate; 3, intraislet infiltrate; 4, intraislet infiltrate associated with β-β-cell destruction. Twenty islets were examined in each pancreas, and the mean score was calculated by dividing the total score by the number of islets examined.

Quantification of β-cell mass and immunohistochemisty

Pancreatic ducts of Wistar and BB rats at 5 and 7 wk of age were cannulated and perfused with 15 ml of 4% formaldehyde. After this, the pancreas was resected, weighed, and postfixed in 50% formaldehyde/picric acid (vol/vol) for 24 h at 4 C. The fixed pancreas was dehydrated, paraffin embedded, and longitudinal 10-μm microtome sections were obtained.

To calculate β-cell mass, insulin was stained by immunohistochemical techniques using a mouse antirat insulin monoclonal antibody and a peroxidase conjugated goat antimouse IgG antibody and revealed with diaminobenzidine kit (Sigma-Aldrich). To determine β-cell mass, the insulin-positive areas were evaluated using a microscope equipped with a digital camera and the image analysis Cell D software (Olympus, Hamburg, Germany). The investigators were blinded with respect to the provenance of the samples. β-Cell mass values were calculated by multiplying the insulin-positive area/total pancreatic area ratio by the total pancreas weight.

To study MAPK activation, pancreas sections, obtained as described above from rats aged 5 and 7 wk, were stained using antibodies directed against phosphorylated forms of JNK, p38, and ERK, biotin conjugated goat antimouse and goat antirabbit IgG antibody. Samples were revealed with a diaminobenzidine kit. The presence and sites of the molecules under study were determined by examining a mean of 10 randomly chosen islets per animal in each experimental group.

Treatment protocol

Randomly grouped BB rats received an ip injection of vehicle (0.5% dimethylsulfoxide/saline) containing anti-IFN-γ monoclonal antibody (100 μg/kg · wk), Exendin-4 (4186.6 ng/kg · d), and Exendin-4+anti-IFN-γ at the same concentrations and doses. Treatment began at 5 wk of age and concluded at 7 and 11 wk of age.

Intraperitoneal glucose tolerance test (IPGTT) assay

Wistar, BB, and thymectomized BB rats were fasted overnight (16–18 h), and a blood sample was collected from the tail vein (fasting or 0-min sample). Then, an ip injection of 40% solution of glucose was administered (2 g/kg), followed by blood sampling at 15, 30, 60, and 120 min after the glucose administration. Glycemia was measured with an automatic glucose monitor (Accucheck Optimun; Roche Diagnostic).

Statistical analyses

Results are presented as means ± SEM of measurements performed in at least three animals. Statistical comparisons were per-

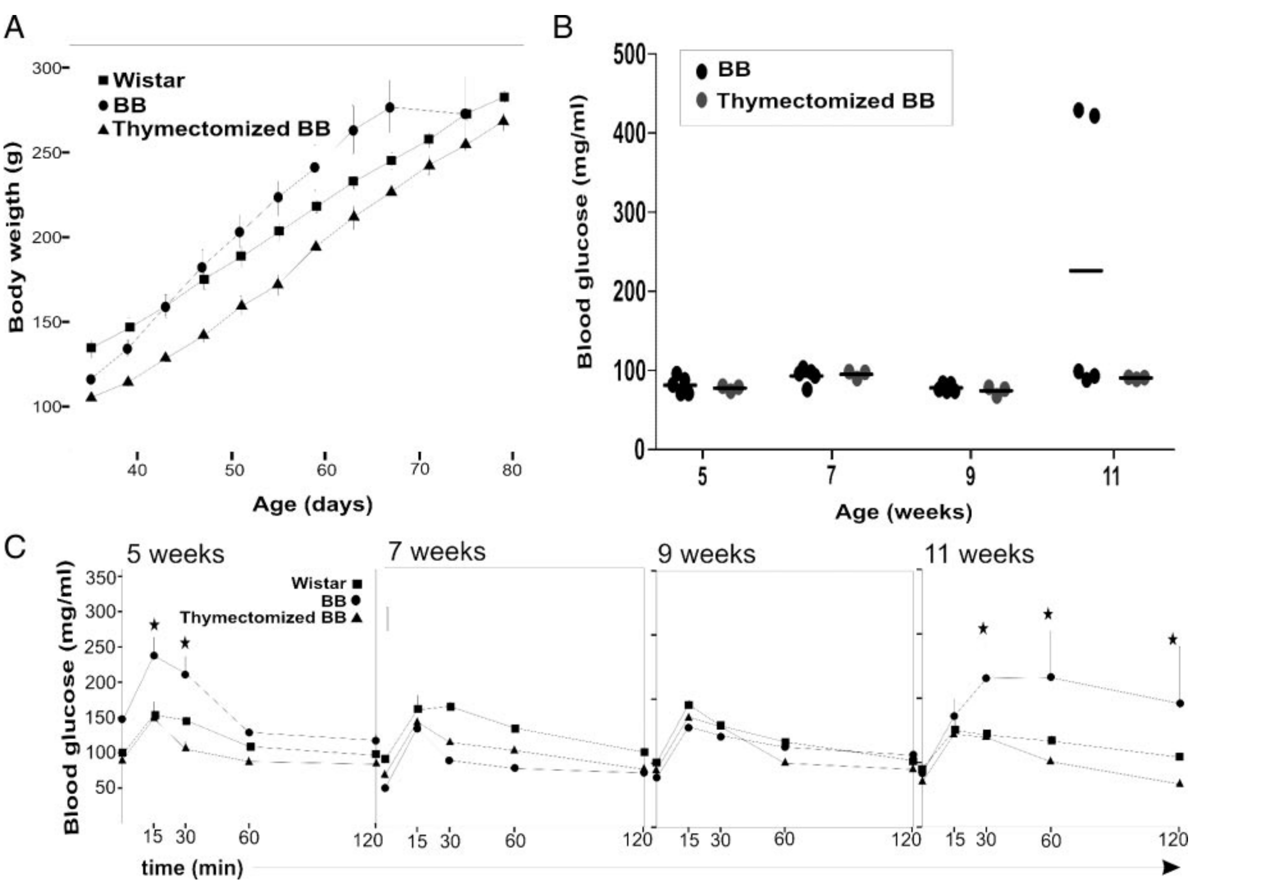

FIG. 1. A, Body weights recorded in Wistar (■), BB (●), and thymectomized BB (▲) rats between 5 and 11 wk of age. The values are presented as means ± SEM of weight (g) in the period between 5 and 7 wk of age. B, Random blood glucose levels were determined in BB (*black circle*) and thymectomized BB (*gray circle*) at 5, 7, 9, and 11 wk of age. The results are presented as means ± SEM of glycemia (mg/ml). Values were obtained from five Wistar and BB rats and three thymectomized BB rats. C, IPGTT was performed in Wistar (■), BB (●), and thymectomized BB (▲) rats at 5, 7, 9, and 11 wk of age. The results are presented as means ± SEM of glycemia (mg/ml) at the stated times postglucose injection. *, $P \leq 0.05$.

formed either by Mann-Whitney test or by ANOVA. All *P* values less than or equal to 0.05 were considered statistically significant.

Results

Body weight and glucose homeostasis during insulitis were determined in BB and control rats (thymectomized BB and Wistar) during insulitis stage at 5, 7, 9, and 11 wk of age. Body weight increases in BB rats between 5 and 11 wk of age were similar to the body weight changes in the control groups (Fig. 1A). Random blood glucose showed similar values in BB rats and the control groups except at 11 wk of age when two out of five animals in BB rat group reached high random glucose levels (Fig. 1B). The IPGTT also displayed an altered curve in BB rats at 11 wk of age, in contrast to Wistar and thymectomized BB rats (Fig. 1C). In addition, transitory glucose intolerance was noted in BB rats at 5 wk of age.

Characterization of insulitis stage in BB rats

Pancreatic mononuclear cell infiltration was assessed at 5, 7, 9, and 11 wk of age in thymectomized and non-thymectomized BB rats. An increase in infiltration score in BB rats from 5 wk of age (and thereafter) was noted, whereas no infiltration occurred in thymectomized BB rats (Fig. 2, A and B).

Time-course changes in β-cell apoptosis and proliferation during insulitis stage

β-Cell proliferation and apoptosis were evaluated in pancreatic tissue sections in Wistar, BB, and thymectomized BB rats at 5, 7, 9, and 11 wk of age. Wistar and thymectomized BB rats showed consistent β-cell proliferation rates along the time-course of the study. However, BB rats showed lower β-cell proliferation rates from 5 to 11 wk of age, compared with control rats (Fig. 3A).

With respect to β-cell apoptosis, an important enhancement was observed from 7 to 11 wk of age in BB rats compared with control rats (Fig. 3B). Of note is that a halt in proliferation occurs as the first step in β-cell homeostasis alteration at 7 wk of age, before an increase in apoptosis.

Changes in β-cell mass during the insulitis stage

The effect of β-cell proliferation inhibition on β-cell mass was tested in Wistar and BB rats at 5 and 7 wk of age.

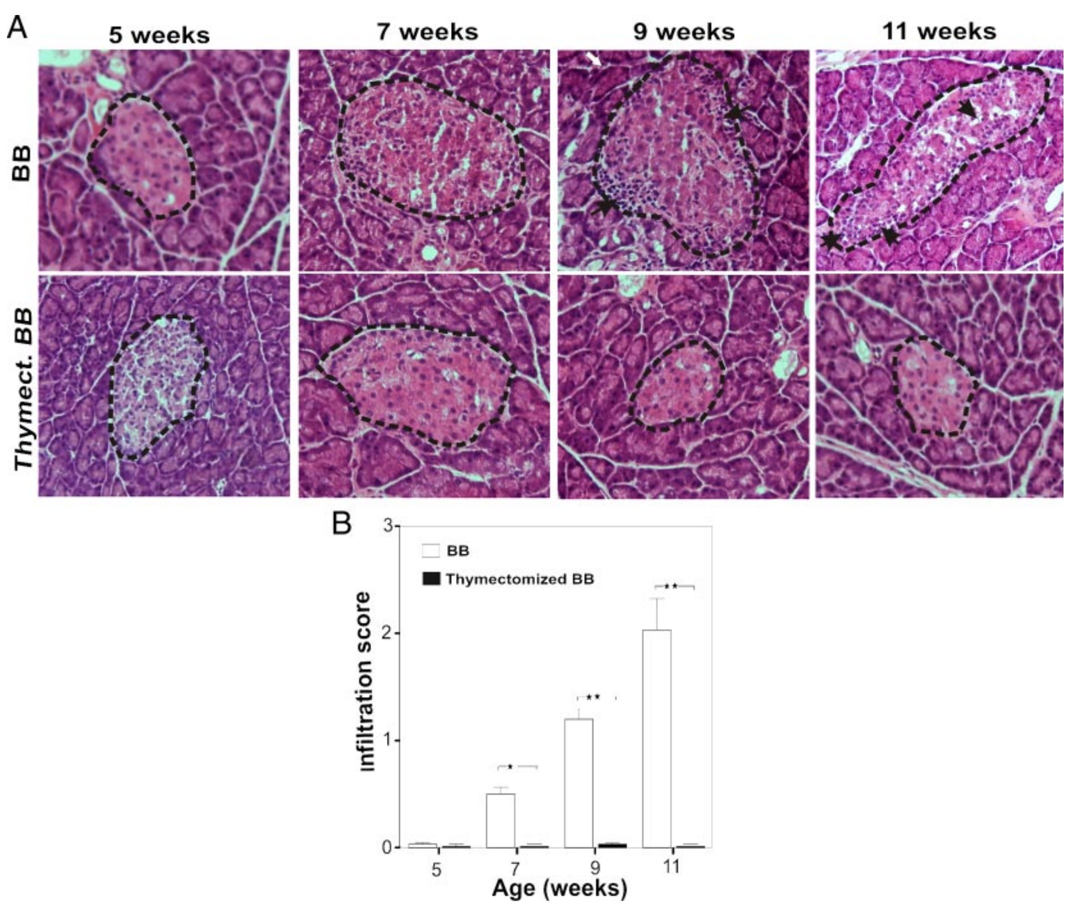

FIG. 2. Characterization of insulitis stage in BB rats. Infiltration scores were determined in Harris' H&E-stained pancreatic sections from BB and thymectomized BB rats at 5, 7, 9, and 11 wk of age. A, Representative images of stained sections showing pancreatic islets (*dotted line*) and mononuclear cells (*arrows*). B, Results represent means ± SEM of infiltration scores. Values were obtained from five BB rats and three thymectomized BB rats. *, $P \leq 0.05$; **, $P \leq 0.01$.

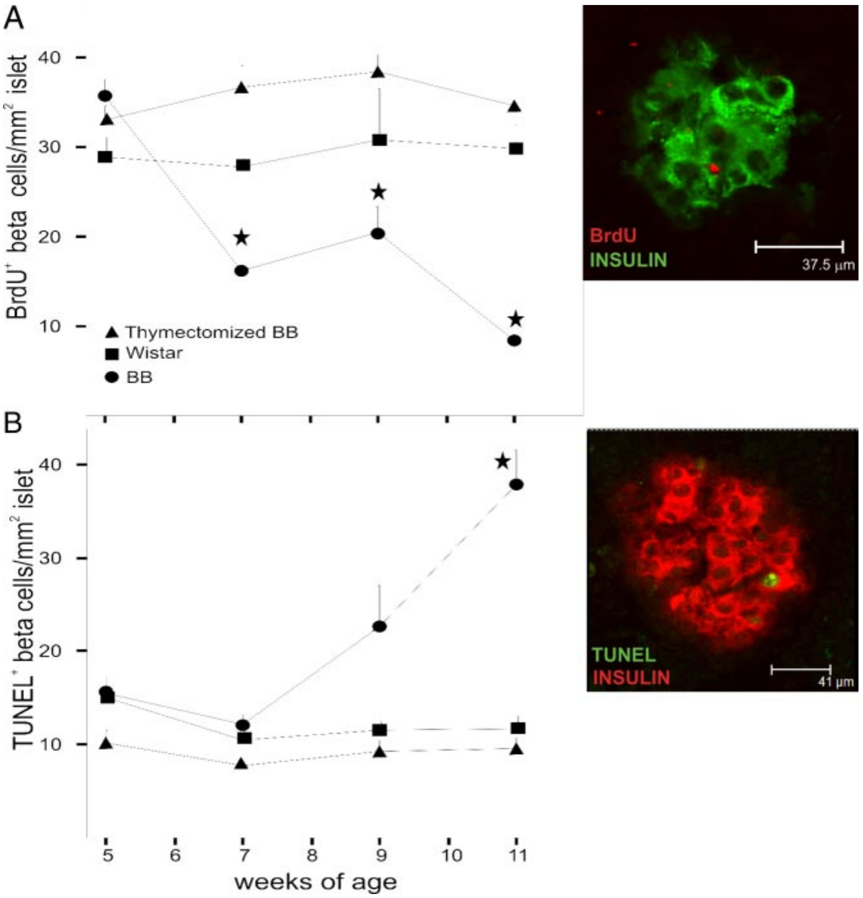

FIG. 3. Time-course of changes in apoptosis and proliferation during insulitis stages. Proliferative (A) and apoptotic (B) β-cells were quantified at 5, 7, 9, and 11 wk of age in pancreas sections from Wistar (■), BB (●), and thymectomized BB (▲) rats. Values are presented as means ± SEM of BrdU+/insulin+ (A) or TUNEL+/insulin+ (B) cells per islet area (mm^2) in a mean of five animals. *Panels alongside the graph* show representative images of BrdU and TUNEL islet staining. *, $P \leq 0.05$.

and suggests that, at this period of time (between 5 and 7 wk of age), islet development in BB rats is impaired.

Changes in MAPK activation profile during insulitis stage

Study of phosphorylated isoforms of ERK1/2, JNK, and p38, components of MAPK signaling pathway in pancreatic sections of Wistar, BB, and thymectomized BB rats at 5 and 7 wk of age, showed changes in their presence/absence and/or intracellular location. P-ERK1/2 maintained its expression level between 5 and 7 wk of age in islets of the Wistar and thymectomized BB rats. However, in BB rats, expression in islets was only observed at 5 wk, and expression had disappeared at 7 wk of age. P-JNK displayed a low expression induction in BB rats at 7 wk of age, in contrast to an absence of expression in the Wistar and thymectomized BB rats at both these times. In addition, the phosphorylated form of p38 showed a constitutive weak expression in cytoplasm of Wistar, BB, and thymectomized BB rat islets at the studied times. A translocation to the nucleus was observed only in BB rat islets at 7 wk of age (Fig. 5).

Figure 4 shows that Wistar rats had a significant increase in ß-cell mass between 5 and 7 wk of age due, probably, to body growth. This increment was not observed in BB rats

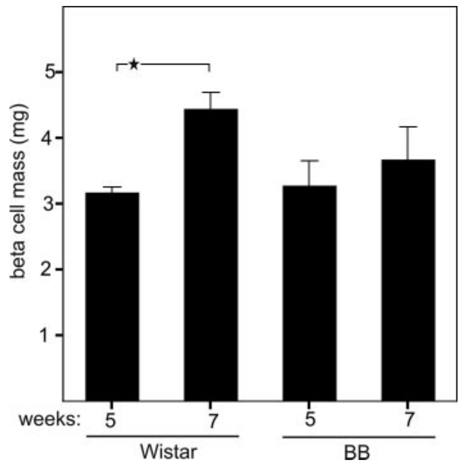

FIG. 4. β-Cell mass was determined in pancreatic sections from Wistar and BB rats at 5 and 7 wk of age. β-Cell mass is presented in the *bar graph* as means ± SEM of values calculated as the ratio of insulin-positive area/total pancreatic area multiplied by the total pancreas weight. Values are obtained from three animals. *, $P \leq 0.05$.

Role of proinflammatory cytokines and effect of Exendin-4 on β-cell proliferation and apoptosis during insulitis stage

Previous studies of our group showed that IFN-γ action contributed to the antiproliferative effect of proinflammatory cytokines (14). To test the role of IFN-γ on β-cell proliferation and apoptosis in our model, BB rats were treated with monoclonal anti-IFN-γ antibody from 5–7 and 11 wk of age, whereas the control group received only the vehicle. Anti-IFN-γ administration partially recovered β-cell proliferation at 7 wk, but no effect was observed at 11 wk (Fig. 6A). A significant decrease in β-cell apoptosis was observed at 11 wk in anti-IFN-γ treated rats, compared with vehicle-only-treated rats (Fig. 6B).

To explore the effect of an activator of β-cell proliferation during insulitis stage, the analog of GLP-1, Exendin-4, was administered alone and in combination with anti-IFN-γ in animals between 5, 7, and 11 wk of age. β-Cell proliferation and apoptosis were measured and compared with control BB rats treated with vehicle alone. As shown in Fig. 6, Exendin-4

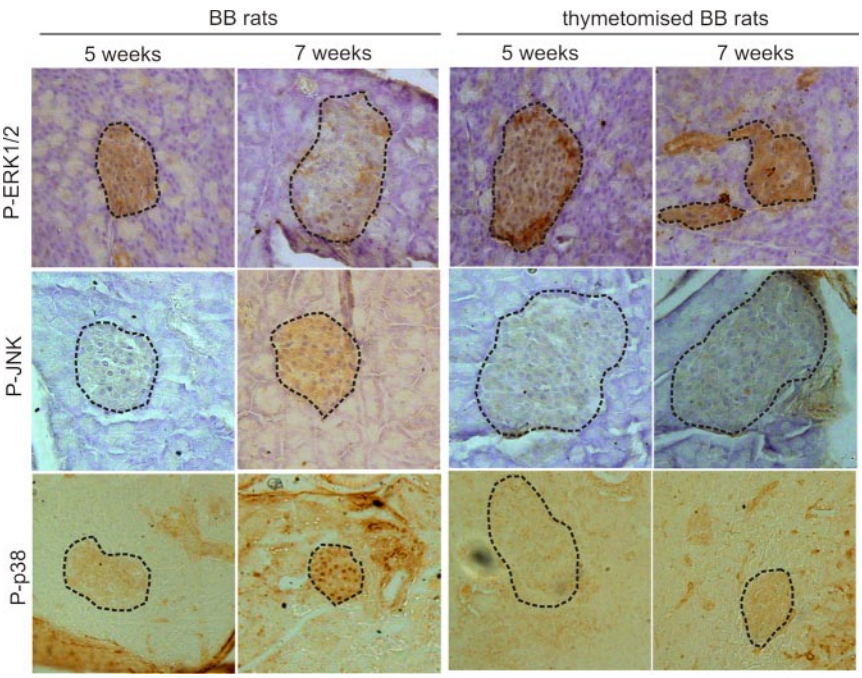

FIG. 5. Phosphorylated (P) forms of ERK1/2, JNK, and p38 stained in pancreatic sections of BB and thymectomized BB rat at 5 and 7 wk of age and studied under a light microscope (×10 objective). Representative images of sections of pancreatic islets (*dotted line*) and stained for protein (*brown*). Eosine counterstaining can be observed in P-ERK/12 and P-JNK.

induced a recovery in β-cell proliferation at all the time points of the study, although the effect at 11 wk was limited and nonsignificant. Combined Exendin-4 and anti-IFN-γ treatment showed a higher effect than either of the drugs alone and, at 7 wk, β-cell proliferation was almost completely recovered (Fig. 6A).

Exendin-4 induced a notable decrease in β-cell apoptosis, which was not modified with Exendin-4 and anti-IFN-γ administered in combination (Fig. 6B).

Effect of Exendin-4 on infiltration levels during insulitis stage

To test the effect of Exendin-4 and anti-IFN-γ, alone and in combination, on infiltration levels, H&E-stained sections obtained from treated and control BB rats were evaluated, and infiltration scores were calculated. Figure 7 depicts the significant improvement in infiltration scores attributed, mainly, to Exendin-4 at 11 wk of age.

Discussion

Our experiments were designed to analyze *in vivo* β-cell proliferation during insulitis stage in BB rats, a rodent model for T1DM. Wistar rats, a colony from which BB rats are produced, and thymectomized BB rats free from diabetes, were used as control animals (26). A constant rate of proliferation was observed in control rats between 5 and 11 wk of age. However, in BB rats, β-cell proliferation was inhibited from 5 wk of age. This indicates a new phase before apoptosis in which β-cell replication is altered.

Although islet infiltration is very slight between 7 and 8 wk of age, periductular mononuclear infiltration and phagocytic inflammatory macrophages have been described in islets as well as exocrine tissue of BB rats (28, 29). In addition, proinflammatory cytokines such as IL-1β and IFN-γ, as well as counter-regulatory cytokines such as

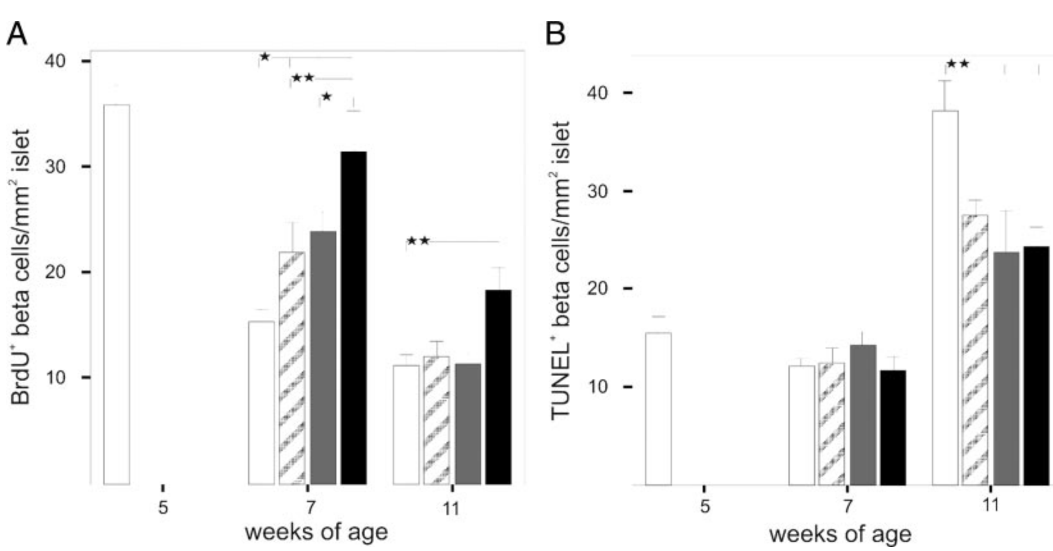

FIG. 6. Effect of anti IFN-γ and Exendin-4 administration (alone or in combination) on β cell proliferation and apoptosis during insulitis stage. β-Cell proliferation (A) and apoptosis (B) were quantified in pancreatic sections of BB rats at 5, 7, and 11 wk of age treated with anti-IFN-γ (*hatched bars*), Exendin-4 (*gray bars*), Exendin-4+anti-IFN-γ antibody (*black bars*), and vehicle-only (*white bars*). Data are expressed as the mean ± SEM of BrdU/insulin (A) or TUNEL/insulin (B) positive cells per islet area (mm²). Results are the mean of five animals. *, $P \leq 0.05$; **, $P \leq 0.01$.

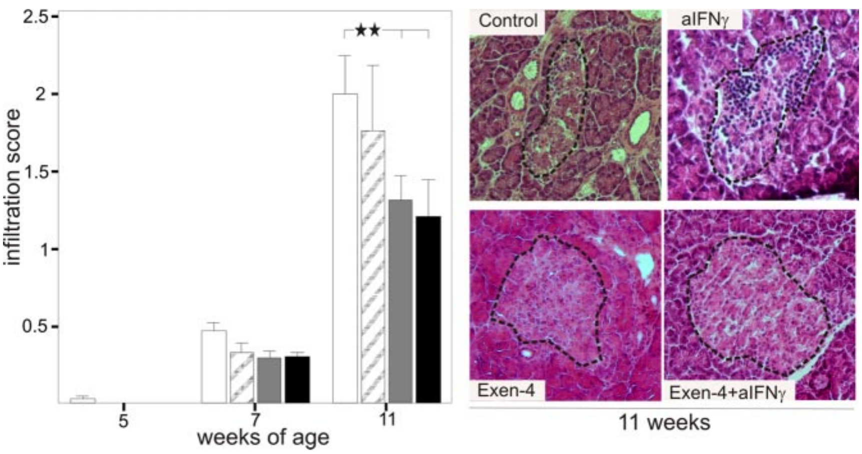

FIG. 7. Effect of anti-IFN-γ and Exendin-4 on infiltration levels. Infiltration scores were determined in Harris' H&E-stained pancreatic sections of BB rats aged 5, 7, 9, and 11 wk treated with anti-IFN-α antibody (*hatched bars*), Exendin-4 (*gray bars*), Exendin-4+anti-IFN-γ antibody (*black bars*), and excipient-only BB rats (*white bars*). Results are expressed as the means ± SEM of infiltration scores derived from a mean of five animals. Panel shows representative images of stained pancreas sections at 11 wk of age. **, $P \leq 0.01$.

IL-6 and IL-10, have been identified in pancreatic tissue. These cytokines can be produced by cells that constitute the infiltrate (23, 24) or by islet cells (30), and their presence exert a considerable influence on infiltrate progression. Presence of proinflammatory cytokines in BB pancreatic tissue from early stage of insulitis (5 wk of age) has been found by our group (our unpublished observations) and is concordant with that previously described. We found a progressively decreased β-cell proliferation beginning at 5 wk of age, before the commencement of the apoptosis phenomenon. The possibility of proliferation inhibition due to apoptosis of proliferating β-cell could be excluded because halting of proliferation is observed to occur before the onset of apoptosis. These alterations of β-cell proliferation in BB rat islets might be the source of the reduced insulin content and β-cell volume that has been described as preceding insulitis (15, 31, 32). To investigate this proposal, we measured β-cell mass in BB and Wistar rats between the ages of 5 and 7 wk. Interestingly, no increase was observed in β-cell mass in BB rats between 5 and 7 wk of age, in contrast with Wistar rats whose β-cell mass showed a significant increment over the same period. Considering that body weight progression is similar in BB and Wistar rats, this result would indicate that islet development is altered. Molecular mechanisms underlying this observed event were evaluated by determining the activation level of ERK1/2, JNK, and p38 (components of MAPK l) between 5 and 7 wk of age. Similar to previous results from our group (14), there was an inhibition of ERK1/2 activation in BB rat islets at 7 wk of age. This phenomenon could explain, at least in part, the halting of proliferation observed in the same period. On the other hand, there were changes over this time period with respect to the other MAPK members studied, *i.e.* JNK phosphorylation and phosphorylated p38 cytoplasm-to-nucleus translocation. Because these proteins are activated in response to stress stimuli, including IFN-γ and IL-1β (33), these changes could constitute the first molecular events triggered by proinflammatory cytokines leading to β-cell apoptosis.

Previous studies from our group have shown that proinflammatory cytokines drive the decrease in β-cell proliferation in cultured islets. To test IFN-γ involvement in the loss in β-cell replication, animals were treated with monoclonal anti-IFN-γ antibody from 5–7 and 11 wk of age. A recovery of β-cell proliferation at 7 wk of age was observed; the lack of effect between 7 and 11 wk of age could be due to severe insulitis and β-cell apoptosis at these times. In addition, long-term treatment with antibodies may induce an anti-idiotypic response in restraining the antibody effect (34).

Exendin-4 is an analog of GLP-1 used to treat type 2 diabetes (35). Exendin-4 activates β-cell replication by increasing cAMP production (36) and inhibiting β-cell apoptosis (37). We tested the effect of Exendin-4, alone or in combination with the anti-IFN-γ antibody, on β-cell proliferation during the insulitis stage. Exendin-4 on its own almost completely reversed the proliferation decrease at 7 wk of age. The effect is lost at 11 wk when a high β-cell apoptosis is observed. This result is in agreement with a modest delay in the onset of diabetes with a recovery of β-cell mass and improved glucose tolerance that has been recently reported with Exendin-4 treatment in the nonobese diabetic mouse model (38). Combined treatment with Exendin-4 and anti-IFN-γ showed increases in β-cell proliferation at higher rates at 7 and 11 wk of age than either of these compounds individually. This effect can be due to the signaling pathway inhibition by cytokines and activation by Exendin-4 not being the same as the antiapoptotic effect of Exendin-4, which is especially significant at 11 wk of age. Exendin-4 on its own, but not anti-IFN-γ, decreased the infiltration score at 11 wk of age, the time at which infiltration was more evident. Only an infiltration-activating role has been previously described for Exendin-4 when it had been observed to be expressed constitutively in tissues in which it is not normally expressed (39). Hence, the action of Exendin-4 on infiltration levels, rather than an immunomodulatory action, appears to be the result of apoptosis protection by maintaining islet architecture and preventing islet infiltration. Surprisingly, the blockage of IFN-γ, a putative immunoregulatory molecule, induces no effects on islet infiltration. This is in agreement with data from a recent report in which anti-IFN-γ treatment induced changes in the infiltrate composition but not in its progression (40).

In conclusion, we describe, in an *in vivo* system, a new stage in the process of β-cell mass loss before death by apoptosis and the onset of diabetes, characterized by the halting of β-cell proliferation. IFN-γ has a key role in this process, probably in combination with the other proinflammatory cytokines present in the pancreatic microenvironment during insulitis (IL-1β and TNF-α). Although the results do not provide explanations of the mechanisms by which proinflammatory cytokines exert their antiproliferative effects on β-cell replication, they do show that this effect can be prevented, or delayed. Knowledge of these mechanisms are of considerable importance in designing therapeutic approaches to the prevention of type 1 diabetes via action, initially, on β-cell regeneration and, subsequently, on β-cell apoptosis.

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