

Cure of Overt Diabetes in NOD Mice by Transient Treatment With Anti-Lymphocyte Serum and Exendin-4

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Treatment of overtly diabetic NOD mice with anti-lymphocyte serum (ALS), a polyclonal anti-T-cell antibody, abrogates autoimmunity and achieves partial clinical remission. Here we investigated whether the addition of exendin-4, a hormone that stimulates insulin secretion and β -cell replication and differentiation, improves induction of remission by ALS. Transient treatment of overtly diabetic NOD mice with ALS and exendin-4 achieved complete remission in 23 of 26 mice (88%) within 75 days, accompanied by progressive normalization of glucose tolerance, improved islet histology, increased insulin content in the pancreas, and insulin release in response to a glucose challenge. Syngeneic islets transplanted into mice cured by treatment with ALS plus exendin-4 remained intact, and cotransfer of lymphocytes from cured mice delayed diabetes induction by adoptive transfer, suggesting the long-lasting presence of autoimmune regulatory cells. Although ALS alone also achieved reversal of diabetes, the frequency of remission was low (40%). No treatment or exendin-4 alone failed to produce remission. These results show that exendin-4 synergistically augments the remission-inducing effect of ALS. The addition of β -cell growth factors, such as exendin-4, to immunotherapy protocols with anti-T-cell antibodies presents a potential novel approach to the cure of patients with new-onset type 1 diabetes. *Diabetes* 53:1700–1705, 2004

Nonobese diabetic (NOD) mice spontaneously develop diabetes, which has features similar to those of human type 1 diabetes, a polygenetic disease resulting from an inadequate amount of β -cell mass caused by T-cell-mediated autoimmune destruction of β -cells (1–3). Anti-T-cell immunotherapy given early, before the appearance of insulinitis, partially prevents the development of diabetes (4–6). However, treatment of diabetes-prone, yet clinically healthy, individuals, particularly juveniles, with immunosuppressive drugs is not a desirable form of therapy because of the potential

complications associated with the chronic use of such drugs. In contrast, the transient use of immunosuppression in already diabetic patients may be justified if the therapy has the potential to achieve a complete cure of diabetes. We have previously demonstrated (7) that the treatment of overtly diabetic NOD mice with anti-lymphocyte serum (ALS), a polyclonal anti-T-cell antibody, induces a long-term abrogation of autoimmunity and, in ~50% of treated female mice, achieves a permanent clinical remission. Reversal of hyperglycemia, however, was a slow process, requiring 75–105 days.

Exendin-4 is a long-acting agonist of glucagon-like peptide (GLP)-1 (8–11), a potent intestinal insulinotropic hormone that augments insulin secretion in rodents as well as in both type 1 (12) and type 2 (8,13–15) human diabetic subjects. Both GLP-1 and exendin-4 have been shown to promote replication and differentiation of β -cells in vivo (16,17) and in vitro (18). Thus, we hypothesized that the addition of exendin-4 to ALS might augment the remission-inducing effects of ALS in treating new-onset diabetes in NOD mice.

RESEARCH DESIGN AND METHODS

Nine-week-old female NOD mice (NOD/MrkTac) were purchased from Taconic (Germantown, NY). After 13 weeks of age, mice were tested twice a week for urine glucose levels. Once urine glucose levels became positive, mice were additionally tested for a nonfasting blood glucose level every 2–3 days. Mice with >17 mmol/l (300 mg/dl) blood glucose levels in three consecutive measurements performed over a 7-day interval were considered diabetic. Remission was defined as blood glucose levels consistently remaining at <11 mmol/l (200 mg/dl).

Treatment protocol. ALS was prepared by immunizing rabbits with lymph node cells harvested from NOD, C3H/He, DBA/2, and (C57BL/6xA)F1 mice as previously described (19). ALS (0.5 ml) was injected intraperitoneally into diabetic NOD mice on day 0 (the third day of hyperglycemia) and day 3. Exendin-4 (Bachem Bioscience, King of Prussia, PA) was administered intraperitoneally at 12 nmol/kg for 4 consecutive days twice (days 0–3 and 7–10). Following the above-described treatment protocols, no further ALS or exendin-4 was administered. All diabetic mice were treated daily with 0.8 units of insulin (4:6 mixture of regular and NPH insulin; Eli Lilly, Indianapolis, IN) until glycemic control was achieved.

Intraperitoneal glucose tolerance test. Glucose (2 g/kg) was administered intraperitoneally to conscious animals after overnight fasting. Blood samples were collected from the tail vein into heparinized capillary tubes. Blood glucose levels were determined with a glucometer. Plasma insulin levels were measured using a commercial enzyme-linked immunosorbent assay kit (Crystal Chem, Chicago, IL). The detection limit of the optimized assay is 156 pg/ml, and the reactivity with mouse insulin is 105%.

Total pancreas insulin content. The freshly dissected pancreas was weighed, snap frozen, and stored at -80°C until assay for insulin as described (20). Briefly, the pancreas was homogenized in acid/ethanol (0.1 N HCl in 100% ethanol) and stored overnight at -80°C . The homogenates were centrifuged at 14,000g for 5 min, and the supernatant was dried under vacuum, resuspended in 1 ml H_2O , and serially diluted in TE (10 mmol/l Tris pH 7.4, 1 mmol/l EDTA). The amount of insulin was measured by radioimmunoassay.

Histology. The pancreas was fixed in a solution containing 70% ethanol, 2.5% formaldehyde, 5% acetic acid, and 25% water for 3 days, processed, and

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ALS, anti-lymphocyte serum; GLP, glucagon-like peptide; IPGTT, intraperitoneal glucose tolerance test; PDX, pancreas duodenum homeobox; STZ, streptozotocin.

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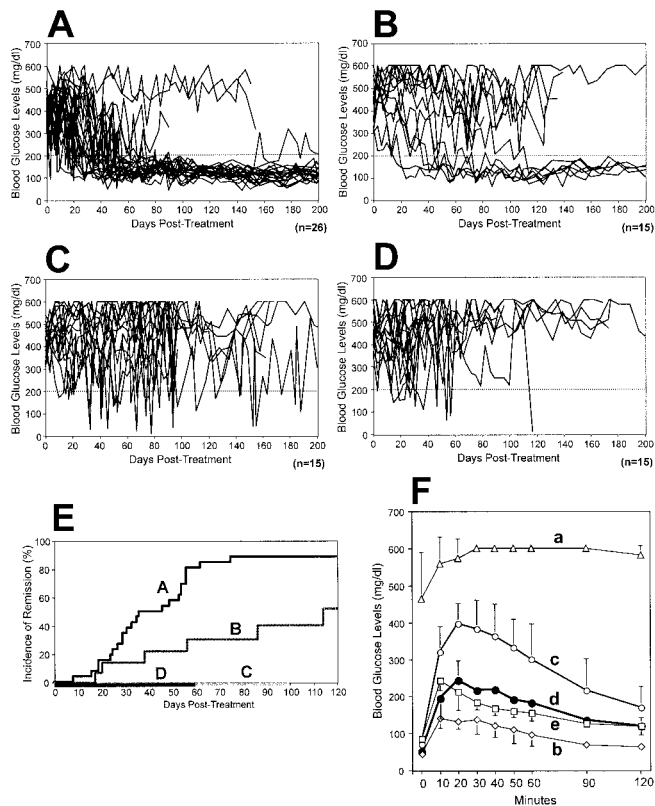


FIG. 1. Immunotherapy in overtly diabetic NOD mice. *A–D*: Changes in blood glucose levels. Overtly diabetic NOD mice were treated with ALS and exendin-4 (*A*), ALS alone (*B*), or exendin-4 alone (*C*) or were untreated (*D*). Treatment started within 2 weeks after blood glucose levels rose to >17 mmol/l. All diabetic mice were given insulin once a day until the mice became euglycemic (<11 mmol/l). *E*: Incidence of remission. Lines *A–D* correspond to the four groups described above. Incidence was calculated by Kaplan-Meier estimates. *F*: IPGTT. *Line a*: Diabetic NOD mice ($n = 4$). *Line b*: Nondiabetic 9-week-old NOD mice ($n = 6$). *Line c*: Euglycemic NOD mice 80 days after the initiation of ALS and exendin-4 treatment (52 ± 5 days after remission) ($n = 8$). *Line d*: Euglycemic NOD mice 200 days after the initiation of ALS and exendin-4 treatment ($n = 7$). *Line e*: Naive C57BL/6 mice ($n = 6$).

embedded in paraffin. At every 100- μ m interval, sections with 5- to 7- μ m thickness were cut, deparaffinized, and stained in hematoxylin and eosin. Additional sections were stained for insulin and glucagon using a polyclonal rabbit anti-insulin antibody and a polyclonal goat anti-glucagon antibody. Twelve to 58 islets from each pancreas were examined to evaluate the degree of mononuclear cell infiltration. The severity of infiltration was classified into four degrees, as previously reported (21): 0, no infiltration; 1, polar islet infiltration; 2, peri-islet infiltration; 3, intraislet infiltration; and 4, extensive intraislet infiltration.

Islet transplantation. Islets were prepared from NOD/SCID mice as previously described (7), and 300 islets were transplanted into the renal subcapsular space of the euglycemic mice that underwent remission. The mice were killed 60 days later, and islet grafts were examined histologically.

Adoptive cell transfer. Adult NOD/SCID mice were used as recipients. Splenocytes were prepared from overtly diabetic NOD females (diabetogenic cells) and long-term cured NOD mice after ALS and exendin-4 treatment (putative immunoregulatory cells). NOD/SCID mice were injected with either 10^7 diabetogenic cells alone or a mixture of 10^7 diabetogenic cells and 2×10^7 putative immunoregulatory cells. Development of diabetes was determined by continuous monitoring of blood glucose levels three times a week.

Statistical analysis. Statistical significance was evaluated by the Kaplan-Meier life-table method as well as one-way ANOVA and Student's *t* test. Differences with $P < 0.05$ were deemed significant.

RESULTS

Treatment of diabetic NOD mice. Diabetic NOD mice were randomly divided into four groups according to treatment regimens: ALS and exendin-4, ALS alone, ex-

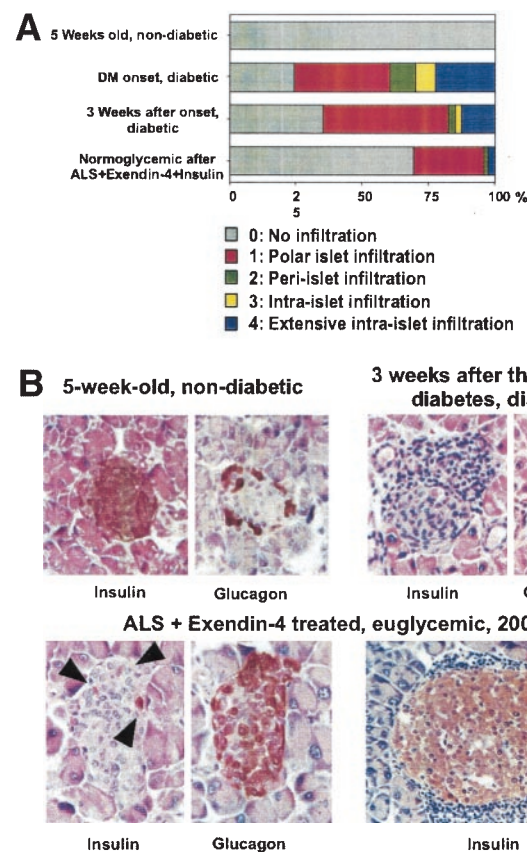


FIG. 2. Histology of the pancreas. *A*: Insulinitis scores. The severity of mononuclear cell infiltration was scored from 0 through 4. *B*: Insulin and glucagon staining of islets. The lower left panel shows a typical small islet that contains very few β -cells. The arrows indicate the cells with positive insulin staining. The lower right panel shows a typical large islet that is enriched in β -cells. Such large, β -cell-rich islets were found in isolated groups and were typically surrounded by peri-islet infiltrates.

endin-4 alone, and no treatment. ALS was administered twice after the onset of diabetes, while exendin-4 was administered for 4 days in two consecutive cycles, with 3 days of rest between the cycles. After completion of these transient treatment protocols, no further treatment with ALS and/or exendin-4 was given. All four groups were given daily insulin therapy that was stopped after blood glucose levels became <11 mmol/l. Treatment with a combination of ALS and exendin-4 resulted in complete and persistent remission in 23 of 26 treated mice (Fig. 1A). In these mice, remission was achieved within 75 days (mean 37 ± 4) after initiation of therapy (Fig. 1E). Once stable euglycemia had been achieved, these mice remained euglycemic thereafter except for one mouse that showed occasional hyperglycemia after 80 days of continuous euglycemia. Treatment with ALS alone also achieved remission, but at a lower frequency (6 of 15 treated mice; $P = 0.004$, by Kaplan-Meier estimates compared with the ALS and exendin-4 group) and somewhat slower pace (55 ± 16 days, range 17–114) (Fig. 1B). Treatment with exendin-4 alone failed to produce any disease remission (Fig. 1C), whereas no spontaneous reversal of diabetes was seen in untreated (insulin alone) mice (Fig. 1D). In all groups, management of blood glucose in mice that failed to undergo remission was extremely difficult. Three of 26 mice in the ALS and exendin-4 group, 8 of 15 in the

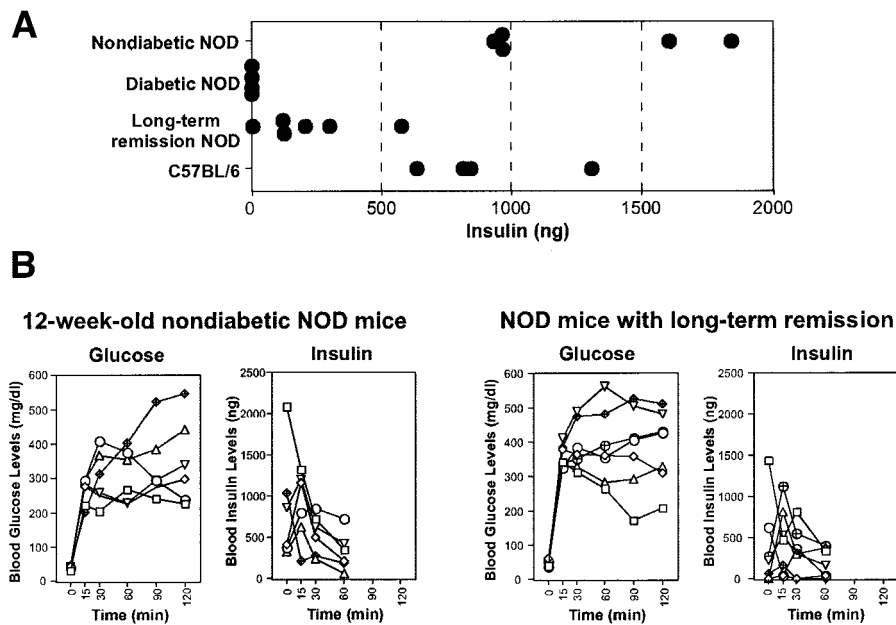


FIG. 3. Insulin content and secretion. **A:** Insulin content of the whole pancreas. **B:** Insulin secretion during IPGTT with a high-dose (2 g/kg) glucose challenge. Each symbol in both blood glucose and blood insulin graphs represents the same mouse.

ALS alone group, and 13 of 15 mice in both the exendin-4 alone and no treatment groups died or needed to be killed between 91 and 157 days (mean 133 ± 21 days after initiation of treatment), 14 and 146 days (87 ± 18), 19 and 172 days (101 ± 15), and 30 and 175 days (77 ± 14), respectively. These results demonstrated that exendin-4 synergistically augmented the effect of ALS to induce survival and cure of diabetes in overtly diabetic NOD mice.

Glucose homeostasis challenges. We determined the degree of glycemic control by intraperitoneal glucose tolerance tests (IPGTTs). Euglycemic NOD mice 80 days after initiation of ALS and exendin-4 treatment (or 52 ± 5 days after remission, $n = 8$) showed a significantly improved IPGTT, with blood glucose levels peaking after 20 min at 400 mg/dl and gradually lowering to 170 mg/dl at 120 min (Fig. 1F). The same group of mice exhibited almost normal IPGTT results 200 days after treatment, except that the peak glucose levels were seen at 20 min rather than at 10 min, as exhibited by naive C57BL/6 mice. Diabetic NOD mice sustained high blood glucose levels (>400 mg/dl) throughout 120 min. Interestingly, 9-week-old nondiabetic NOD mice showed much lower peak glucose levels than age-matched C57BL/6 mice. NOD mice that had been free of diabetes after ALS treatment showed an almost identical IPGTT pattern to that of ALS and exendin-4-treated mice (data not shown). Thus, glucose metabolism continuously improved after the reversal of hyperglycemia by ALS and exendin-4, despite discontinuation of further therapy.

Histology of the pancreata. By histological examination, mice that underwent remission had significantly more islets free of lymphocytic infiltration compared with diabetic mice either at the time of onset or 3 weeks after the onset (72 vs. 26.3 or 41.6%, respectively; $P < 0.0001$, by one-way ANOVA) (Fig. 2A). The severity of insulinitis in cured mice that underwent remission by transient treatment with ALS and exendin-4 was much less compared with diabetic mice. Immunohistochemical staining revealed that islets of 5-week-old nondiabetic NOD mice showed typical diffuse insulin staining and peripheral

glucagon staining, whereas the majority of islets in diabetic NOD mice showed no insulin staining but positive glucagon staining in the area spared by cellular infiltration (Fig. 2B). Two types of histologies emerged in the examination of islets in pancreata harvested from cured mice 200 days after immunotherapy. One type of islet had either no or only a low degree of peripheral insulinitis and contained only a few (4–8) insulin-positive cells scattered throughout the islets. Glucagon-positive cells were distributed diffusely all over these islets. Another group of islets were large and showed positive insulin staining throughout the islet, with a peripheral mononuclear cell infiltration. Remarkably, these islets were clustered in relatively small sections of the pancreas and could only be identified by examination of many histological sections.

Insulin content and secretion. Because of the difficulty in measuring β -cell mass by morphometry, we measured the amount of insulin contained in extracts of the whole pancreata harvested from the mice in the different treatment categories. The pancreata of NOD mice that underwent remission contained 220 ± 82 ng of insulin (range 1–575; $n = 6$) compared with $1,266 \pm 189$ ng (928–1,840; $n = 5$) in the pancreas of 12-week-old nondiabetic NOD mice or 892 ± 144 ng (632–1,304; $n = 4$) in the pancreas of naive C57BL/6 mice (Fig. 3A). In sharp contrast, the pancreata of diabetic NOD mice contained virtually no insulin (2 ± 1 ng; $n = 4$). Thus, the pancreata of cured mice had a greater β -cell mass compared with those of diabetic mice. The mice were challenged by an IPGTT. At 15 min after glucose challenge, blood insulin levels in cured mice were 457 ± 155 ng (41–1,129; $n = 7$) compared with 894 ± 172 ng (223–1,329; i.e., $n = 6$) in 12-week-old nondiabetic mice ($P = 0.09$, by Student's *t* test). Some of the cured mice showed minimum insulin release after glucose challenge despite maintenance of morning euglycemia. Diabetic NOD mice had no detectable blood insulin levels at any time point following glucose challenge (data not shown).

Immunologic effect of ALS. To assess the immunologic effect of ALS, four mice were transplanted at 200 days

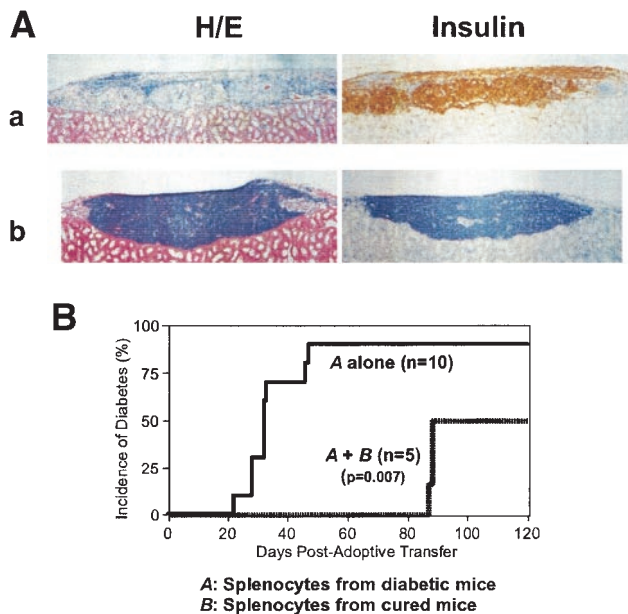


FIG. 4. Islet transplantation and adoptive cell transfer. **A:** Islets from NOD/SCID mice were transplanted into the renal subcapsular space in euglycemic mice (*row a*) or one mouse with occasional hyperglycemia (*row b*) at 200 days after treatment with ALS and exendin-4. Mice were killed 60 days after transplantation, and grafts were examined histologically by hematoxylin and eosin (H/E) and insulin staining. Representative histologies are shown. **B:** Development of diabetes after adoptive cell transfer. Either splenocytes from diabetic mice (diabetogenic cells) alone or a mixture of diabetogenic cells and splenocytes from ALS and exendin-4-treated, cured mice were injected into NOD/SCID mice.

after treatment with ALS and exendin-4 with islets prepared from NOD/SCID mice. Mice were killed 60 days after transplantation, and grafts were examined histologically. In three mice that were euglycemic at the time of transplantation, islet grafts remained intact (Fig. 4A, *row a*). A localized low-degree mononuclear cell infiltration was seen only outside of transplanted islets. In sharp contrast, in one mouse with lingering hyperglycemia at the time of transplantation (Fig. 4A, *row b*), the islet graft was completely covered by mononuclear cells, suggesting the recurrence of autoimmune diabetes (Fig. 4A, *row b*). We also determined whether mice that were diabetes free long after transient ALS and exendin-4 treatment harbored immunoregulatory cells that were capable of suppressing autoimmunity. Using a standard adoptive transfer model, we injected splenocytes prepared from overtly diabetic NOD mice (diabetogenic cells) into NOD/SCID mice with or without splenocytes obtained from mice that underwent remission after ALS and exendin-4 treatment (putative immunoregulatory cells). As shown in Fig. 4B, cotransfer of cells from cured mice inhibited induction of diabetes by diabetogenic cells. Similar results were obtained with lymphocytes prepared from mice given ALS alone (data not shown). These results demonstrate that ALS induces immunoregulatory cells that are capable of inhibiting autoimmunity long after the cessation of treatment.

DISCUSSION

Here we showed that treatment of new-onset diabetic NOD mice with a combination of ALS and exendin-4 achieved complete remission in 90% of treated mice. Although treatment of diabetic mice with ALS alone also

achieved reversal of overt diabetes, as we have previously shown (7), the frequency of remission was much lower and progression to remission tended to be slower compared with ALS and exendin-4 treatment. Both untreated mice and mice treated with exendin-4 alone failed to produce any disease remission. Thus, exendin-4 synergistically augmented the effect of ALS to induce survival and cure of diabetes in overtly diabetic NOD mice. Once stable euglycemia had been achieved, virtually all of the mice remained euglycemic (<11 mmol/l) throughout the study period (up to 200 days), despite earlier discontinuation of further therapy. In contrast to the rapid restoration of euglycemia after ALS and exendin-4 treatment, improvement of glucose metabolism was a slow process, as illustrated by abnormal IPGTT results in cured mice 80 days after the initiation of therapy, indicating that the islet mass early after remission was sufficient to maintain a euglycemic state, yet not sufficient to control an excessive glucose load during IPGTT. By 200 days posttreatment, IPGTT results returned to near normal. This circumstance reflects an increased insulin content in the pancreata of long-term cured mice to the level of ~20% of that of the nondiabetic NOD pancreas, which is in contrast to the pancreata of diabetic mice that did not contain any measurable insulin. However, some cured mice had no measurable insulin in the pancreas or showed minimum insulin release after glucose challenge, despite maintenance of morning euglycemia for a long period, probably attributable to marginal β -cell mass and exhaustive use of insulin for the maintenance of glycemic control.

Remission induced by ALS and exendin-4 therapy was accompanied by progressive normalization of glucose tolerance, improvement of islet histology, presence of increased amounts of insulin in the pancreas, and the release of insulin in response to a glucose challenge. These changes point to an increased β -cell mass in cured mice as diabetic NOD mice showed very little or no insulin staining and an absence of measurable insulin in the pancreas, probably due to an extensive loss of β -cells. The increase in β -cell mass can result from an increase in β -cell size (hypertrophy) and/or an increase in numbers of β -cells by neogenesis and/or the replication of preexisting β -cells. Although increased insulin production stimulated by exendin-4 may lead to β -cell hypertrophy, it is difficult to envision that hypertrophy alone can account for the long-lasting favorable effect on β -cell mass and glycemic control. Progressive but slow improvement of glucose tolerance in cured mice also suggests involvement of other mechanism(s).

The unusual histology and distribution of the islets in mice cured of diabetes by combined treatment with ALS and exendin-4 deserves comment. Many of the islets were small and essentially devoid of β -cells and markedly enriched in glucagon-producing α -cells. Some islets contained none or only a few (i.e., 2–4) β -cells. Other islets, located in isolated lobes of the pancreas, were large and contained a full complement of β -cells. It is tempting to speculate that the islets composed almost entirely of α -cells are in the processes of generating new β -cells by recapitulating the ontogeny of pancreas development, whereby in the mouse, α -cells first appear by embryonic day 9.5 (e9.5), followed by the appearance of β -cells 3 days

later at e13.5. The larger islets may represent more fully differentiated, mature islets enriched in β -cells. Such a speculative mechanism, if such exists, implies that the treatment of the mice with ALS and exendin-4 may result in the stimulation of the differentiation of some form of stem/progenitor or precursor cells resident within the islets after the mature β -cell population has been destroyed by the autoimmunity.

Was a regenerative process involved in the beneficial effect of exendin-4? At this point, we do not have any concrete evidence that exendin-4 promoted β -cell regeneration. Whereas exendin-4 exerts its β -cell-regenerating function by increasing the expression of pancreas duodenum homeobox (PDX)-1 on β -cell precursor cells (14,22,23), we failed at any time points after treatment to detect any specific increase in PDX-1 mRNA by RT-PCR or protein expression by Western blot in the pancreata of mice given exendin-4 with or without ALS. The levels of PDX-1 expression were equivalent to those in the pancreata of diabetic mice given only insulin. We were also unable to immunohistologically demonstrate an increase in bromodeoxyuridine or anti-Ki67 staining, which is indicative of cell proliferation, in any regions of the pancreas, i.e., ductal, periductal, or parenchymal areas. These results suggest that rapid β -cell regeneration may not account for the beneficial effect of exendin-4.

Exendin-4 has been shown to influence β -cell mass via the regulation of susceptibility to apoptotic cell death (24). Coadministration of exendin-4 with streptozotocin (STZ) significantly reduced STZ-induced β -cell apoptosis in vivo, whereas exendin-4 directly reduced cytokine-induced apoptosis in purified rat β -cells in vitro. Moreover, adult diabetic NOD mice contain endogenous precursor cells capable of giving rise to new islet structures after the underlying autoimmune disease is eliminated or suppressed (25,26). Pancreata of STZ-induced diabetic mice also contain precursor cells that proliferate to become insulin-producing cells following syngeneic bone marrow transplantation (27). Taken together, we postulate the following mechanism for the remission-inducing effect of ALS and exendin-4. ALS, by effectively eliminating autoimmune effector T-cells (7), allowed endogenous precursor cells to slowly give rise to increased β -cell mass sufficient to maintain normoglycemia. This slow process is illustrated by long intervals that are required for the induction of remission in mice given ALS alone, particularly when ALS treatment was delayed after diabetes onset (7). When exendin-4 was coadministered with ALS, it exerted a "rescuing influence" by preventing apoptotic death of quiescent β -cells still present in the pancreata of diabetic mice, resulting in more surviving β -cells and precursor cells to further expand. Stimulation of increased insulin production and/or a low degree of regenerative/replicative process of β -cells by exendin-4, albeit undetectable by RT-PCR and Western blot, might also be involved. In untreated mice or mice given exendin-4 alone, however, autoimmunity persisted and any increased β -cell mass was probably destroyed by the existing autoimmune effector mechanism.

ALS and exendin-4 were administered only for a short period of time (ALS, twice in 4 days; exendin-4, eight times in 11 days) at the onset of diabetes, and yet they achieved

long-term suppression of autoimmunity and continuous improvement of glycemic control. We have previously postulated that ALS eliminates autoreactive effector T-cells but spares autoimmune regulatory cells, thus creating a long-lasting immunoregulatory cell-dominant condition (7). Indeed, we showed in our present study that elicitation of autoimmune diabetes in NOD/SCID mice by adoptive transfer of diabetogenic lymphocytes was prevented by the cotransfer of splenocytes prepared from ALS-treated, long-term, diabetes-free NOD mice, suggesting the persistent presence of immunoregulatory cells in ALS-treated mice long after the cessation of treatment. Characterization of the cells mediating immunoregulation is currently in progress. Gradual improvement of glycemic control in mice long after cessation of treatment indicates that exendin-4 also exerted long-lasting beneficial influences on β -cell mass and glycemic control. A similar "memory effect" of GLP-1 and exendin-4 has previously been reported by other investigators (16,17,28–30).

Successful induction of remission in overtly diabetic NOD mice with anti-T-cell antibody, either polyclonal ALS (7) or monoclonal anti-CD3 antibody (31), led to a recent clinical trial (32) attempting to induce remission in patients with new-onset diabetes. Whereas treatment with humanized anti-CD3 monoclonal antibody mitigated the deterioration in insulin production and improved metabolic control in some patients during the first year of treatment, complete remission as seen in the NOD mouse model of type 1 diabetes has not been achieved. The results presented here suggest that the addition of a GLP-1 agonist such as exendin-4 to transient immunotherapy with anti-T-cell antibodies may enhance the probabilities of a permanent cure for new-onset type 1 diabetes. Both ALS and exendin-4 have already been tested in humans: polyclonal antilymphocyte/antithymocyte preparations have been used clinically for years and synthetic exendin-4 (exenatide; Amylin Pharmaceuticals, San Diego, CA) is currently in a phase III clinical trial for the treatment of type 2 diabetes (33,34).

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