Increased β-Cell Proliferation and Reduced Mass Before Diabetes Onset in the Nonobese Diabetic Mouse

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To determine whether loss of β-cell mass and function in the NOD mouse occurs gradually, beginning after the onset of insulitis, or abruptly, just before the onset of overt diabetes, β-cell mass and rates of β-cell proliferation and insulin secretory responses from the perfused pancreas were measured in NOD and control NOD/Scid mice at 8–9, 13, and 18 weeks of age. Of the NOD mice, 11 and 70% had diabetes (fasting blood glucose >8.3 mmol/l) at 13 and 18 weeks of age, respectively. β-cell mass in 8-week-old NOD mice was 69% of control mice ($P > 0.05$), but the rate of 5-bromo-2-deoxyuridine uptake was greater, suggesting a compensatory proliferative response to ongoing autoimmune β-cell destruction. Despite an increase in the rate of β-cell proliferation, β-cell mass was significantly reduced by 42% in 13-week-old nondiabetic NOD mice and by 73% in 18-week-old diabetic NOD mice. Insulin secretory responses to glucose and arginine demonstrated reductions of similar magnitude. In 18-week-old diabetic NOD mice, insulin secretion was reduced to a greater degree than β-cell mass, suggesting the presence of β-cell dysfunction in addition to reduced mass. These results suggest that in the NOD mouse, β-cell destruction begins soon after the onset of insulitis. Despite a compensatory β-cell proliferative response, β-cell mass progressively falls and is significantly reduced by 13 weeks despite normal blood glucose concentrations. Diabetes may be present when residual β-cell mass represents 30% of control levels. *Diabetes* 48:989–996, 1999

Antibodies against various islet antigens appear in the serum of individuals predisposed to the development of type 1 diabetes before the onset of clinical hyperglycemia (1,2). These antibodies are assumed to reflect the presence of an autoimmune inflammatory response in the pancreatic islet (insulitis) that culminates in destruction of β-cells (1,3,4), with diabetes onset when ~90% of the β-cells have been destroyed. Because therapeutic interventions during this preclinical phase of type 1 diabetes offer the most realistic opportunities for disease prevention, a complete understanding of the evolution of β-cell dysfunction and reduced β-cell mass during this preclinical period is essential.

Animal models have provided key insights into the pathophysiology of type 1 diabetes (5–8), and the nonobese diabetic mouse has been particularly valuable in this regard. In susceptible strains, insulitis develops around 4 weeks of age and leads to β-cell destruction resulting in overt hyperglycemia in ~80% of female and 20% of male NOD mice by 30 weeks of age (5,9). Thus, in the NOD mouse, diabetes onset is preceded by a preclinical phase in which autoimmune insulitis in associated with normal blood glucose concentrations (9,10). In these respects, the NOD mouse is an appropriate model of human type 1 diabetes.

The time at which β-cell mass begins to fall before the onset of type 1 diabetes is controversial. One view, based on the progressive reduction in insulin secretory responses to glucose that occurs in predisposed humans, is that there is a gradual and progressive fall in β-cell mass and insulin secretion beginning soon after the onset of insulitis (3). A contrasting view, which suggests a sudden wave of β-cell destruction just before the onset of overt diabetes, is based on the observation that the phenotype of splenocytes in NOD mice changes from Th2 cells to more destructive Th1 cells just before the onset of hyperglycemia (11). The optimal time for initiation of preventive immunotherapy could be influenced by establishing which of these two models is valid. The present studies were therefore carried out to measure β-cell mass and insulin secretory function at key time points during the development of diabetes in the NOD mouse, with particular emphasis on the period after the onset of insulitis, but before the onset of overt hyperglycemia.

RESEARCH DESIGN AND METHODS

Animals. Female NOD-LT/J mice (Jackson, Bar Harbor, ME) were studied at 8–9 (nondiabetic), 13 (nondiabetic), and 18 (diabetic) weeks of age. In these animals, insulitis begins around 4 weeks of age (10), and diabetes onset is usually from 15 weeks onward. Age-matched female NOD/Scid mice served as controls. These animals have the same genetic background as NOD mice, but because of the presence of severe combined immunodeficiency, they do not develop autoimmune diabetes. Animals were purchased at 6 weeks of age, housed in the animal facility of the University of Chicago with a 12-h light-dark cycle, and fed a standard chow (Purina 5001; Ralston-Purina, St. Louis, MO). All studies were performed on nonfasted mice with the exception of blood glucose testing. Mice were weighed and killed using methods approved by the animal care and use committee of the University of Chicago.

Measurement of blood glucose. Blood glucose measurements were performed after a 4-h fast by tail blood sampling (Hemocue, Angelholm, Sweden). In a subset of NOD-LT/J mice ($n = 6$ at 8–9 weeks, $n = 14$ at 13 weeks, and $n = 8$ at 18 weeks), glucose tolerance testing was performed after 2 g/kg i.p. injection of glucose.

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Brd-U, 5-bromo-2-deoxyuridine; DAB, 3,3′ diamino benzidine; KRB, Krebs-Ringer buffer; SGP, slope of glucose potentiation.

TABLE 1
Weights and blood glucose levels in NOD and NOD/Scid mice

	n	Weight (g)	Blood glucose (mmol/l)
NOD LT/J mice			
8–9 weeks	11	21.1 ± 0.7*	5.4 ± 0.4
13 weeks	29	23.3 ± 0.2	9.6 ± 1.7‡
18 weeks	14	24.7 ± 0.5	19.4 ± 4.7†
NOD/Scid mice			
8–9 weeks	13	20.8 ± 0.3*	6.7 ± 0.2
13 weeks	25	23.9 ± 0.4	7.1 ± 0.1
18 weeks	13	23.3 ± 0.3	7.5 ± 0.2

Data are *n* or means ± SE. *$P < 0.01$ compared with 13- and 18-week-old mice. †$P < 0.01$ compared with all other groups (includes four nondiabetic mice with average blood glucose of 7.4 ± 0.3 mmol/l compared with 24.3 ± 6.0 mmol/l in the diabetic mice). ‡Includes five diabetic mice (average blood glucose 22.4 ± 8.3 mmol/l compared with 6.9 ± 0.2 mmol/l in the nondiabetic mice).

Measurement of β-cell mass. Pancreases were obtained from 8- to 9-, 13-, and 18-week-old NOD and NOD/Scid mice for measurement of β-cell mass and replication rates. Pancreases were quickly resected, cleared of fat and lymph nodes, weighed, fixed in Bouin's solution, and embedded in paraffin. β-cell mass was measured by point-counting morphometry of insulin immunostained pancreatic sections (5–7 μm) as described previously for rat islets (12). A polyclonal guinea pig anti-porcine insulin primary antibody was used, followed by a peroxidase-labeled secondary antibody (Vectastain ABC kit; Vector, Burlingame, CA), developed with 3,3′ diamino benzidine (DAB) (Sigma, St. Louis, MO) and counterstained with hematoxylin. All sections were blinded before quantitation and read by one observer (A.J.P.). β-Cell mass was calculated as previously described (12).

Measurement of β-cell replication rates. Serial 5–7 μm thick pancreatic sections were used to study β-cell replication rates as previously described (12–14). Mice received intraperitoneal injections of 5-bromo-2-deoxyuridine (Brd-U) (Sigma), 100 mg/kg body wt, 6 h before death. Briefly, sections were double-stained for Brd-U and for non-β islet endocrine cells. Brd-U staining was accomplished with a mouse anti-Brd-U monoclonal antibody followed by a peroxidase-conjugated anti-mouse immunoglobulin G. DAB with nickel enhancer was used as the chromogen (Cell Proliferation Kit; Amersham Life Sciences, Arlington Heights, IL). Non-β-cell islet endocrine staining was achieved using a cocktail of primary antibodies to glucagon, somatostatin, and pancreatic polypeptide (Linco, St. Charles, MO). Sections were labeled with peroxidase-conjugated secondary antibody as described above, developed with DAB, and counterstained with hematoxylin. On these sections, the mantle of non-β islet endocrine cells have a red-brown cytosolic staining, while the Brd-U$^+$ cells have black nuclei. β-cell nuclei were counted at 912× magnification. All nuclei, or the first 1,000 β-cell nuclei, were counted in each section, and data were expressed as the percentage of Brd-U$^+$ β-cells per total number of β-cells per 6 h. All sections were blinded at the time of quantitation.

Although endocrine cells are usually clearly distinguishable from inflammatory cells on light microscopy, it is theoretically possible that Brd-U$^+$ cells within the islets could be lymphocytes rather than β-cells. Therefore, double immunohistochemical staining for insulin and Brd-U was performed on a subset of the 13-week-old NOD mice in order to compare β-cell replication rates measured using this technique with those obtained by staining non-β islet endocrine cells.

Insulin secretion from the in situ perfused pancreas. To explore the relationship between changes in insulin secretion and changes in β-cell mass, insulin secretion from the in situ perfused pancreas was measured. The pancreas was perfused in a humidified temperature-controlled chamber as previously described (13). The perfusate consisted of oxygenated Krebs-Ringer buffer (KRB) containing 0.25% bovine serum albumin and a variable concentration of glucose. The slope of glucose potentiation (SGP) of the insulin secretory response to arginine has been proposed as the best physiological measure of functional β-cell mass. Experiments were performed in 13-week-old mice to measure the SGP of the insulin secretory response to arginine. In these experiments, 20 mmol/l arginine was administered, initially in the presence of 5 mmol/l glucose, and subsequently in the presence of 20 mmol/l glucose. The average insulin secreted during perfusion of arginine in the presence of 5 mmol/l glucose was subtracted from that secreted in the presence of arginine and 20 mmol/l glucose, and the difference was divided by the change in glucose concentration (15 mmol/l) to give the SGP.

Additional experiments were performed in mice at each time point to measure the insulin secretory response to a ramp increase in glucose. The perfusion system used three peristaltic pumps (Gilson Minipuls 2; Gilson, Middleton, WI), two of which were computer controlled, allowing the perfusate glucose concentration to increase progressively from 2 to 26 mmol/l over 100 min while maintaining a constant total flow rate of 1 ml/min. After 100 min, 20 mmol/l arginine was perfused in the continued presence of 26 mmol/l glucose for a further 25 min. In all experiments, the pancreas was perfused with KRB/2 mmol/l glucose for a 30-min equilibration period before initiation of sample collection. Insulin concentrations (nanomoles per liter) were measured in the effluent perfusate in the 1st min and every 10th min thereafter.

Assay methods. Insulin concentrations were measured by a double antibody radioimmunoassay using a rat insulin standard. The intra-assay coefficient of variation for this technique is 7%. All samples were assayed in duplicate.

Statistical analysis. Results are expressed as means ± SE. In insulin secretion experiments, the average insulin concentration of effluent perfusate during perfusion of glucose or arginine was calculated for each experiment, and the group means were compared. Statistical analysis was performed using a one-way analysis of variance, and Duncan's multiple range test was used for post hoc comparisons. Because the data were not normally distributed, they were analyzed either after log transformation or using the nonparametric Wilcoxon rank-sum test, where appropriate. Differences were considered to be significant at $P < 0.05$.

RESULTS

Ages, weights, and blood glucose levels. The NOD LT/J and NOD/Scid mice were matched for age at each time point studied. Mouse weight increased with age in each group ($P < 0.01$). Intraperitoneal glucose tolerance testing was performed on a subset of NOD mice at each time point. There was a significant correlation between the glucose measurements made before (fasting) and 2 h after the glucose injection in the group as a whole ($r = 0.86$, $P = 0.0001$). The average 2-h blood glucose measurement in mice with a fasting blood glucose level of <8.3 mmol/l was 7.4 ± 0.3 mmol/l (fasting 6.4 ± 0.3 mmol/l, $n = 19$). In contrast, the average 2-h blood glucose level in mice with a fasting glucose of >8.3 mmol/l was 27.2 ± 3.9 mmol/l (fasting 17.3 ± 4.6 mmol/l, $n = 9$), $P < 0.05$ compared with the 2-h blood glucose in mice with a fasting glucose of <8.3 mmol/l. Mice that had a fasting blood glucose between 8.3 and 11.2 mmol/l (a level often used to define diabetes in NOD mice) had a blood glucose of 19.4 ± 3.2 mmol/l at 2 h after glucose injection (fasting 9.3 ± 0.5 mmol/l, $n = 4$), $P < 0.05$ compared with the level in mice with a fasting glucose of <8.3 mmol/l. Therefore, NOD-LT/J mice were divided into diabetic and nondiabetic subgroups based on fasting blood glucose (levels >8.3 mmol/l were taken to be indicative of diabetes). Of the NOD mice, 11% (5 of 28) had diabetes as judged by a fasting blood glucose >8.3 mmol/l at 13 weeks of age, while 70% (10 of 14) of the animals studied had diabetes by 18 weeks of age. As a result, the average blood glucose levels in the 18-week-old NOD LT/J mice were significantly higher than in all other groups (Table 1). At 8 weeks of age, fasting blood glucose levels were similar in the NOD and NOD/Scid mice. Although blood glucose in the 13-week-old NOD mice tended to be slightly higher, this

TABLE 2
β-Cell mass and β-cell replication rates

	n	β-Cell mass (mg)	% β-Cell BrdU⁺/6 h
NOD LT/J mice			
8–9 weeks	6	1.09 ± 0.3	0.54 ± 0.1*
13 weeks	11	0.75 ± 0.1†	0.74 ± 0.1‡
18 weeks	6	0.35 ± 0.13§	ND
NOD/Scid mice			
8–9 weeks	6	1.59 ± 0.2	0.09± 0.01
13 weeks	12	1.30 ± 0.2	0.07 ± 0.02
18 weeks	8	1.28 ± 0.2	0.14 ± 0.05

Data are means ± SE or *n*. *$P < 0.01$ compared with 8- to 9-week-old NOD/Scid mice ($n = 5$). †$P < 0.05$ compared with 13-week-old NOD/Scid mice (includes nondiabetic mice only). ‡$P < 0.001$ compared with 13-week-old NOD/Scid mice ($n = 5$, includes nondiabetic mice only). §$P < 0.05$ compared with 18-week-old NOD/Scid mice (includes diabetic mice only). ND, not determined; there were too few β-cells to calculate meaningful β-cell replication rates.

group included five diabetic mice (blood glucose 22.4 ± 8.3 mmol/l). The average blood glucose in the 13-week-old nondiabetic NOD mice was 6.9 ± 0.2 mmol/l, which was similar to the age-matched NOD/Scid mice. Diabetic NOD LT/J mice at 13 weeks of age and those that did not have diabetes at 18 weeks of age were analyzed as separate groups and compared with both age-matched NOD/Scid mice and age-matched nondiabetic and diabetic NOD mice, respectively. Diabetes did not develop in NOD/Scid mice. The prevalence of diabetes in the NOD LT/J mice in the current study is similar to that reported in other studies (9).

β-Cell mass. Pancreatic weight was similar in NOD and NOD/Scid mice at all three ages. β-Cell mass tended to decrease in NOD/Scid mice from 1.59 ± 0.2 at 8–9 weeks of age to 1.30 ± 0.2 at 13 weeks of age ($P > 0.05$), but there was no change between 13 and 18 weeks of age (1.28 ± 0.2). In the NOD mice, there was a gradual loss of β-cell mass with time (Table 2). At 8–9 weeks of age, the average β-cell mass was 1.09 ± 0.3 mg, which represented 69% of the value in the NOD/Scid mice ($P > 0.05$). At 13 weeks of age, the average β-cell mass had fallen to 0.75 ± 0.1 mg in the nondiabetic mice (58% of NOD/Scid mice value, $P < 0.03$) and to 0.38 ± 0.2 mg in the diabetic mice (29% of the level in the NOD/Scid mice, $P < 0.03$, and 51% of the level in the nondiabetic NOD mice, $P > 0.05$). By 18 weeks of age, β-cell mass in the diabetic NOD mice represented 27% of the value in age-matched NOD/Scid mice ($P < 0.05$). Two NOD mice that did not develop diabetes by 18 weeks of age had β-cell mass measurements (0.72 and 2.45 mg) that were similar to those seen in NOD/Scid mice (range in age-matched NOD/Scid mice: –0.79 to 2.17 mg). β-cell mass in the diabetic 18-week-old NOD mice was significantly lower than in the younger mice ($P = 0.05$ and $P < 0.05$ compared with 13-week-old nondiabetic and 8-week-old NOD mice, respectively). Representative examples of histological sections of NOD mouse pancreas at the three ages studied are shown in Fig. 1.

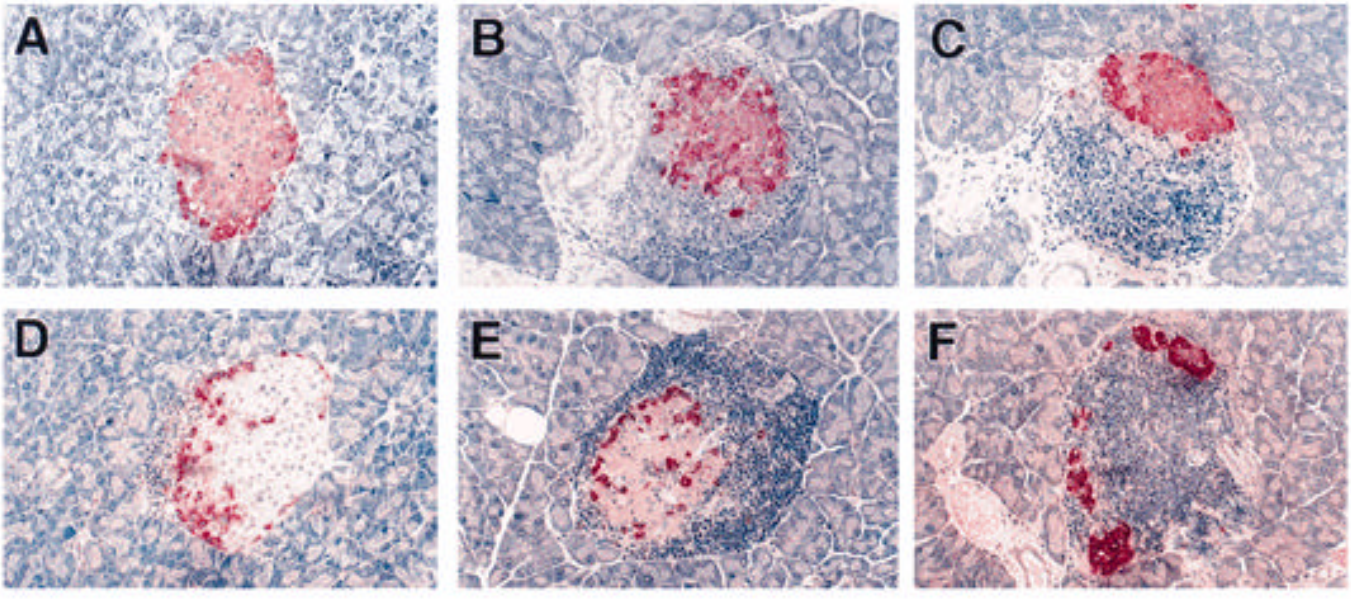

FIG. 1. Pancreatic immunohistochemistry. Paraffin-embedded 5- to 7-μm sections from NOD mice at 8–9 (*A* and *D*), 13 (*B* and *E*), and 18 (*C* and *F*) weeks of age were immunostained using either an antibody directed against insulin (*A–C*) or a cocktail of antibodies directed against glucagon, somatostatin, and pancreatic polypeptide (*D–F*). The insulin staining demonstrates the progressive loss of β-cells over the course of the study, and the cocktail staining demonstrates the preservation of the non-β islet endocrine cells.

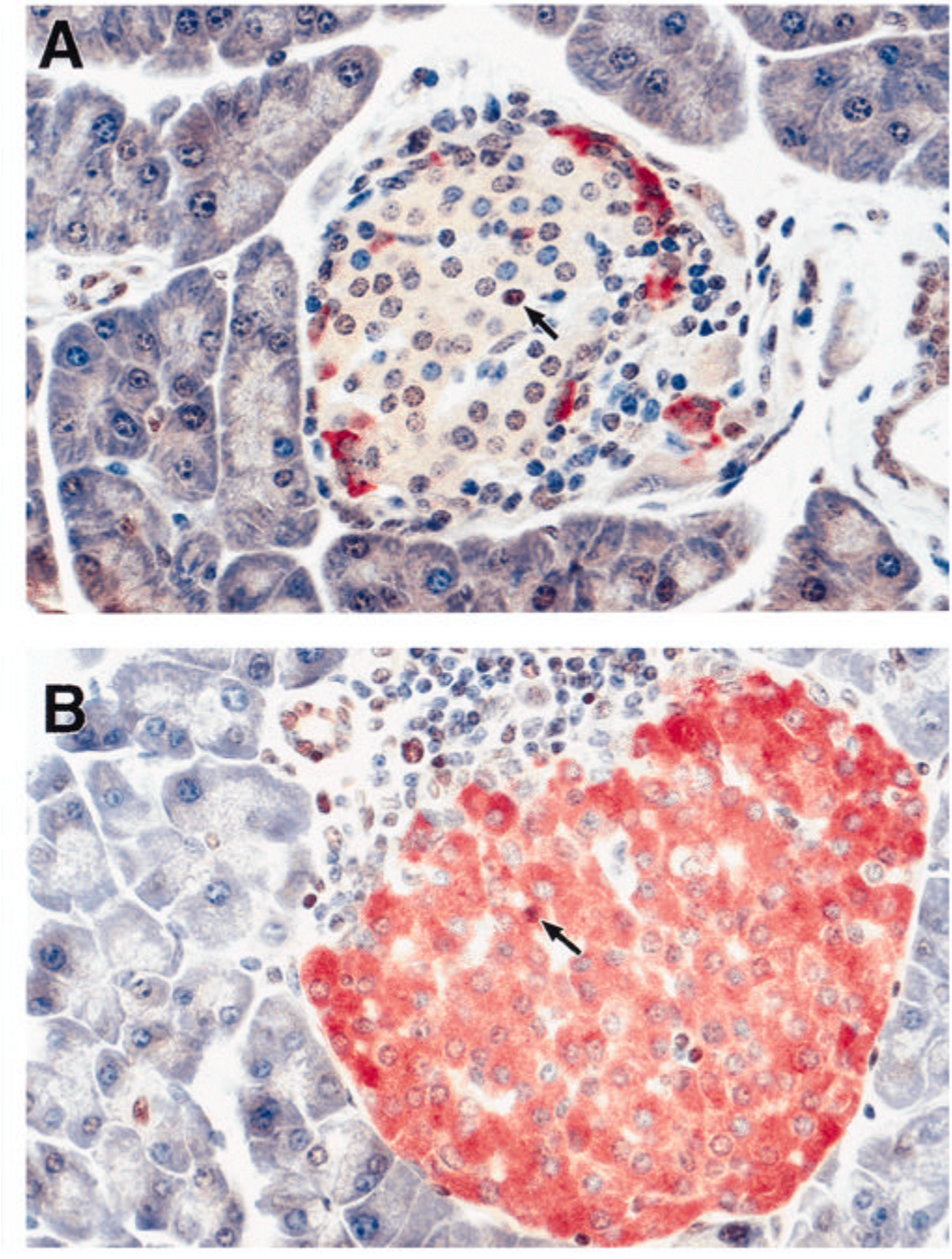

FIG. 2. Brd-U immunostaining of pancreatic sections from a 13-week-old NOD mouse. *A*: This section has been immunostained using a cocktail of antibodies directed against glucagon, somatostatin, and pancreatic polypeptide and an antibody directed against Brd-U. *B*: This section has been stained with antibodies directed against insulin and Brd-U. The darker Brd-U$^+$ nuclei are identified by the arrows.

β-Cell replication rates. β-Cell replication rates were significantly greater in nondiabetic NOD mouse pancreases than in pancreases from age-matched NOD/Scid mice at 8–9 and at 13 weeks of age (Table 2). At 8 weeks of age, Brd-U incorporation was 6-fold higher in NOD than in NOD/Scid mice ($P < 0.01$), and this difference was even more pronounced at 13 weeks of age when the rate of replication was more than 10-fold higher in NOD mice ($P < 0.001$). At 18 weeks of age, there were so few β-cells remaining in the diabetic NOD pancreas that it was not possible to calculate replication rates accurately. To exclude the possibility that the β-cell replications rates measured indirectly by staining for non-β islet endocrine cells were falsely elevated because of the inclusion of Brd-U$^+$ lymphocytes, sections from 13-week-old nondiabetic NOD mice were also stained with antibodies directed against both insulin and Brd-U. The β-cell replication rate in the 13-week-old nondiabetic NOD mice measured using this technique was 0.67 ± 0.05, a value not statistically different from that obtained by staining non-β islet endocrine cells. These values represent a 10-fold higher replication rate than that observed in the age-matched NOD/Scid mice ($P < 0.05$).

Insulin secretory responses to glucose and arginine. Experiments were performed in 13-week-old mice to measure the SGP of the insulin secretory response to arginine

(Fig. 3). The average insulin concentration of the effluent perfusate during administration of 20 mmol/l arginine in the presence of 5 mmol/l glucose in 13-week-old nondiabetic NOD mice (3.0 ± 0.4 nmol/l) was 43% of the level in the age-matched NOD/Scid mice (6.9 ± 3.9 nmol/l, $P < 0.03$). In contrast, the insulin secretory response to 20 mmol/l glucose in NOD mice (1.2 ± 0.3 nmol/l) was not significantly different from age-matched NOD/Scid mice (1.5 ± 0.2 nmol/l). When arginine was administered in the presence of 20 mmol/l glucose, the average insulin secretory response in the NOD mice was 46% of the level in the control mice (6.7 ± 1.2 compared with 14.7 ± 2.8 nmol/l, $P < 0.003$). The average SGP was 40.8 ± 8.8 pmol · $mmol^{-1}$ · l^{-1} in the NOD mice compared with 87.2 ± 15.2 pmol · $mmol^{-1}$ · l^{-1} in the NOD/Scid mice ($P < 0.02$).

Additional experiments were performed to measure the insulin secretory response from the perfused pancreas to a ramp increase in glucose and to arginine (Fig. 4*A–C*). There was no significant difference between the average perfusate insulin concentrations in response either to the ramp increase in glucose from 2 to 26 mmol/l or to arginine administration in the presence of 26 mmol/l glucose, in the NOD/Scid mice at 8, 13, and 18 weeks of age. In nondiabetic NOD mice, the responses to the ramp increase in glucose concentration were 85 and 87% of that in age-matched NOD/Scid mice at 8 and 13 weeks of age, respectively ($P > 0.05$), and the responses to 20 mmol/l arginine in the presence of 26 mmol/l glucose were 79 and 72% of age-matched control values, respectively ($P > 0.05$). In contrast, there was no detectable insulin secretory response to glucose or arginine by the pancreas of the one diabetic NOD mouse perfused at 13 weeks of age. In 18-week-old diabetic NOD mice, the insulin secretory responses to glucose and arginine were 3 and 4%, respectively, of age-matched NOD/Scid levels ($P < 0.05$). The insulin secretory responses to glucose in pancreases from two 18-week-old NOD mice that did not satisfy the criteria for diabetes were 0.06 and 3.0 nmol/l (range in age-matched NOD/Scid mice: –1.1 to 7.3 nmol/l), and the responses to arginine were 1.4 and 27.0 nmol/l, respectively (range in NOD/Scid mice: 10.3–55.7 nmol/l).

To determine whether the reduction in insulin secretion in nondiabetic NOD mice was only due to the reduction in β-cell mass, insulin secretory responses to glucose and arginine were adjusted by dividing insulin concentrations in each mouse by the average β-cell mass of the group. When the SGP of the insulin secretory response to arginine was adjusted to take into account the difference in β-cell mass in 13-week-old mice, the levels were not statistically different in the two groups (58.3 ± 12.6 vs. 67.0 ± 11.7 nmol · $mmol^{-1}$ · l^{-1}· mg^{-1} in the NOD and NOD/Scid mice, respectively, $P > 0.05$). The insulin secretory responses to the ramp increase in glucose and to arginine administered in the presence of 26 mmol/l glucose, even when adjusted for β-cell mass (Fig. 4*D–F*) were significantly reduced at the 18-week time point. The fact that adjustment for β-cell mass at 18 weeks does not correct for the reduction in insulin secretion indicates the presence of an additional functional secretory defect.

DISCUSSION

The present study was undertaken to measure β-cell mass and insulin secretory function at key time points during the development of diabetes in the NOD mouse. The overall goal was to shed new light on the controversy regarding whether β-cell mass in autoimmune diabetes is lost in a gradual but progressive manner, from the time of onset of insulitis or soon thereafter (3), or whether β-cell mass remains relatively constant in the nondiabetic period and falls precipitously just before diabetes onset (11). The natural history of diabetes in the NOD mouse makes it an ideal model with which to resolve this question, because the animals who ultimately develop diabetes go through a prediabetic period from ~4 weeks of age, the age at which insulitis is consistently present, until 14–15 weeks of age, the age at which the incidence of diabetes starts to increase. In the present study, only 11% of the NOD mice had diabetes at 13 weeks of age, and this had increased to 70% by 18 weeks of age. We were therefore able to compare β-cell mass and measure the β-cell proliferative response to the presence of insulitis in nondiabetic animals at ages 8 and 13 weeks as well as diabetic animals at age 18 weeks. The availability of NOD/Scid mice to serve as controls allowed us to control for age-related changes in these parameters resulting from the NOD background.

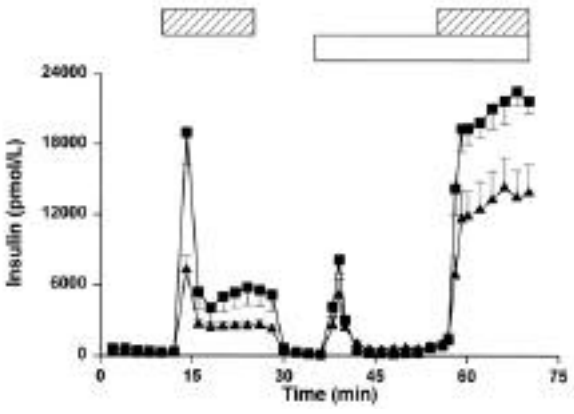

FIG. 3. Insulin secretory responses to a step increase in glucose and arginine concentration in the in situ perfused pancreas. Experiments were performed in pancreases from 13-week-old NOD/Scid mice (■—■, $n = 7$) and NOD mice (▲—▲, $n = 7$). After a 40-min period (30-min equilibration and 10-min basal sampling period) during which the pancreases were perfused with KRB containing 5 mmol/l glucose, 20 mmol/l arginine was added in the presence of 5 mmol/l glucose (10–25 min, ▨). The arginine was allowed to wash out (26–35 min) before 20 mmol/l glucose (□) was perfused between 36 and 55 min, followed by 20 mmol/l arginine (▨) in the continued presence of 20 mmol/l glucose. Data are presented as means ± SE.

Our results demonstrate that at 8 weeks of age, β-cell mass in NOD mice was 69% of the value in NOD/Scid mice. Although this decrease was not statistically significant, the presence of ongoing autoimmune destruction is strongly suggested by the presence of a statistically significant β-cell proliferative response, as judged by an increase in Brd-U incorporation into new β-cells. It thus appears that even at 8 weeks of age there is ongoing destruction of β-cells in the NOD mouse, although the extent of this process is masked by an increase in the rate of β-cell proliferation, which maintains β-cell mass within a relatively normal range. By age 13 weeks, despite a continued β-cell proliferative response, β-cell mass had fallen significantly in the nondiabetic NOD mice and represented 70% of the value in 8- to 9-week-old mice and 58% of the value in age-matched NOD/Scid mice. At 18 weeks of age, however, after diabetes onset, β-cell mass

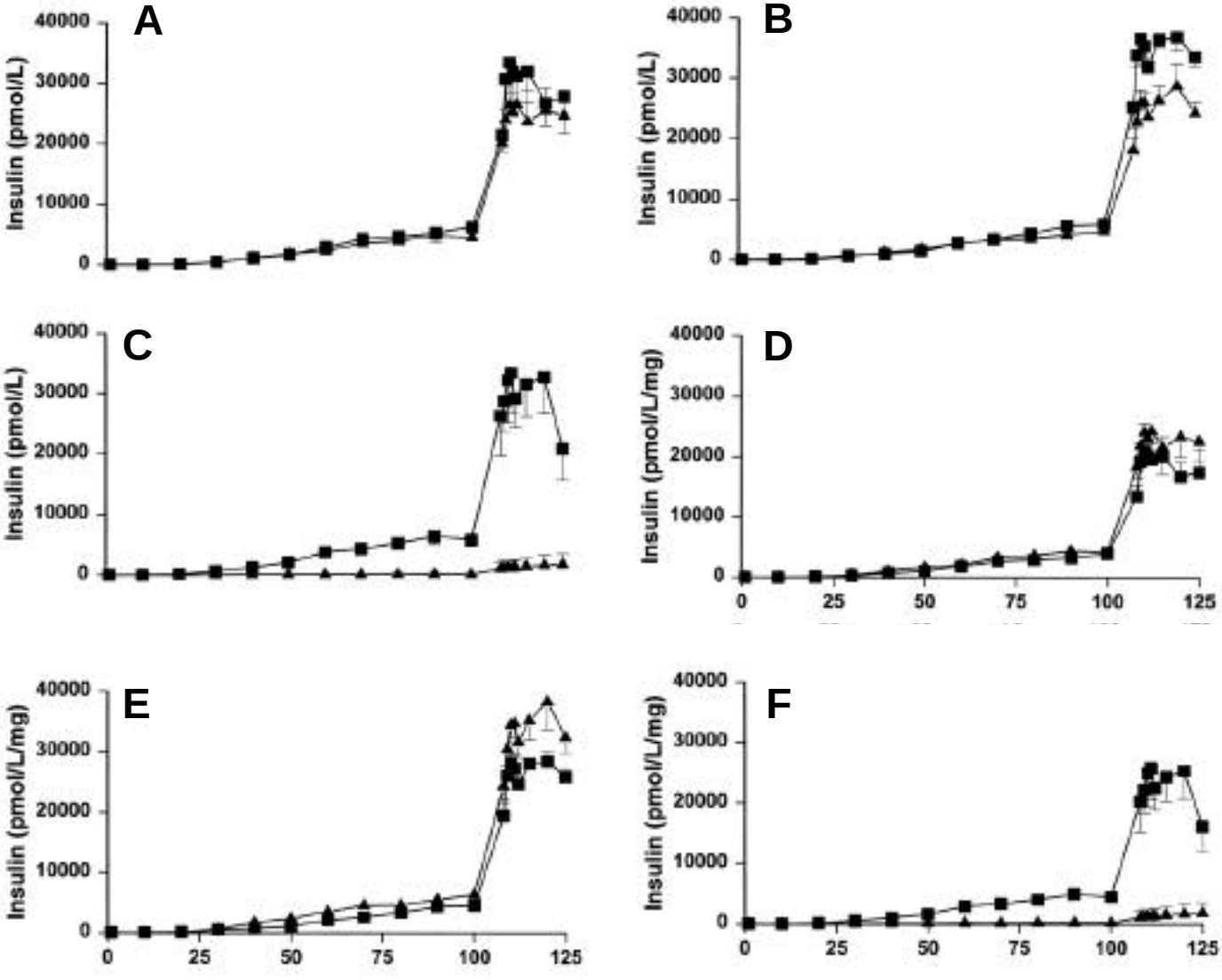

FIG. 4. Insulin secretory responses to a ramp increase in glucose concentration and arginine in the in situ perfused pancreas. During the first part of each experiment, the perfusate glucose concentration was progressively increased from 2 to 26 mmol/l (1–100 min), and thereafter 20 mmol/l arginine was added in the continued presence of 26 mmol/l glucose (100–125 min). *A–C*: Data from 8- to 9-, 13-, and 18-week-old NOD/Scid mice (■—■, $n = 7$, $n = 4$, and $n = 6$) and NOD mice (▲—▲, $n = 5$, $n = 5$, and $n = 4$), respectively. The average insulin concentration of the effluent perfusate during the ramp increase from 2 to 26 mmol/l glucose was significantly reduced in the 18-week-old NOD mice, although there was a trend to reduction in the 8- to 9- and 13-week-old mice that was not significantly different. *D–F*: The same data adjusted by dividing the insulin secretion data from each mouse by the average β-cell mass measured in a separate group of mice.

had fallen further in the NOD mice and now represented only 27% of the value in age-matched control mice. These results indicate that in the NOD mouse, diabetes may develop when residual β-cell mass is significantly greater than has previously been suggested.

A previous study demonstrated progressive loss of β-cell mass associated with increasing severity of insulitis in the NOD mouse using semiquantitative methods to grade both parameters (15). However, the current study is the first to quantify β-cell mass in the NOD mouse histomorphometrically. This method allows more reliable quantification of β-cell mass, which may be important in studying relationships with parameters such as β-cell insulin secretory responses.

The finding of increased β-cell proliferation together with reduced β-cell mass suggests increased destruction of β-cells. This is consistent with evidence from other studies that have demonstrated that apoptosis mediates the autoimmune destruction of β-cells (16,17). The cause of the increased β-cell replication rates is unclear. Glucose level is the principal factor regulating β-cell mass (18), although other factors, such as growth hormone and prolactin, also stimulate β-cell replication (19). Hyperglycemia has been hypothesized to stimulate increases in β-cell mass in other animal models of diabetes, such as the 90% pancreatectomized rat (20) and the Zucker diabetic fatty rat (12). Because the nondiabetic NOD mice had significantly increased β-cell replication rates despite normal blood glucose levels (Table 1 footnote), some other factor must be responsible for stimulating β-cell replication. At some point (between 13 and 18 weeks of age in most NOD mice), the ability of β-cell replication to compensate for the depletion of β-cell mass fails. Just as the factors stimulating increases in β-cell mass are unclear, the reason

why compensatory β-cell replication ultimately fails to keep pace with destruction despite the additional stimulus of hyperglycemia is not apparent.

It has previously been suggested that the SGP of the insulin secretory response to arginine is the most accurate reflection of functioning β-cell mass. The present studies confirmed this assertion. In 13-week-old NOD mice, the reduction in β-cell mass compared with the NOD/Scid mice (42%) was similar in magnitude to the reduction in the SGP to arginine (53%). A similar conclusion was suggested by the results of a previous study demonstrating that the progressive reduction in the insulin secretory response by the perfused pancreas to glucose and arginine correlates with evidence of increasing islet inflammation (5). However, the current study is the first to systematically examine the relationship of insulin secretion to β-cell mass in this model of type 1 diabetes. In contrast to the SGP of the insulin secretory response to arginine, the response to a graded glucose perfusion was relatively well retained and was not significantly reduced in the NOD mouse pancreas when compared with NOD/Scid mice at either 8 or 13 weeks of age. The reason for these differences is not clear, but it appears that the relatively prolonged exposure of the NOD mouse pancreas to gradually increasing glucose concentrations during the ramp experiments potentiates the insulin secretory response to both glucose and subsequently administered arginine. In contrast to the situation at 13 weeks, when insulin secretion was appropriate for the level of the β-cell mass, in 18-week-old NOD mice, insulin secretory responses to glucose and arginine were markedly attenuated even when adjusted for the measured β-cell mass. This suggests that at this stage a functional secretory defect has developed in the NOD mice in addition to the reduction in β-cell mass. Insulin content was not measured, and it is possible that immunohistochemistry could overestimate β-cell mass by failing to detect reduced insulin content within partially granulated β-cells. However, many previous studies have demonstrated that hyperglycemia can impair β-cell function (glucose toxicity [21–23]), and it is likely that the impaired β-cell function in the pancreases of 18-week-old NOD mice was aggravated by the presence of glucose toxicity. Such a defect is likely to be partially reversible if normoglycemia is restored. The suggestion of a functional β-cell secretory defect at the onset of diabetes is in keeping with the well-documented "honeymoon" period observed in many patients with type 1 diabetes, when euglycemia is restored with insulin therapy, and with the observation that diabetes in the NOD mouse can be reversed for extended periods of time by such interventions as the administration of anti-CD3 (24). Previous studies have also demonstrated the presence of a functional defect in glucose-stimulated insulin secretion in NOD mice (25,26). Although the mice did not have diabetes, there was a progressive defect in insulin secretion by isolated NOD islets with increasing age that was reversed after in vitro culture of the islets (25).

In summary, a reduction in β-cell mass is evident in nondiabetic NOD mice and is slowly progressive. When diabetes develops, the residual β-cell mass is at least ~30% of control values. The nondiabetic state is characterized by a mild to moderate decrease in β-cell mass, and insulin secretion remains appropriate for the level of the β-cell mass. β-cell replication rates are markedly increased, and although this compensatory mechanism initially maintains β-cell mass and insulin secretion at a relatively normal level, it ultimately fails, resulting in the development of diabetes. After diabetes onset, insulin secretion is decreased to a greater extent than β-cell mass, presumably as a result of the toxic effects of elevated glucose levels on β-cell function. Greater understanding of the mechanisms responsible for the initial stimulation and ultimate failure of β-cell replication and of the insulin secretory defect present at the onset of diabetes may lead to novel therapies to delay or prevent the onset of type 1 diabetes.

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