

Early, Selective, and Marked Loss of Sympathetic Nerves From the Islets of BioBreeder Diabetic Rats

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To discover whether islet sympathetic nerves are damaged during the autoimmune destruction of islet B-cells, we immunostained sections of pancreas from BioBreeder (BB) diabetic rats, using antibodies against vesicular monoamine transporter 2 (VMAT2), a marker of sympathetic nerve terminals. We found a marked decrease in the VMAT2-positive fiber area in the islets of BB rats that had been diabetic for only 1–2 weeks compared with their nondiabetic controls. In contrast, there was no significant decrease in the VMAT2-positive fiber area in the exocrine pancreas in these early diabetic BB rats. Furthermore, streptozotocin-diabetic rats showed no decrease in VMAT2-positive fiber area in their islets compared with controls. The classical diabetic autonomic neuropathy (DAN) that eventually occurs in the heart was not present in BB diabetic rats at this early stage as evidenced by normal cardiac VMAT2 immunostaining and normal cardiac norepinephrine content. Also, in contrast to DAN, this islet neuropathy did not worsen with duration of diabetes. These data provide evidence of a heretofore unrecognized early sympathetic islet neuropathy (eSIN). Because eSIN occurs selectively in the islet, is rapid in onset, and is associated with autoimmune but not chemically induced diabetes, it is distinct from DAN in location, time course, and mechanism. *Diabetes* 51:2997–3002, 2002

The sympathetic nerves of the pancreas can inhibit insulin release from the islet B-cells (1,2) and stimulate glucagon release from the islet A-cells (1,2). For example, activation of these nerves is sufficient to mediate the glucagon response to insulin-induced hypoglycemia (3). It is interesting that this specific glucagon response is lost in type 1 diabetes (4).

Type 1 diabetes in humans is associated with an autoimmune attack on islet B-cells involving a variety of cytokines, including interleukin 1 and tumor necrosis factor- α (5). These two cytokines are also neurotoxic (6) and therefore could injure islet nerves during the autoimmune attack on islet B-cells. Injured adult sympathetic nerves, in turn, are known to increase their dependence on

nerve growth factor (NGF) for function and survival (7). Islet B-cells seem to be the source of the growth factors that influence both the sympathetic and sensory innervation of the islet (8), a specificity ascribed to NGF itself. Indeed, at least in vitro, islet B-cells seem to make NGF mRNA as demonstrated by RT-PCR and NGF protein as demonstrated by immunohistochemistry (9). In addition, they secrete NGF as demonstrated by bioassay and enzyme-linked immunosorbent assay (9). These observations suggest the possibility that the autoimmune attack on the B-cells during the development of type 1 diabetes will lead to a loss of the islet sympathetic nerves, first by injuring these nerves and then by depriving them of now critical neurotrophic support.

To determine whether the hypothesized loss of islet sympathetic nerves actually occurs, we first needed a marker of the sympathetic innervation of the pancreatic islet. We chose to do immunohistochemistry for the vesicular monoamine transporter 2 (VMAT2), a transporter expressed on the synaptic vesicles of sympathetic nerves (10) and therefore a useful marker for their nerve terminals.

To quantify islet sympathetic nerves, we first identified islets by the glucagon staining around their rim and then counted the VMAT2-positive fiber area within the islet. To validate this method, we used the sympathetic neurotoxin 6-hydroxydopamine (6-OHDA) to destroy islet sympathetic nerves. Thereafter, we used the same method to determine whether there was an early loss of islet sympathetic nerves in an animal model of autoimmune type 1 diabetes, the BB diabetic rat. The results of this study suggest that we have discovered a heretofore unrecognized and highly selective form of neuropathy in autoimmune diabetes that is distinct from classical diabetic autonomic neuropathy (DAN). We call it early sympathetic islet neuropathy (eSIN).

RESEARCH DESIGN AND METHODS

Animals and treatments. Male and female diabetes-resistant (DR) BioBreeding (BB) rats congenic for the lymphopenia gene (*llyp*) on rat chromosome 4 (11) were mated to produce the BB rats used in this study. All BB rats were typed for the *llyp* gene (11) at ~30 days of age. Diabetes-prone rats (homozygous *llyp/llyp*) all developed diabetes between 55 and 90 days of age and then started daily insulin treatments. DR rats (+/+ or +/*llyp*) all remained nondiabetic. The BB DR rats were matched for age with BB diabetic rats and used as controls.

Two groups of male Wistar rats (Simonsen Labs, Gilroy, CA), weighing 200–350 g, were treated with either systemic 6-OHDA (100 mg/kg i.v.) in 20% ascorbic acid or saline 1 week before tissue fixation and harvest. Another two groups of male Wistar rats were treated with a single dose of streptozotocin (STZ) dissolved immediately before injection in saline, in which pH was set at

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6-OHDA, 6-hydroxydopamine; DAN, diabetic autonomic neuropathy; eSIN, early sympathetic islet neuropathy; NE, norepinephrine; NGF, nerve growth factor; STZ, streptozotocin; VMAT2, vesicular monoamine transporter 2.

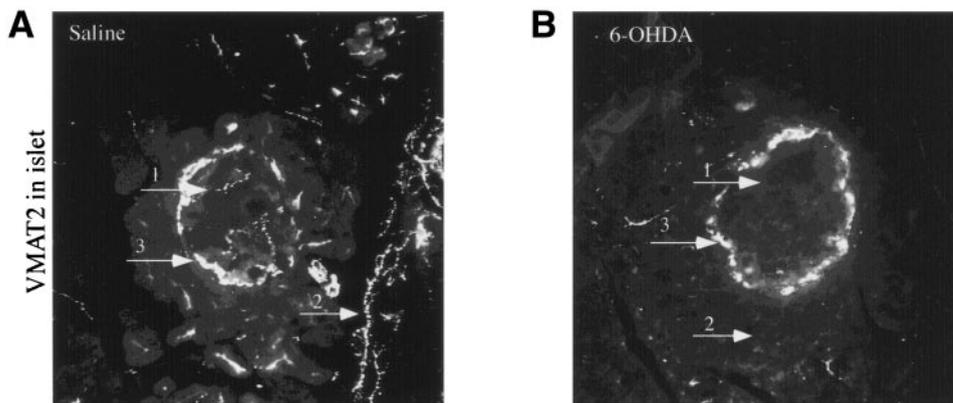


FIG. 1. Glucagon and VMAT2 staining of an islet in the pancreas of a saline-treated (A) and a 6-OHDA-treated (B) Wistar rat. Islets were identified by glucagon staining around the rim of the islet (arrow 3). The number of VMAT2-positive fibers in both the islet (arrow 1) and exocrine pancreas (arrow 2) seem to be reduced in the animals that were treated with systemic 6-OHDA.

3.0 by the addition of citric acid. STZ-treated rats were considered diabetic when their fed plasma glucose levels were >350 mg/dl. They were not treated with insulin. One week later, the rats were anesthetized, perfused, and killed, as described below.

All rats included in these studies were certified as healthy by the Veterinary Medical Officer and exhibited normal grooming and feeding behavior. All research involving animals was conducted in a facility that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Administration.

Tissue fixation. Rats were deeply anesthetized with pentobarbital (60 mg/kg i.p.), received a thoracotomy, and were perfused through the left ventricle first with 300 ml of saline and then with 300 ml of 4% paraformaldehyde. For avoiding the impact of potential regional differences in innervation within a tissue, all pancreatic samples were harvested from the splenic lobe and all heart samples were taken from the left distal ventricle. These samples were immersed in 4% paraformaldehyde for 12 h for postfixation and then placed in 25% sucrose in 0.01 mol/l phosphate buffer (pH 7.4) for overnight dehydration. The tissue was then embedded in mounting medium (Tissue-Tek, Miles, Elkhart, IN), frozen in dry ice, and stored at -80°C until it was sectioned and stained. Tissue destined for norepinephrine (NE) content analysis was first harvested either before whole-body fixation or from nonfixed animals and then snap-frozen on dry ice and stored at -80°C until analysis.

Immunohistochemistry and quantification. Two separate $16\text{-}\mu\text{m}$ sections, separated by $80\ \mu\text{m}$, were obtained from each tissue of each animal. These cryostat sections were mounted on slides coated with chrome alum and dried in the air for 1 h. The dried specimens were immersed in 0.25% Triton X-100 in PBS for 1 h and then incubated with mouse anti-glucagon monoclonal antibody (Lot 106H4808, 1:1,000; Sigma Chemical Company, Saint Louis, MO) for 12 h. After the slides were rinsed twice for 20 min with 0.01 mol/l PBS, they were incubated with rabbit anti-VMAT2 antibody (H-V004, 1:1,000; Phoenix Pharmaceuticals) for 12 h. The immunoreactive glucagon cells were demonstrated by FITC-conjugated rabbit anti-mouse antibody (1:100; Jackson ImmunoResearch Laboratories). The VMAT2 immunoreactive nerves were labeled by Cy3-conjugated goat anti-rabbit antibody (1:200; Jackson ImmunoResearch Laboratories).

Pancreatic islets were identified by glucagon staining around their rim and outlined by the operator to allow the computer-assisted image analysis system to calculate islet area in pixels. Five to eight islets in each of two sections were chosen for measurement. Sympathetic nerve fibers within the islet were identified by VMAT2 immunostaining. The VMAT2-positive fiber area of each islet was calculated by the image analysis system after the operator traced each fiber segment by hand. The operator quantifying the VMAT2-positive fiber area was blinded to animal groups and treatments.

To quantify sympathetic nerves in the exocrine pancreas, a fixed area ($0.35\ \text{mm}^2$) of splenic pancreas, devoid of islets, was chosen for observation. The VMAT2-positive fiber area was calculated as above. An identical approach was used to quantify the sympathetic innervation of the distal left ventricle of the heart.

To assess pancreatic and cardiac NE content, frozen tissue (~ 1 g) was homogenized and boiled in 1 N of acetic acid (10 ml/g tissue, 10 min). The homogenate was centrifuged twice (10,000 rpm, 20 min), and the supernatant was dried and reconstituted in 2 ml of assay buffer. The reconstituted extract was stored at -20°C until assayed. Plasma NE was measured in duplicate with a sensitive and specific radioenzymatic assay (12). The intra- and interassay coefficients of variation for the tissue catecholamine assay in this laboratory are 6 and 12%, respectively.

Statistical analysis. Each treatment group was compared with its own control group using a two-sample test. Comparisons between different treatment groups were made using ANOVA with a post hoc Scheffe *F* test. All data are expressed as mean \pm SE.

RESULTS

Effect of 6-OHDA on islet VMAT2-positive nerve fibers. VMAT2-positive nerve fibers were found both in the islets and in the exocrine tissue of Wistar rat pancreas (Fig. 1A). Rats that were pretreated with the sympathetic neurotoxin 6-OHDA had the expected marked decrease (98%) of VMAT2-positive fibers in islets (Figs. 1B and 2; Table 1). 6-OHDA treatment also reduced the VMAT2-positive fiber area in the exocrine pancreas by 88% (343 ± 20 pixels/ mm^2 [$n = 6$] vs. saline $2,813 \pm 461$ pixels/ mm^2 ; $P < 0.005$; Fig. 1B) and decreased total pancreatic NE content by 90% (6-OHDA 34 ± 7 ng NE/g pancreas [$n = 6$] vs. control 330 ± 43 ng/g [$n = 6$]; $P < 0.0005$).

Loss of islet VMAT2-positive fibers in early BB diabetes. We next tested the hypothesis that autoimmune type 1 diabetes is associated with an early loss of sympathetic fibers in the islet. We found a marked decrease of VMAT2-positive fiber area in the islets of BB rats with 1–2 weeks of early diabetes (Fig. 3B) compared with those from BB rats resistant to diabetes (Figs. 3A and 4; Table 1). In contrast, VMAT2-positive fibers could still be found easily throughout the exocrine pancreas of early BB diabetic rats (Fig. 3B), and their VMAT2-positive fiber area in the exocrine pancreas was similar to that of their early

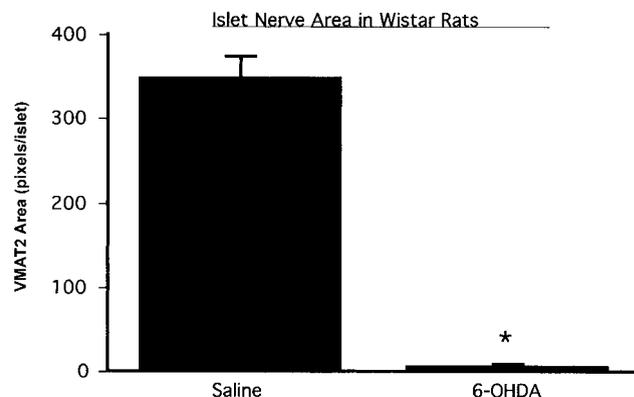


FIG. 2. Area in pixels of VMAT2-positive nerve fibers in the islets of Wistar rats that were pretreated with saline or 6-OHDA. Data are expressed as mean \pm SE. *Significantly different from saline-treated control; $P < 0.005$.

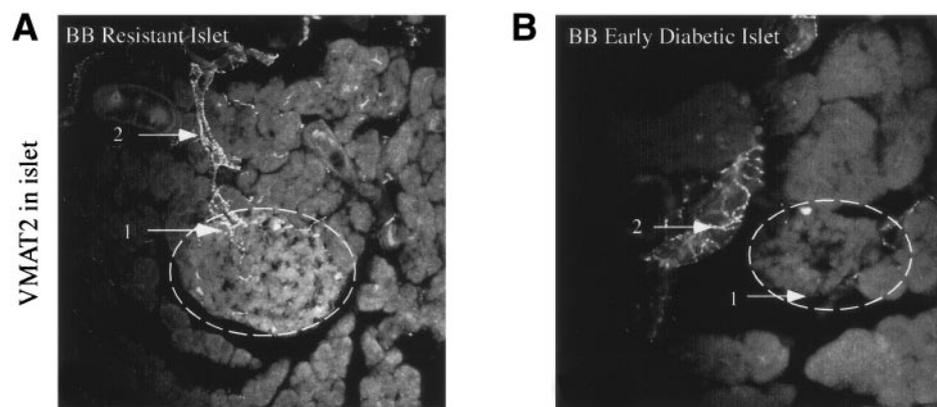


FIG. 3. VMAT2 staining of an islet in the pancreas of an early DR (A) and an early diabetic (B) BB rat. Islet area is shown by a dashed oval. The number of VMAT2-positive fibers seem to be reduced in the islet (arrow 1) but not in the exocrine pancreas (arrow 2) of the early BB diabetic rat (B). Early BB diabetic rats had diabetes for 1–2 weeks.

BB DR controls (Table 2), as was total pancreatic NE content (Table 2). Likewise, VMAT2-positive fiber area in the left ventricle of the heart and heart NE content were not significantly different between early BB diabetic rats (Fig. 5B) and their early BB DR controls (Fig. 5A; Table 2). Thus, VMAT2-positive fibers were selectively depleted in the pancreatic islets of early BB diabetic rats.

To determine whether this marked loss of islet sympathetic nerves was due simply to loss of islet tissue, we administered the chemical B-cell toxin STZ. VMAT2-positive fiber area in the islets of STZ diabetic rats was not significantly decreased compared with their saline-treated controls (Table 1).

Finally, to verify that the islet-specific loss of sympathetic fibers did not precede BB diabetes, we also quantified VMAT2-positive fibers in the islets of three prediabetic BB rats. These rats were homozygous *lyp/lyp* yet too young to develop diabetes. Their islets showed no decrease in VMAT2-positive fiber area compared with their age-matched BB DR controls (Table 1).

Selective loss of islet VMAT2-positive nerve fibers in late BB diabetes. To determine whether eSIN progresses after 1–2 weeks of diabetes, we studied late BB diabetic rats (1–4 months' duration). We found a marked loss of VMAT2-positive fibers from the islets of late BB diabetic rats that was similar to the marked loss of early BB diabetic rats (Fig. 4; Table 1). The decrease of VMAT2-positive fibers in late BB diabetic pancreas again seemed to be restricted to the islet because neither VMAT2-positive fiber area in the exocrine pancreas nor total pancreatic NE content of late BB diabetic rats was de-

creased compared with that of their late BB DR controls (Table 2). Likewise, VMAT2-positive fiber area in the heart of late BB diabetic rats was not decreased compared with their late BB DR controls (Table 2). Thus, VMAT2-positive fibers were selectively depleted in the pancreatic islets of late BB diabetic rats.

DISCUSSION

Effect of 6-OHDA on islet VMAT2. 6-OHDA treatment produced a near total loss of islet VMAT2-positive fibers. 6-OHDA treatment also eliminated islet neuropeptide Y immunoreactive fibers (data not shown), an independent marker of sympathetic fibers (13) in pancreatic islets (14). These data suggest that islet VMAT2-positive fibers are a specific marker for islet sympathetic nerve terminals.

VMAT2-positive fibers were also seen in the exocrine portions of the pancreas of saline-treated rats. 6-OHDA also produced an 88% decrease of exocrine pancreatic VMAT2-positive fiber area, remarkably similar to the 90% decrease of total pancreatic NE content, the most commonly used index of sympathetic denervation. These data suggest that VMAT2-positive fibers are also a specific marker for the sympathetic nerve terminals in the exocrine pancreas.

Decrease of islet VMAT2-positive nerve fibers in the early BB diabetic rat. In agreement with our hypothesis that autoimmune type 1 diabetes is associated with eSIN,

TABLE 1
Sympathetic innervation of pancreatic islet

Group	n	Islet VMAT2 fiber area*
Saline	4	348 ± 26
6-OHDA	6	6 ± 3†
Early BB DR	4	375 ± 115
Early BB diabetic	7	58 ± 15†
Late BB DR	4	388 ± 106
Late BB diabetic	7	42 ± 13†‡
Saline	4	348 ± 26
STZ diabetic	4	465 ± 51
BB DR	3	206 ± 10
BB prediabetic	3	217 ± 30

Data are mean ± SE. *Units = (pixels/islet); † $P < 0.005$ vs. controls; ‡ $P = NS$ vs. early BB diabetic.

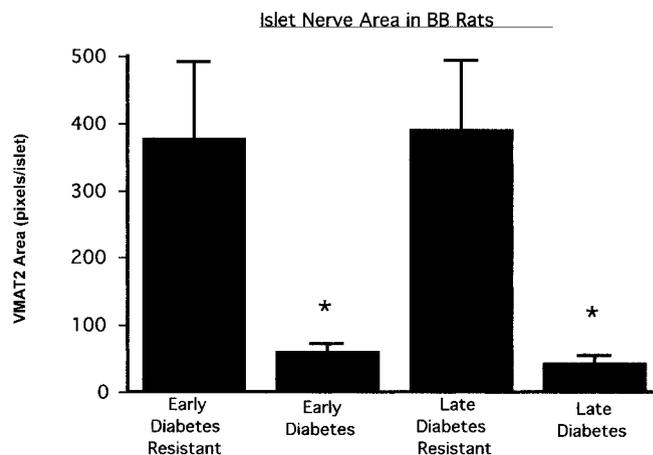


FIG. 4. Area in pixels of VMAT2-positive nerve fibers in the islets of BB rats that were early DR, early diabetic (1–2 weeks), late DR, or late diabetic (1–4 months). Data are expressed as mean ± SE. * $P < 0.005$ vs. DR controls.

TABLE 2
Sympathetic innervation of exocrine pancreas and heart

Group	Exocrine VMAT2 fiber area*	<i>n</i>	Pancreatic NE content†	<i>n</i>	Heart VMAT2 fiber area*	<i>n</i>	Heart NE content†	<i>n</i>
Early BB DR	2,841 ± 655	4	132 ± 7	7	6,898 ± 963	7	223 ± 27	5
Early BB diabetic	3,671 ± 429	7	108 ± 9	5	5,517 ± 958	4	175 ± 20	4
Late BB DR	5,612 ± 571	5	169 ± 31	5	8,009 ± 539	3	—	—
Late BB diabetic	5,761 ± 835	7	202 ± 21	6	10,240 ± 1,257	6	—	—

Data are mean ± SE. *Units = (pixels/mm²); †units = (ng/g weight).

we found a marked decrease in islet VMAT2-positive fiber area in early BB diabetic rats compared with their early BB DR controls. In contrast to the marked loss of islet sympathetic nerves seen in the early BB diabetic rat, there was no loss of sympathetic fibers in the exocrine pancreas. Thus, the neuropathy observed in the early BB diabetic rat is islet specific. Because islets compose only 1–2% of total pancreatic volume, the selective loss of islet sympathetic nerves did not decrease total pancreatic NE content. Thus, the quantitation of VMAT2-positive fiber area allows the detection of an islet-specific sympathetic neuropathy in autoimmune diabetes that more classical techniques may have missed.

To determine whether eSIN might precede diabetes, we examined prediabetic BB rats, which are homozygous for the lymphopenia mutation yet too young to develop diabetes. They had an islet VMAT2-positive fiber area that was similar to that of their age-matched BB DR controls. Therefore, it seems that the onset of diabetes, not the predisposing genes, causes eSIN in the BB rat.

To determine whether the loss of islet sympathetic nerves observed in early BB diabetic rats might be due simply to the marked loss of islet tissue associated with the destruction of islet B-cells, we administered STZ to produce a chemical form of islet tissue destruction. Rats treated with STZ had no decrease of islet VMAT2-positive fiber area compared with their saline-treated controls, despite the induction of diabetes indicating major B-cell loss. Therefore, it seems that a major loss of islet tissue per se is not sufficient to cause the marked loss of islet sympathetic nerves seen in rats with autoimmune diabetes.

Thus, other factors present in the BB diabetic but not the STZ diabetic rat must cause eSIN. One possible factor is cytokine-induced injury of islet sympathetic nerves secondary to the autoimmune attack on islet B-cells that occurs in BB but not STZ diabetic rats. Another possible

factor is islet NGF deficiency. Because others have found that the islet B-cells produce the sympathetic neurotrophin NGF (9), at least in vitro, we hypothesize that it is the combination of the cytokine-induced sympathetic nerve injury followed by loss of B-cell-derived neurotrophic support that leads to eSIN in the early BB diabetic rat. Additional studies involving measurements of both islet cytokines and islet NGF are necessary to test this hypothesis.

Two previous studies had suggested some islet sympathetic neuropathy in animal models of diabetes. One study reported a decrease of NE fluorescence in the islets of diabetic Chinese hamsters (15), but the neuropathy seemed to be inversely related to metabolic control and may, therefore, be due to a generalized DAN rather than to eSIN. Likewise, another study reported a decrease of monoamine oxidase-positive fibers after 6 weeks of diabetes in alloxan-treated Wistar rats (16). The present report contrasts with the two reports above in suggesting a severe, early, and selective loss of sympathetic nerves in an autoimmune model of type 1 diabetes, the BB diabetic rat. However, the previous report of Tominaga et al. (17) shares some similarities with the present study. They showed loss of contact between the sympathetic nerve terminals and the islet A-cells in the BB diabetic rat as demonstrated by electron microscopy. They did not determine whether this decreased contact was due to loss of nerve terminals or simply retraction of nerve terminals from the A-cell. They also found an impaired glucagon response to glucopenia in the perfused pancreata of early BB diabetic rats and suggested that impaired sympathetic stimulation of the A-cell contributed to this impaired glucagon response (17). Because we have previously shown that activation of the sympathetic nerves of the pancreas is sufficient to mediate the glucagon response to insulin-induced hypoglycemia (3), it is tempting to speculate that the eSIN we describe here may cause the loss of

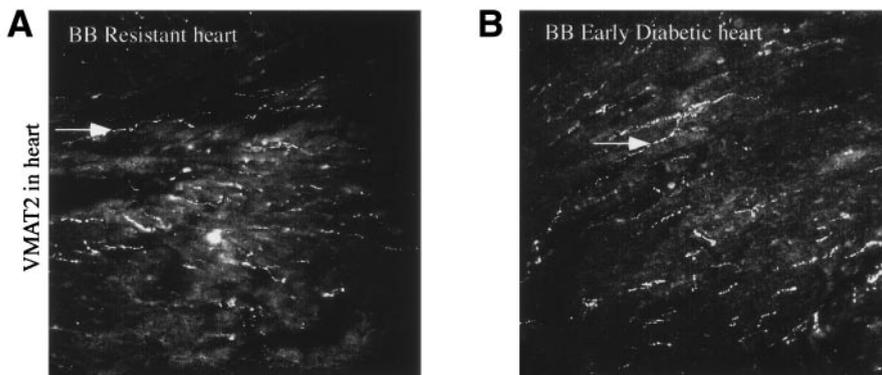


FIG. 5. VMAT2 fiber staining in the distal left ventricle of the heart in early DR (A) or early diabetic (B) BB rats. Numerous VMAT2-positive fibers were identified in the early DR as well as early diabetic BB rat (arrows).

the glucagon response to insulin-induced hypoglycemia that occurs in the first week of diabetes in the BB rat (18). However, it is important to emphasize that the present study provides only anatomical evidence for islet sympathetic denervation and that functional studies are needed to address the above hypothesis. As a first step, preliminary studies to assess the functional impact of eSIN in early BB diabetic rats have been conducted and indeed suggest impaired glucagon responses, at least to sympathetic nerve stimulation (19).

Although eSIN is an early and islet-specific form of diabetic neuropathy, much later and more widespread loss of small nerve fibers has been widely reported in the extrapancreatic tissues in human type 1 diabetes and animal models thereof. For instance, loss of myelinated and unmyelinated axons is clearly established in distal symmetric sensory or sensorimotor nerves (20–22), and loss of nerve fibers from the greater splanchnic nerve (23), the mesenteric nerves (24), the celiac ganglia (24,25), the intrinsic (26) and extrinsic (27) innervation of the intestine, and the esophagus (28) has been reported. These neuropathies appear in animals and patients who have had diabetes ranging from several months to years and are usually attributed to classical DAN, secondary to chronic hyperglycemia. In contrast, the islet-specific sympathetic neuropathy reported here occurs at the onset of autoimmune diabetes. Thus, we hypothesize that eSIN is distinct from DAN in timing, location, and mechanism.

Sympathetic innervation of the heart in early BB diabetic rats. We tested the above hypothesis by examining the sympathetic innervation of the heart, which has received intensive study because DAN of the heart is a complication of type 1 diabetes that has been invoked as a cause of sudden death in individuals with diabetes (29). For example, loss of sympathetic nerve fibers in the heart of BB diabetic rats (28 weeks' duration) was inferred from morphometric quantification of catecholamines (30). STZ-diabetic rats also exhibited sympathetic denervation of the left ventricle of the heart after 6 months of diabetes (31). In our study, we found no significant decrease of either the VMAT2-positive fiber area or the NE content of the left ventricle of early BB diabetic rats. These data suggest that DAN had not yet developed in early BB diabetic rats and, therefore, that eSIN is distinct from DAN, both in onset and cause.

Finally, we determined that eSIN does not progress after 1–2 weeks of diabetes: late BB diabetic rats (1–4 months' duration) had a decrease of islet VMAT2-positive fiber area that was remarkably similar to that seen in the early BB diabetic rat. This comparison suggests that eSIN is fully developed very soon after the onset of diabetes. In contrast, DAN takes months or years to develop and is thought to be progressive (32).

In conclusion, these quantitative data provide evidence for a previously unrecognized type of diabetic neuropathy that is specific to the islet and affects sympathetic nerves. It is fully established in the first 2 weeks after the onset of autoimmune type 1 diabetes in the BB rat and therefore seems to be distinct from classical DAN in onset and cause. We have named it early sympathetic islet neuropathy (eSIN).

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