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Original Research

Development of Standardized Insulin Treatment Protocols for Spontaneous Rodent Models of Type 1 Diabetes

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Standardized protocols for maintaining near-normal glycemic levels in diabetic rodent models for testing therapeutic agents to treat disease are unavailable. We developed protocols for 2 common models of spontaneous type 1 diabetes, the BioBreeding diabetes-prone (BBDP) rat and nonobese diabetic (NOD) mouse. Insulin formulation, dose level, timing of dose administration, and delivery method were examined and adjusted so that glycemic levels remained within a normal range and fluctuation throughout feeding and resting cycles was minimized. Protamine zinc formulations provided the longest activity, regardless of the source of insulin. Glycemic control with few fluctuations was achieved in diabetic BBDP rats through twice-daily administration of protamine zinc insulin, and results were similar regardless of whether BBDP rats were acutely or chronically diabetic at initiation of treatment. In contrast, glycemic control could not be attained in NOD mice through administration of insulin twice daily. However, glycemic control was achieved in mice through daily administration of 0.25 U insulin through osmotic pumps. Whereas twice-daily injections of protamine zinc insulin provided glycemic control with only minor fluctuations in BBDP rats, mice required continuous delivery of insulin to prevent wide glycemic excursions. Use of these standard protocols likely will aid in the testing of agents to prevent or reverse diabetes.

Abbreviations: BBDP, BioBreeding diabetes-prone; BBDR, BioBreeding diabetes-resistant; NOD, nonobese diabetic; PZI, protamine zinc insulin; T1D, type 1 diabetes; VAF, viral-antibody–free; ZT, Zeitgeber time.

Clinical trials to prevent or reverse type 1 diabetes (T1D) are predicated on preclinical study data obtained from animal models of the disease to determine agents that exhibit efficacy and translational potential. However, according to findings published over the past several years (summarized in references 2, 17, and 31), not all preclinical T1D studies are created equal. Without a standardized screening process, the hundreds of candidate therapeutic agents in development cannot be evaluated critically for translational potential. One parameter that varies considerably from report to report in T1D reversal studies is the insulin treatment provided to diabetic NOD mice. To address the need for standardized preclinical screening of new therapeutics, the National Institute for Diabetes and Digestive and Kidney Diseases has developed the Type 1 Diabetes Preclinical Testing Program.[2,35] Under this program, a central contract testing facility (Biomedical Research Models) bridged the gap between discovery of potential therapeutics and clinical testing for efficacy in prevention or reversal of T1D. Using 2 of the best characterized models of T1D, the BioBreeding diabetes-prone (BBDP) rat and the nonobese diabetic (NOD) mouse, we sought to develop standardized protocols for the treatment of diabetes with insulin to provide the best glycemic control throughout the fed and nonfed states. We began by housing these models in a viral-antibody–free (VAF) barrier facility, we created study designs approved by a scientific advisory board consisting of leaders in the field, and we performed studies by using standard operation procedures.

The standard of care in patients with T1D is to attempt to maintain near-normal glucose levels, by providing exogenous insulin therapy several times daily via injection or pump after rigorous monitoring of glycemic levels and by appropriately coordinating insulin dosing with food intake. Current blood glucose control in diabetic rodent models focuses on maintaining the diabetic animal in a state of moderate hyperglycemia, with normal weight gain in the absence of severe ketonuria. This state is achieved by once-daily injections of titrated insulin doses or by implantation of continuous release insulin pellets;[38] however, insulin types and methods can vary widely between institutions and laboratories, yielding a wide range of glycemic control. Therefore there is marked difference between the stringent glycemic control targeted by humans with diabetes as compared with the relatively loose glycemic control afforded to rodents with diabetes. Despite the many physiologic differences between humans and rodents, glycemic control potentially can be addressed by making insulin treatment in rodents more comparable in terms of glycemic

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control to what is achieved currently in humans, especially given that patients with T1D will continue to administer insulin during treatment with therapeutic agents (for example, antiCD3).[11] The lessening of the frequency, duration, and severity of hyperglycemic events is anticipated to provide the best chance for β cells to rest (function properly) while interventions are tested.[21] Ideally, for these studies, animals should receive sufficient insulin to maintain glycemic levels close to the normal range in control nondiabetic animals.

For these studies, we focused on the 2 most widely used spontaneous rodent models of T1D: the BioBreeding diabetes-prone (BBDP) rat and the nonobese diabetic (NOD) mouse.[1,12] The BBDP strain originated from a colony of outbred Wistar rats that developed spontaneous diabetes at the BioBreeding Laboratories in the 1970s. In the 1980s, the strain was acquired by the University of Massachusetts Medical School. During inbreeding, the BioBreeding diabetes- resistant (BBDR) control strain was established. Both strains are maintained at our facility and represent the most fully inbred (more than 110 generations) and characterized colonies available. BBDP rats develop T1D at 50 to 90 d of age at a frequency of approximately 85% to 90%, with equal frequency in male and female rats; the disease in BBDP rats results from autoimmune insulitis that is mediated primarily by $CD4^+$ and $CD8^+$ T cells and the development of autoantibodies to islet antigen. This insulitis is similar to that in human patients.[18] Insulin therapy is required shortly after onset of hyperglycemia or death will occur due to ketoacidosis.[19] The *Gimap5* mutation in BBDP rats results in a T-cell lymphopenia and is necessary for development of T1D in BBDP rats (along with expression of a MHC class II *RT1 B/D*^{u} allele); adoptive transfer of splenocytes or regulatory T cells from BBDR rats before 35 d of age prevents the onset of diabetes in BBDP rats.[9,28,38] Alternatively, depletion of regulatory T cells from BBDR rats (which are nonlymphopenic) induces T1D in that strain.

The NOD mouse strain originated from selective inbreeding of the Cataract Shionogi mouse strain and was imported from Japan to The Joslin Diabetes Center in 1984. NOD mice are now the most widely used preclinical model of T1D, in part due to the availability of genetic analysis and manipulation as well as the wide array of reagents available for mechanistic studies. The most commonly cited source for NOD mice is The Jackson Laboratory (Bar Harbor, ME), where female NOD mice develop disease at a frequency of 65% to 100% by 30 wk of age, whereas male NOD mice develop disease at a frequency of 35% to 85% (inbred for more than 83 generations). The incidence can vary from year to year[34] and from facility to facility depending on several factors, the most important being housing conditions.[15,26] The incidence of T1D in female NOD mice at our VAF barrier facility has been 65% to 80% over the past 3 y; this frequency can be far lower in nonVAF facilities. Diabetic NOD mice exhibit mild ketoacidosis, which allows them to survive for as long as several weeks after the onset of hyperglycemia without supportive insulin treatment. NOD mice also present with insulin resistance and a distinct stage of insulitis, referred to as peri-insulitis, that is not found in either human T1D or in diabetic BBDP rats.[5,18] Although both NOD mouse and BBDP rat models of T1D have particular advantages and disadvantages, a prudent path of drug development would include the examination of the therapeutic efficacy of novel agents in both models.[2,31]

To standardize and improve current testing protocols, we developed insulin treatment regimens that maintain blood glucose levels near normal levels throughout day and night activities over prolonged periods, as would be expected to occur in interventional clinical trials. We show here that whereas 2 daily injections of insulin to diabetic BBDP rats were sufficient to achieve our goal, diabetic NOD mice required continuous delivery of insulin through the implantation of osmotic pumps.

Materials and Methods

Insulins. Protamine zinc insulin (PZI; a product comprising 90% beef insulin and 10% pork insulin) was obtained from IDEXX Laboratories (Greensboro, NC). Humulin 50/50 (50% human insulin isophane suspension/50% human insulin injection), Humulin 70/30 (70% human insulin isophane suspension/30% human insulin injection) and Humulin R (recombinant DNA origin) were manufactured by Eli Lilly (Indianapolis, IN). Lantus (insulin glargine, recombinant DNA origin) was manufactured by Sanofi–Aventis (Bridgewater, NJ). Prozinc (protamine-zinc–formulated insulin, recombinant DNA origin) was manufactured by Boerhinger Ingelheim (Ridgefield, CT). Sterile diluents, when used, were obtained from the insulin manufacturers. Insulin was injected subcutaneously in the skin overlying the pectoral muscles.

Animals. Male BBDP/Wor//Brm and BBDR/Wor//Brm rats were obtained from our inhouse breeding colony (Biomedical Research Models, Worcester, MA). Female NOD/ShiLtJ mice (age, 4 to 6 wk) were purchased from The Jackson Laboratory (stock no. 001976). Rats were housed in conventional polycarbonate cages with wire food–water racks and filter-top lids, and mice were housed either in microisolation or ventilated racks within an AAALAC-accredited VAF barrier facility. Periodic testing of sentinel rats and mice was performed to assure the absence of common rodent viruses and other pathogens. Rats and mice used on studies had no evidence of any disease unrelated to diabetes. All insulin administrations were performed by subcutaneous injection, except when insulin was provided by using pumps. All animals received food (rats, Purina 7012 autoclaved diet; mice, irradiated Lab Diet 5LG4; Purina Mills, Gray Summit, MO) and acidified drinking water ad libitum. Animals were maintained in accordance with the guidelines of the Biomedical Research Models IACUC and the *Guide for the Care and Use of Laboratory Animals*.[10]

Diagnosis of diabetes. Within our VAF barrier facility, the normal range of nonfasted blood glucose levels is 86 to 162 mg/dL for BBDR rats at 56 to 63 d of age and 61 to 130 mg/dL for nondiabetic NOD mice at 10 wk of age.

Beginning at 50 d of age, BBDP rats were screened twice weekly for glycosuria (CliniStix, Bayer HealthCare, Diabetes Care Division, Elkhart, IN). When a positive glycosuria test was observed, a serum or blood glucose test was performed to confirm diabetes onset. Diabetes onset was defined as a positive glycosuria test (4+) followed by a nonfasted serum or whole-blood glucose level of greater than 250 mg/dL. Measurements were performed by using a GM7 Analox Analyzer (Analox Instruments, London, UK) or Contour handheld glucometer (Bayer; range, 10 to 600 mg/dL).

Beginning at 10 wk of age, NOD mice were bled twice weekly via tail nick, and blood glucose levels were measured using a Contour handheld glucometer (Bayer). Mice with blood glucose levels of at least 250 mg/dL were retested the following day. Dia-

betes onset was defined as a nonfasted blood glucose level of at least 250 mg/dL for 2 consecutive days.

Insulin treatment. BBDP colony rats that became diabetic were either euthanized or maintained on a once-daily dose of 0.9 U PZI per 100 g administered at approximately Zeitgeber time (ZT) 5 to ZT6 until study enrollment; this regimen is the standard of care to maintain diabetic rats in a moderate state of glycosuria with weight gain.[38] Once enrolled onto an insulin-control study (after 4 to 65 d of receiving standard insulin treatment; $n = 8$ to 12 per group), diabetic BBDP rats received either a single dose (0.9 U per 100 g) of insulin at approximately ZT8 to ZT10, once-daily doses of PZI insulin (0.9 U per 100 g at ZT8), or twice-daily doses of PZI insulin (0.7 to 0.9 U per 100 g at ZT8 and 0.5 to 0.6 U per 100 g at ZT20).

Diabetic NOD mice ($n = 7$ to 12 per group; duration of diabetes, 7 d or less) were treated with either a single injection of 0.6 U of Lantus or PZI or 1.5 U of Humulin 50/50 or Humulin 70/30 at ZT13. Alternatively, diabetic NOD mice were treated with twice-daily injections of PZI (0.6 U at ZT13 and 0.2 U at ZT1). ALZET mini osmotic pumps (model 1002) were purchased from Durect (Cupertino, CA). Pumps were filled Humulin R diluted to 33.34, 41.67, or 50.00 U/mL (corresponding to dose levels of 0.20, 0.25, and 0.30 U daily, respectively; $n = 12$ to 20 mice per group) by using sterile diluent, primed for at least 4 h in sterile saline in a 37 °C water bath, and implanted subcutaneously according to the manufacturer's instructions. For insertion of pumps, mice were anesthetized briefly with isoflurane. The insertion site was shaved and disinfected with povidone–iodine and alcohol. Wounds were closed with sterile wound clips and monitored until healed and the clips removed. No mice exhibited signs of incorrect pump insertion or infection at the insertion site. Blood glucose levels were monitored at ZT8 to ZT9 daily and every 3 h on days 3, 7, and 14 after pump implantation.

Diabetes management. Diabetic BBDP rats ranged in age from 50 to 120 d. Initial insulin dose levels were based on the individual rat's body weight, serum or blood glucose level, and onset. Subsequent insulin doses were adjusted based on daily glycosuria and ketonuria tests (measured by using KetoStix [Bayer]) as well as body weight measurements; these results were used as determinants of treatment effectiveness. Insulin dose levels were increased by 0.2 U for every 10- to 15-g increase in body weight. In the event of severe ketoacidosis (3+ to 4+ ketonuria), lactated Ringer solution with sodium bicarbonate (9 mL lactated Ringer solution with 1 to 2 mL 8.4% sodium bicarbonate per rat) once daily via subcutaneous injection as needed. In the event of aglycosuria, a serum or blood glucose level was measured. Severe hypoglycemia (less than 40 mg/dL) was treated by providing an intraperitoneal injection of 1 mL 50% dextrose, followed by a decreased dose of insulin (30% to 50% of the original dose) at ZT11 (after a second serum or blood glucose measurement) together with a subcutaneous injection of a mixture of 1 mL 50% dextrose and 9 mL lactated Ringer solution per rat. Marked hypoglycemia (40 to 60 mg/dL) was treated by providing a decreased dose of insulin (20% to 30% of the original dose) at ZT11 (after a second serum or blood glucose measurement) together with a subcutaneous injection of a mixture of 1 mL 50% dextrose and 9 mL lactated Ringers solution per rat. Mild hypoglycemia (60 to 80 mg/dL) was treated by providing a dose of insulin (equivalent to the original dose) at ZT11 together with a subcutaneous dose of lactated Ringer solution (10 mL per rat). No rats required removal from study because of unresolved clinical signs.

Diabetic mice ranged in age from 12 to 20 wk and were monitored periodically for nonfasted blood glucose levels as described. For studies using NOD mice, hypoglycemia was defined as nonfasted blood glucose levels lower than 40 mg/dL, and severe hypoglycemia was defined as nonfasted blood glucose levels lower than 25 mg/dL. Mice exhibiting a blood glucose level of 40 mg/dL or less with or without clinical signs of diabetes management (for example, more than 10% body weight loss, lethargy, rough hair coat) were provided lactated Ringers solution with 5% dextrose (1 mL per mouse) once daily via subcutaneous injection as needed. Mice exhibiting a blood glucose level of 25 mg/dL or less without clinical signs of diabetes management were provided lactated Ringers solution with 5% dextrose once daily as needed. Mice with blood glucose levels of 41 to 499 mg/dL were not provided fluid therapy unless clinical signs of diabetes management were present. Mice with a blood glucose 25 mg/dL or less or 500 mg/dL or greater with clinical signs of diabetes management were removed from study and euthanized. Because diabetic NOD mice may exhibit only mild ketoacidosis in the absence of insulin therapy, they therefore were not monitored for ketonuria.[16] No mice required removal from study because of unresolved clinical signs.

ZT. Observation and insulin dosage time points are presented in ZT, where ZT0 is lights-on (0300) and ZT12 is lights-off (1500). The lights-off hours (ZT12 to ZT24–ZT0) are indicated by gray shading (Figures 1 through 4), except in long-term studies.

Statistical analysis. All values are reported as mean ± SEM. Statistically significant differences in blood glucose levels between groups were determined by 2-way ANOVA with the Bonferroni multiple comparison test. Statistically significant differences in duration of treatment effectiveness between groups were determined by using the Kruskal–Wallis test with the Dunn multiple comparison test. A P value less than 0.05 was considered statistically significant. All analyses were performed by using Prism 5 (GraphPad Software, La Jolla, CA).

Results

Maintaining near-normal glycemic levels in diabetic BBDP rats. Similar to humans with T1D, diabetic BBDP rats must be treated daily with insulin to prevent ketoacidosis and death.[8] Before our standard of care was established, preliminary studies found that Humulin 50/50 or Humulin 70/30 (mixtures of intermediate- and short-acting human recombinant insulins) were ineffective, suggesting that long-acting formulations of insulin were needed in rats, perhaps due to their increased metabolic rate compared with that of humans. Figure 1 A shows the serum glucose levels in diabetic BBDP rats undergoing daily treatment with a 1or 2 doses of PZI compared with the serum glucose levels in nondiabetic control BBDR rats. BBDP rats treated with a single dose of PZI showed cyclic rises in serum glucose levels at 10 to 12 h after insulin injection (suggestive of the duration of action of PZI compounded by the diurnal rhythm of endogenous glucose production and the reported peak in blood glucose levels shortly after lights off[3,14]). Spikes in serum glucose of more than 50 mg/dL greater than levels seen in control BBDR rats occurred every day in the once-daily PZI-treated group; these differences were statistically significant ($P < 0.05$) at ZT6 on days 1, 2, and 3, at approximately 24 h after treatment (Figure 1 A). However, rats treated

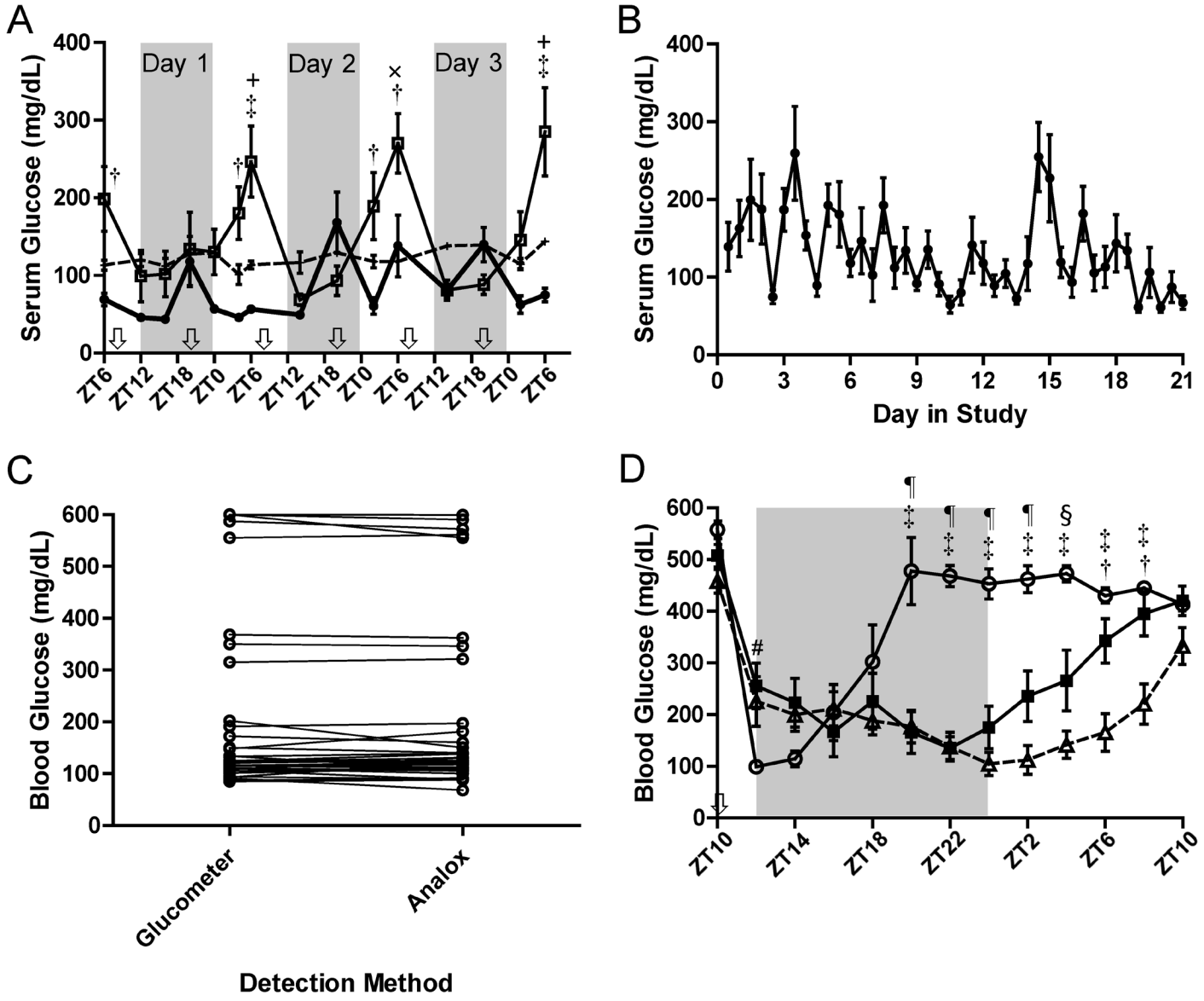

Figure 1. Serum glucose levels in diabetic BBDP/Wor rats after once or twice daily dosing with PZI, Lantus, or Prozinc insulin. (A) Groups of 12 diabetic BBDP rats were treated with either a single daily titrated dose of PZI (at ZT8, thin line and open squares) or 2 daily titrated doses of PZI (at ZT8 and ZT20, thick line and circles), whereas age-matched, nondiabetic male BBDR rats were left untreated ($n = 6$; dashed black line). Serum glucose levels were measured every 6 to 8 h. Significant (†, $P < 0.01$; ‡, $P < 0.001$) differences between values after 2 doses of PZI compared with 1 dose of PZI are shown, as are differences (+, $P < 0.05$; ×, $P < 0.01$) between values for mice given 1 dose of PZI compared with untreated controls. (B) Eleven diabetic BBDP rats were treated with 2 daily titrated doses of PZI (at ZT8 and ZT20) for 3 wk. Serum glucose levels were measured every 12 h. (C) Serum and blood glucose levels were measured from 39 BBDP rats using a single blood collection per rat. (D) Groups of 8 to 10 diabetic BBDP rats were treated with a single injection of either PZI (squares), Lantus (open circles), or Prozinc (dashed line with open triangles) at a dose of 0.9 U/100 g at ZT10. Blood glucose was measured every 2 h for 24 h. Significant (†, $P < 0.01$ between values after Prozinc insulin compared with PZI; ‡, $P < 0.001$ between values after Prozinc insulin compared with Lantus; #, $P < 0.05$, §, $P < 0.01$, and ¶, $P < 0.001$ between values after PZI compared with Lantus) differences are shown. Time of insulin administration is indicated by an arrow.

with PZI twice daily maintained serum glucose levels that were no more than 50 mg/dL higher than the levels seen in control, nondiabetic rats (range, 100 to 140 mg/dL; mean, 122 mg/dL). The differences in glycemic control due to twice-daily compared with once-daily PZI were statistically significant at ZT6 and ZT4 on day 1, ZT6 and ZT2 on day 2, and ZT6 at the beginning and end of day 3. Figure 1 B shows the average serum glucose levels (measured every 12 h) in BBDP rats that were treated with twice-daily PZI for 3 wk. According to these results, we standardized the first (evening) dose of insulin to 80% of 0.9 U/100 g body weight and the second (morning) dose to 67% of the first dose. The insulin dose was increased or decreased by 0.2 U at the next scheduled dosing time when serum glucose levels were rising above or falling below the normal range (86 to 162 mg/dL) ± 50

mg/dL from Figure 1 A. Average blood glucose level readings fell within the normal range ± 50 mg/dL 93% of the time except at 3.5, 14.5, and 15 d after initial dose administration. Similar results were obtained whether BBDP rats were acutely (duration, less than 5 d) or chronically (duration, 15 to 19 d) diabetic (data not shown).

Measuring glucose in whole blood compared with serum. Real-time assessment of glycemic status has many advantages, especially as it relates to clinical care. Previously, glycemic levels in diabetic rats were determined by using clinical chemistry analyzers. Although believed to be more accurate than using handheld glucometers, the clinical chemistry method is time-consuming and requires a considerable volume of blood. Because of this blood-volume requirement, diabetes studies in mice almost exclusively use handheld glucometers, which typically require only a single drop of blood. To determine whether glucometer measurements of whole-blood glucose levels were equivalent to serum glucose levels, we examined both parameters in a cohort of 39 BBDP rats representing a wide range of glucose levels. Differences between readings from handheld and clinical chemistry analyzers ranged from 0% to 35% in individual rats, but the average difference between the 2 analyzers for the study population of 39 BBDP rats was 1.3% and was not statistically significant (P = 0.8533, Mann–Whitney test, Figure 1 C) between methods.

Replacing PZI for treatment of diabetic BBDP rats. The manufacture of PZI was discontinued recently, prompting the need to identify an effective alternative in diabetic rats. We tested PZI against Lantus (a long-acting human recombinant insulin glargine) and Prozinc (a new protamine-zinc–formulated human recombinant insulin). Treatment with PZI in this experiment (Figure 1 D) exhibited a similar profile as that previously (Figure 1 A). Treatment with Lantus quickly decreased blood glucose levels to approximately 100 mg/dL within 2 h after administration, although this effect was short-lived, and blood glucose in BBDP rats was near their pretreatment levels by ZT20 (9 h after insulin administration) and remained hyperglycemic during the rest of the monitoring period. Prozinc mimicked PZI for the first 12 h after administration (ZT22); BBDP rats treated with Prozinc continued to exhibit blood glucose levels below 200 mg/dL until 20 h after treatment (ZT6). Although the immediate decrease in blood glucose levels at ZT12 was significantly ($P < 0.05$) greater in Lantus-treated rats than in rats given either Prozinc or PZI, long-term glycemic levels were significantly ($P < 0.05$) lower in response to Prozinc and PZI than Lantus between ZT20 to ZT8 and ZT20 to ZT4, respectively (Figure 1 D). The mean duration of treatment effectiveness (defined as the last time point with a blood glucose level of less than 200 mg/dL) was longer after either Prozinc ($P < 0.001$) or PZI ($P < 0.05$) than after Lantus (data not shown). These data suggest that Prozinc is a suitable long-acting insulin with similar or even better potency than PZI (as indicated by the significantly [$P < 0.01$] lower blood glucose levels in Prozinc- compared with PZI-treated BBDP rats at ZT6 and ZT8). The reason why a long-acting insulin like Lantus did not exhibit a long duration of action in BBDP rats could be related to either the 3 amino-acid changes in the in A and B insulin chains designed to increase stable hexamer formation and slow absorption[37] or, more likely, may be associated with the protamine zinc formulation found in PZI and Prozinc; this modification also increases product stability and duration of action[20] and is key to insulin effectiveness in diabetic rats.

Variable glycemic control in NOD mice after injectable insulins. Although the NOD mouse is the most common preclinical model for T1D, insulin dosing schedules to achieve glycemic control in diabetic NOD mice vary widely in the type of insulin used (PZI, Ultralente, Lantus, Humulin, and LinBits[13,22,29]), insulin dose (as much as 2 U or more of insulin per approximately 25-g mouse[13,25]), and definition of diabetes onset (from 180 to 400 mg/dL,[29,30,32,33]). To establish a standard insulin treatment protocol for NOD mice, we first evaluated the effect of single injections of various types of insulin on blood glucose levels in diabetic mice (Figure 2). A single injection (0.6 U) of Lantus (described as a long-acting recombinant insulin glargine) immediately reduced blood glucose levels (mean 105 mg/dL) by 1 h after treatment (ZT14). Thereafter, mean blood glucose levels steadily increased to more than 200 mg/dL by 4 h after treatment (ZT17) with a single dose of Lantus and to pretreatment levels by 12 h after treatment (ZT1; Figure 2 A).

We compared PZI (a long-acting insulin used to treat diabetic BBDP rats) with 2 Humulin formulations (50/50 and 70/30) in NOD mice. A single injection of PZI, Humulin 50/50, or Humulin 70/30 reduced blood glucose levels in NOD mice within 1 h after treatment (ZT14; mean blood glucose of 72, 78, and 70 mg/dL, respectively); thereafter, blood glucose levels began to increase at variable rates (Figure 2 B). The mean duration of treatment effectiveness was significantly ($P < 0.05$) longer after PZI than after Humulin 50/50 (data not shown). Mean blood glucose levels were significantly ($P < 0.05$) lower in mice treated with PZI compared with Humulin 50/50 at ZT4, ZT6, and ZT12. Collectively, these data suggest that, among the 4 formulations tested, PZI provided the longest treatment effect in NOD mice, even though Humulin doses were 2.5-fold greater than those of PZI.

Blood glucose response to 2 daily doses of PZI in NOD mice. In light of the preceding results, we chose to continue using PZI insulin but moved to twice-daily injections to provide optimal 24-h glycemic control in NOD mice. Blood glucose levels were measured at 0, 1, 2, 4, 6, 9, and 12 h relative to each insulin administration. Average blood glucose level dropped to within normal glycemic levels (120 ± 50 mg/dL) in the first hour after administration of 0.6 U of PZI and began to rise between 2 and 4 h after treatment (Figure 2 C). By 12 h after the initial treatment, the average blood glucose level was 290 mg/dL. After the second dose of PZI, average glucose levels again dropped within 1 h after injection (ZT2) and continued to decrease until 4 h after treatment, after which point average blood glucose levels began to rise to a maximal level of 256 mg/dL at 12 h after administration (ZT13). Treatment with 0.2 U at 1 h after lights-on resulted in an average blood glucose level equivalent to that seen after treatment with 0.6 U of PZI at 1 h after lights-off. Although all diabetic NOD mice responded to both doses of PZI, the duration of response was limited, and blood sugar levels exceeded 200 mg/dL by 6 to 8 h after treatment.

In a separate experiment, we treated diabetic NOD mice with PZI twice daily for 4 d. All NOD mice responded to each insulin administration (Figure 2 D). As seen in the previous experiment, blood glucose levels dropped to within the normal glycemic range after each insulin administration. By 6 h after each consecutive insulin administration, the average blood sugar level had risen, and it continued to rise until the next insulin administration. However, throughout the entire study period, blood sugar levels in all NOD mice were below 300 mg/dL for at least 50% of the time.

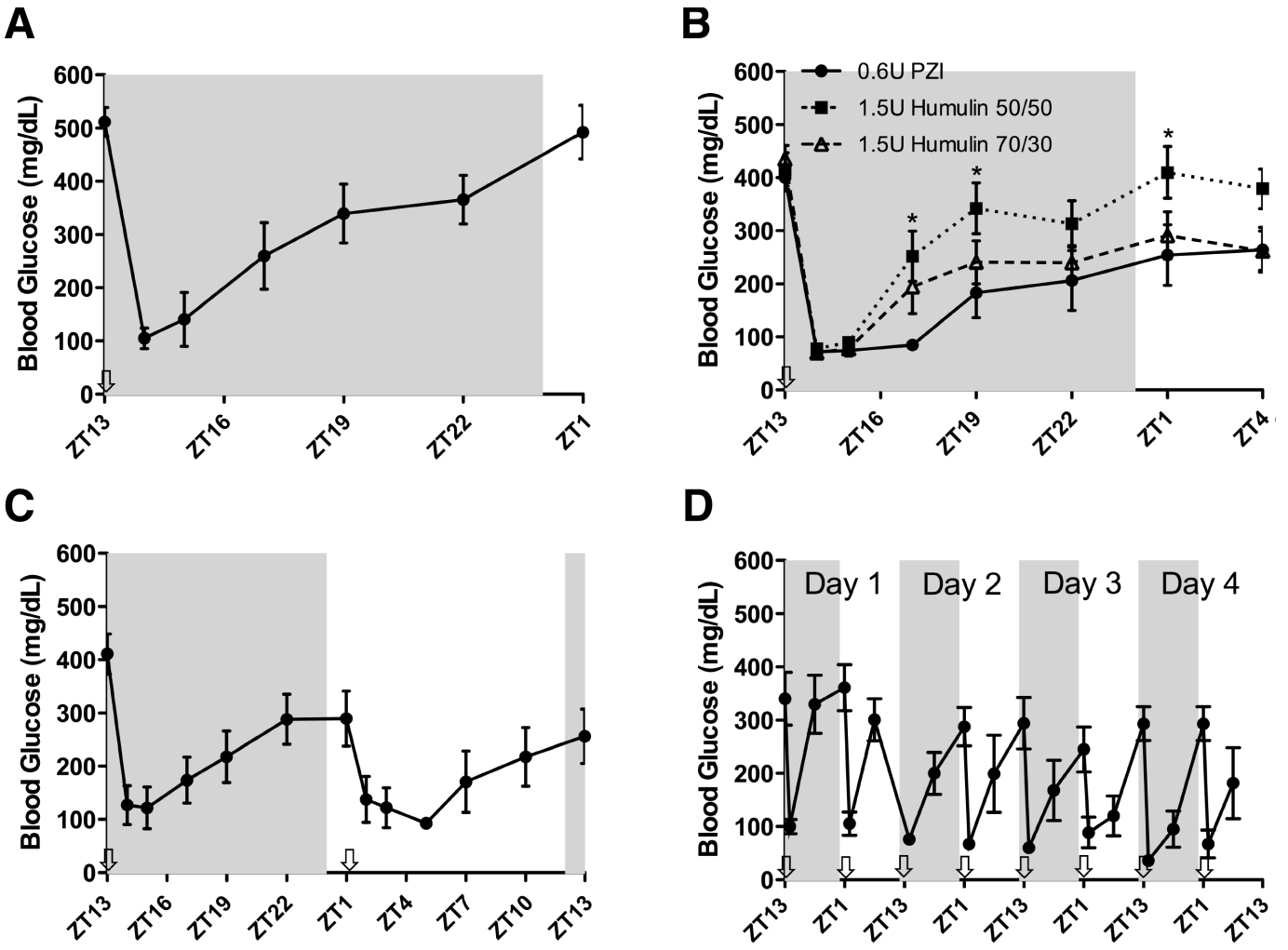

Figure 2. Comparison of Lantus, Humulin, and PZI insulin treatment in recent-onset diabetic NOD mice. (A) Twelve diabetic NOD mice each received a single dose of Lantus at ZT13. Three of the treated mice did not respond and were excluded from analysis. Blood glucose measurements were taken at 0, 1, 2, 4, 6, 9, and 12 h relative to insulin administration. (B) Thirty diabetic NOD mice were randomized into 3 groups, and each mouse received a single dose of PZI, Humulin 50/50, or Humulin 70/30 at ZT13. Blood glucose measurements were taken at 0, 1, 2, 3, 6, 9, 12, and 15 h relative to insulin administration. *, Significant ($P < 0.05$) difference between value after PZI compared with Humulin 50/50. (C) Ten diabetic NOD mice were administered PZI at ZT13 and ZT1. Blood glucose measurements were taken at 0, 1, 2, 4, 6, 9, and 12 h relative to insulin administration. (D) Seven diabetic NOD mice were given PZI at ZT13 and ZT1 for 4 d. Blood glucose measurements were taken at 0, 1, 6, and 12 h relative to insulin administration. Time of insulin administration is indicated by an arrow.

Use of mini osmotic pumps for continuous insulin delivery. Although twice-daily injection of PZI was somewhat effective, glucose levels in diabetic NOD mice still exceeded 200 mg/dL at some point every day. To determine whether multiple smaller doses of insulin would avoid periods of hyperglycemia, we implanted osmotic mini pumps in NOD mice for insulin treatment.

The mean blood glucose level in the mice given 0.2 U daily by pump fluctuated close to 200 mg/dL from day 1 to 9 after pump implantation, gradually increased to 267 mg/dL by day 12, decreased to 203 mg/dL by day 16, and finished at 249 mg/dL. The overall average blood glucose level was 209 ± 10 mg/dL during optimal pump function (Table 1). Glycemic control was never stably within the euglycemic target range of 60 to 180 mg/dL, and the median number of days to achieve a stable blood glucose level below 200 mg/dL was 14 d.

For the 0.25-U group, mean blood glucose levels fluctuated close to 100 mg/dL throughout the study, with a high of 152 mg/dL on day 1 and a low of 89 mg/dL on day 16 (Figure 3). The overall average blood glucose level was 116 ± 10 mg/dL during optimal pump function. Collectively, the mean once-daily blood glucose levels were stably below 200 mg/dL beginning on day 1, and the median number of days to achieve a stable blood glucose level below 200 mg/dL was 4 d (Table 1). Blood glucose was stably within the euglycemic target range between days 1 to 17 after pump implantation.

The mean blood glucose levels in the 0.3-U group also fluctuated close to 100 mg/dL throughout the study, with a high of 181 mg/dL on day 2 and a low of 70 mg/dL on day 14. The overall average blood glucose level was 119 ± 8 mg/dL during optimal pump function (Table 1). Collectively, the mean once-daily blood glucose levels were stably below 200 mg/dL beginning on day 1,

Table 1. Summary of average daily blood glucose (mean ± SEM) for days 1 through 14 after implantation of mice with insulin pumps

Daily insulin dose (U)	Overall average blood glucose level (mg/dL)	Range of blood glucose values (mg/dL)	Median no. of days to achieve stable blood glucose level of less than 200 mg/dL	No. of hypoglycemic events (%)[a]
0	503 ± 12	366–547	not applicable	0 (0%)
0.2	209 ± 10	154–267	14	9 of 269 (3.3%)
0.25	116 ± 6	89–152	4	30 of 276 (10.9%)
0.3	119 ± 8	70–181	2	49 of 276 (17.8%)

[a]Hypoglycemic events are defined as blood glucose levels less than 40 mg/dL.

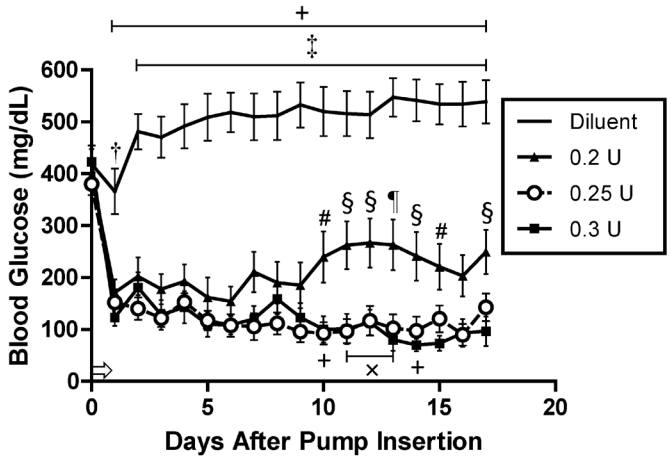

Figure 3. Average daily blood glucose levels in diabetic NOD mice implanted with osmotic pumps containing Humulin R. Diabetic NOD mice (n = 12 to 20 per group) were implanted with ALZET pumps containing either diluent or Humulin R at a concentration to provide for release of either 0.2, 0.25, or 0.3 U insulin over a 24-h time period. Daily blood glucose levels were measured between ZT8 and ZT9. Time of insulin administration is indicated by an arrow. Significant differences between values are indicated as: †, $P < 0.01$ and ‡, $P < 0.0001$ for diluent compared with 0.2 U; + = $P < 0.001$ for diluent compared with 0.25U and diluent compared with 0.3U. +, $P < 0.05$ and ×, $P < 0.01$ for 0.2 U compared with 0.25 U; and #, $P < 0.05$, §, $P < 0.01$, and ¶, $P < 0.001$ for 0.2 U compared with 0.3 U.

and the median number of days to achieve a stable blood glucose level below 200 mg/dL was 2 d. Blood glucose was stably within the euglycemic target range between days 1 to 17 after pump implantation. The implantation of pumps that released diluent only had no effect on blood glucose levels.

During intensive periods of blood glucose monitoring, mean blood glucose levels for the 0.2-U group were only within the normal glycemic target range of 60 to 180 mg/dL at ZT11 on days 3, 4, and 7 (Figure 4); mean blood glucose levels for this group were outside of the euglycemic target range at all other time points. Mean blood glucose levels for the 0.25-U mice were within the normal glycemic target range for all time points except ZT17 and ZT20 on day 3 and ZT17 on day 7. Mean blood glucose levels for the 0.3-U group were within the normal glycemic target range for all time points except ZT14, ZT17, ZT20, and ZT2 on day 3 and ZT17 and ZT20 on day 7. The highest blood glucose levels were seen at 5 h after lights-off (ZT17) on days 3 and 7 and between 5 and 8 h after lights-off (ZT17 to 20) on day 14. The differences between time points within each treatment group were not statistically significant. The frequency of hypoglycemic events was directly related to the insulin dose (Table 1). Despite these events, no study mouse exhibited any clinical symptoms of hypoglycemia or had to be removed from study because of severe hypoglycemia.

Discussion

We describe here the development of 2 protocols to establish effective glycemic control in the 2 most common rodent models of T1D. These protocols were developed so that the testing of therapeutics for the T1D-PTP program can be done by using rodents in which glycemic control closely approximates that of nondiabetic mice and rats and of human patients with T1D (normal range of blood glucose levels in humans is 70 to 130 mg/dL preprandial, less than 140 mg/dL postprandial; normal range in nondiabetic NOD mice is 61 to 130 mg/dL postprandial at 10 weeks of age). Recent studies suggest that the best success for treatment of diabetes might be achieved when therapeutics are administered under conditions of strict glycemic control.[13,23,30] A human clinical trial that is currently enrolling subjects seeks to determine whether strict glycemic control at diabetes onset (achieved through continuous glucose monitoring and linked insulin injection in hospital) can preserve β cell function (Metabolic Control Study, Type 1 Diabetes TrialNet and DirecNet trials consortia). Therefore, standardized protocols designed to provide optimal glycemic control in diabetic NOD mice and BBDP rats potentially may benefit preservation of β cell mass.

For BBDP rats, administration of PZI twice daily was sufficient to obtain control within a range that never exceeded 70 mg/dL above euglycemic levels, albeit with feeding-associated excursions. The best results were obtained when BBDP rats received 80% of a dose equivalent to 0.9 U/100 g body weight at 8 h after lights-on and 67% of the first dose at 8 h after lights-off. Although some fluctuations occurred, glycemic control was achieved for as long as 3 wk. No differences were present between BBDP rats that were acutely diabetic and those with longer durations of disease at the start of administration of twice-daily PZI (data not shown). In addition, we determined that Prozinc insulin was a suitable alternative to PZI for glycemic control in BBDP rats. Although similar insulin regimens have been reported previously,[36,38] we found that the standard protocol described herein allows for flexible insulin dosage that is based on real-time blood glucose levels (as is the practice for human patients) and provides optimal glycemic control in diabetic BBDP rats.

In NOD mice, injections of insulin did not achieve sustained lowering of blood glucose. Despite the use of various insulin formulations purported to have longer durations of action in humans or that had prolonged duration of action in BBDP rats, a suitable duration of action was not achieved in NOD mice. This

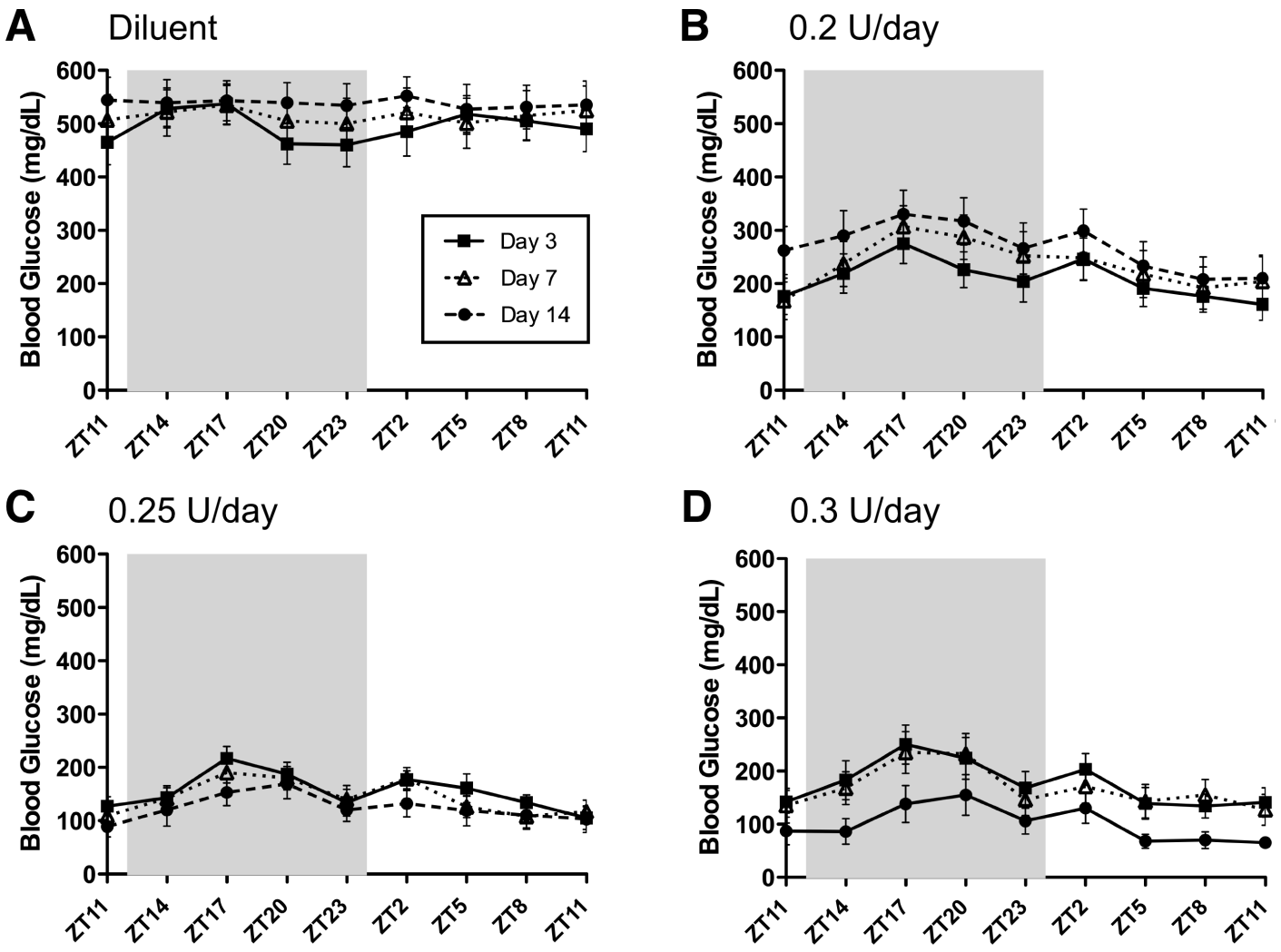

Figure 4. Intensive blood glucose monitoring of diabetic NOD mice implanted with osmotic pumps releasing Humulin R. Mice (from Figure 3) were monitored for blood glucose levels every 3 h for 24 h on days 3, 7, and 14 after pump insertion.

response may be related to an increased metabolic rate in mice compared with rats or the presence of the insulin resistance reported to occur in the NOD strain.[5] Interestingly, mice with a duration of diabetes that exceeded 7 d also did not respond consistently to single-injection insulin treatment (data not shown). For many mice that experienced uncontrolled hyperglycemia for more than 7 d, a single daily injection of insulin at even higher doses was unable to lower blood glucose even in the short term (data not shown). This apparent resistance to insulin therapy, which seems to develop over several days of uncontrolled hyperglycemia, may be related to a continued decline of β cell mass after diabetes onset. This response may help to explain why it is sometimes difficult to use immunomodulators such as anti-CD3 antibody and antilymphocyte serum to reverse diabetes symptoms in NOD mice after diabetes onset.[6,22,23,30] If experiments are not designed to begin insulin treatment within the first few days of onset in NOD mice, subsequent treatment may be ineffective, and attempts to reverse the autoimmune pathology will fail due to severe metabolic disturbances and systemic inflammation in the mice.[5,13,24,27] At least one group has shown that maintenance of normoglycemia throughout the treatment period increased the frequency of reversal in diabetic NOD mice, possibly through the regeneration of β cell mass and function.[7,29]

Compared with injected insulin, osmotic insulin pumps provided superior glycemic control, although diabetic NOD mice did experience events of hypoglycemia. Osmotic pumps offer several advantages: 1) only one pump per animal is required; 2) the pump can be filled with any formulation of insulin; and 3) with a constant release rate, any desired concentration of insulin can be formulated to achieve administration of a precise dose. The variations in blood glucose levels during intensive monitoring periods may have been due to either subtle variations in the volume of insulin loaded into the pumps, insulin release from the pumps, or the diurnal rhythm of endogenous glucose production and blood glucose levels, which reportedly peak shortly after lights-off.[3,14] In addition, the use of osmotic pumps partially alleviates the discrepancy between the insulin doses used in NOD mice, BBDP rats, and human patients with T1D. The protocols described here maintain diabetic NOD mice within the normoglycemic range with minimal fluctuations by using 10 U insulin per

kilogram daily, a dose similar to that required for diabetic BBDP rats (12 U/kg). However, some groups have treated diabetic NOD mice with as much as 2 U insulin daily, a dose equivalent to 80 U/kg in a 25-g mouse.[13,25] These higher doses likely are used to ensure that the mice with be within the normoglycemic range on the following day at the expense of moderate to severe hypoglycemia during the lights-off cycle. How hypoglycemia might affect β cell or immune cell function is unclear, but it might bias study results and hamper the translation of findings from rodent models to human clinical studies (in which hypoglycemia must be avoided). The typical insulin usage in humans is approximately 1 U/kg daily (during insulin detemir or insulin lispro protamine suspension basal-bolus therapy).[4] Standardized protocols such as those we describe here may facilitate effective evaluation of future candidate therapeutic agents in preclinical efficacy trials. More importantly, our standardized protocols achieve glycemic control in rodent models that accurately mimics the tight glycemic control afforded to human patients during clinical trials.

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