Metabolically Inactive Insulin Analog Prevents Type I Diabetes in Prediabetic NOD Mice

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Abstract

The purpose of this study was to determine the relative importance of the metabolic effects of insulin for diabetes prevention by administering insulin or an inactive insulin analog by daily subcutaneous injections to prediabetic mice. A recombinant monomeric human insulin analog, which does not bind to the insulin receptor as a consequence of an alteration of a single amino acid at position 25 of the B chain, was shown to be equally effective at diabetes prevention as was intact insulin. In contrast to native insulin, the insulin analog did not cause hypoglycemia after subcutaneous injection. The insulin analog, however, protected young adult mice from diabetes, even when it was initiated after the onset of extensive lymphocytic infiltration of the islets. Thus, preventative therapy by daily subcutaneous injections of insulin does not require the hypoglycemic response, or binding to the insulin receptor to prevent the onset of type I diabetes. (*J. Clin. Invest.* 1997. 100:1344–1348.) Key words: immunotherapy • synthetic hormones • autoimmunity • type I diabetes mellitus

Introduction

Insulin-dependent (type I) diabetes mellitus is an autoimmune disease that results from destruction of insulin-secreting pancreatic beta cells by both humoral and cell-mediated mechanisms. The nonobese diabetic (NOD) mouse, an animal model of spontaneous diabetes, shares many features of human type I diabetes, including abrupt onset of overt diabetes, presence of lymphocytic infiltration of the pancreatic islet cells before the onset of hyperglycemia (1), presence of antiislet (2, 3) and antiinsulin antibodies before the onset of clinical disease (4), and prevention of disease by immunotherapy (1). Previous studies in the NOD model demonstrated that insulin therapy can decrease the incidence of diabetes when initiated in weanling mice before the onset of significant insulitis (5). Insulin administration by subcutaneous, intravenous, nasal, or oral administration to very young mice has been shown to decrease the incidence of diabetes (5–10). The incidence of diabetes, however, varied in these studies depending on the routes of administration, the use of adjuvants, and the age at therapy initiation. The mechanism for diabetes prevention by insulin is currently unknown, but possible mechanisms include: (*a*) downregulation of islet beta cell antigens as a consequence of metabolic effects of insulin (11, 12); (*b*) induction of regulatory T cells or tolerance to insulin antigenic determinants by daily exposure to insulin; and (*c*) alteration of lymphocyte function as a direct effect of insulin binding to insulin receptors on lymphocytes (13–15). The purpose of this study was to determine the relative importance of metabolic effects versus immunological effects of insulin for diabetes prevention by administering insulin or an inactive insulin analog by daily subcutaneous injections to prediabetic NOD mice. The insulin analog, B25 Asp, does not bind to the insulin receptor or IGF-I receptor as a consequence of the alteration of a single amino acid (see Fig. 1 [16, 17]). Drejer et al., using competitive binding assays with hepatoma cells and adipocytes, demonstrated that the Asp B25 analog has a binding affinity of only 0.05% to the insulin receptor, and $< 0.005\%$ to the IGF-I receptor (17). We demonstrate that this non-glucose-altering insulin analog was equally effective at diabetes prevention as was native insulin. The analog protected young adult mice from diabetes, even when it was initiated after the onset of extensive lymphocytic infiltration of the islets. Thus, preventative therapy by daily subcutaneous injections of insulin does not require the hypoglycemic response, or binding to the insulin or IGF-I receptor, to prevent the onset of type I diabetes.

Methods

Animals. NOD/MrkTacfBR mice obtained from Taconic Farms, Inc. (Germantown, NY) were housed under specific pathogen-free conditions. Only female mice were used for these studies, having a spontaneous incidence of diabetes of 64% at 20 wk, and 80% at 36 wk, with a median onset at diabetes at 26 wk (range: 16–52 wk).

Treatment. Female NOD mice were divided into three groups: vehicle ($n = 29$), insulin ($n = 34$), or the inactive insulin analog group ($n = 26$). The monomeric insulin analog, B25 Asp, produced by recombinant DNA methods, has a single amino acid alteration at position B25 of human insulin (Fig. 1) (16), and was kindly provided by Jens Brange (Novo-Nordisk A/S, Bagsvaerd, Denmark). The therapy was initiated at 12 wk of age. The animals received 0.1 ml subcutaneously each day of either 5 μg (0.125 U) human neutral protamine hagedorn insulin (Eli Lilly & Co., Indianapolis, IN), 5 μg (0.125 U) insulin analog B25Asp in NPH diluent (16), or vehicle (hormone-free suspen-

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1. *Abbreviation used in this paper:* NOD, nonobese diabetic.

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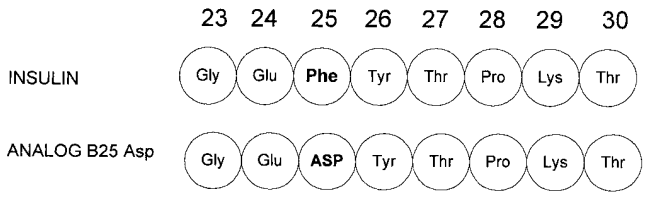


Figure 1. Primary structure of insulin analog B25Asp. The monomeric insulin analog is identical to human insulin in both the A chain and B chain, except for a single amino acid substitution of an aspartic acid for phenylalanine at position B25.

sion diluent fluid: 0.16% m-cresol, 0.065% phenol, 1.6% glycerin, 0.2% sodium phosphate, and water [Novo Nordisk Pharmaceuticals, Princeton, NJ]). All animals were evaluated for diabetes onset by monitoring blood glucose at 2–4-wk intervals. Insulin was withheld for 2 d to avoid masking diabetes onset by insulin therapy, and blood glucose was then monitored. In addition, the animals were monitored carefully when receiving their daily injections. If polyuria or weight loss was noted, blood glucose was evaluated. Any elevated blood glucose levels were confirmed by repeat analysis, with two consecutive blood glucoses > 16.7 mmol/liter (300 mg/dl) considered as IDDM onset. Diabetes-free survival was calculated using the product limit or Kaplan-Meier method with Prism software (GraphPAD Software for Science, San Diego, CA). Analysis of variance were performed using SigmaStat software (Jandel Scientific, San Rafael, CA).

Degree of insulitis. Randomly selected animals from each group were killed, and the pancreas of each was examined in a double-blind fashion for insulitis. Histological analysis of the pancreas was performed using the technique of Serreze et al. (18) with minor modifications. Pancreas tissue was fixed in formalin, stained with hematoxylin and eosin, and sectioned at three nonoverlapping levels. Six to ten islets per mouse were individually scored by two independent observers using a semiquantitative scale ranging from 0–4 as follows: 0, normal islet with no sign of T cell infiltration; 1, islet associated with perivascular, periductal leukocytic infiltration only; 2, more extensive periislet infiltration, but with lymphocytes with $< 25\%$ islet destruction; 3, $> 25\%$ islet destruction; and 4, complete islet destruction (18). An insulitis score for each mouse was obtained by dividing the total score for each mouse by the number of islets examined.

Results

In vitro studies demonstrate that the insulin analog, B25Asp, binds minimally to the insulin receptor as demonstrated by adipocyte receptor–binding assays (16, 19). To confirm that this analog was inactive in vivo, we evaluated the blood glucose response after subcutaneous administration of either the insulin analog or an equal dose of native human insulin in prediabetic NOD mice. As expected, B25Asp analog did not lower blood glucose, while the native insulin significantly reduced blood glucose levels for up to 4 h after subcutaneous injection compared to the hormone-free insulin diluent (Fig. 2 *A*). Serum insulin levels achieved by subcutaneous injection of 5 μg of the monomeric insulin analog or human NPH insulin were evaluated up to 8 h after injection (Fig. 2 *B*). Even though comparable serum levels of insulin and the analog were observed with a peak level occurring 2 h after injection (Fig. 2 *B*), the analog did not cause hypoglycemia (Fig. 2 *A*).

In previous studies we demonstrated that the daily subcutaneous injection of 5 μg of human insulin to young adult NOD mice significantly reduced the incidence of diabetes, even when initiated after the development of extensive insulitis (10). To test the hypothesis that insulin prevents diabetes by mechanisms not requiring binding to the insulin receptor, the ability of daily subcutaneous injections of insulin or B25Asp analog to prevent diabetes was evaluated in young adult female NOD mice (age 12 wk). Before initiating therapy, histological evaluation of representative mice confirmed that they had extensive lymphocytic infiltration of their islets at 12 wk of age (1.36 ± 0.87, $n = 4$). The onset of diabetes in the different treatment groups was ascertained by blood glucose monitoring (Fig. 3, *A–C*). In mice treated with vehicle alone, diabetes onset was noted as early as 14 wk, and mice continue to develop diabetes from ages 14–44 wk (Fig. 3 *A*). In contrast, there were fewer mice to develop diabetes in both the insulin and analog-treated mice (Figs. 3, *B* and *C*, Fig. 4). None of the insulin or

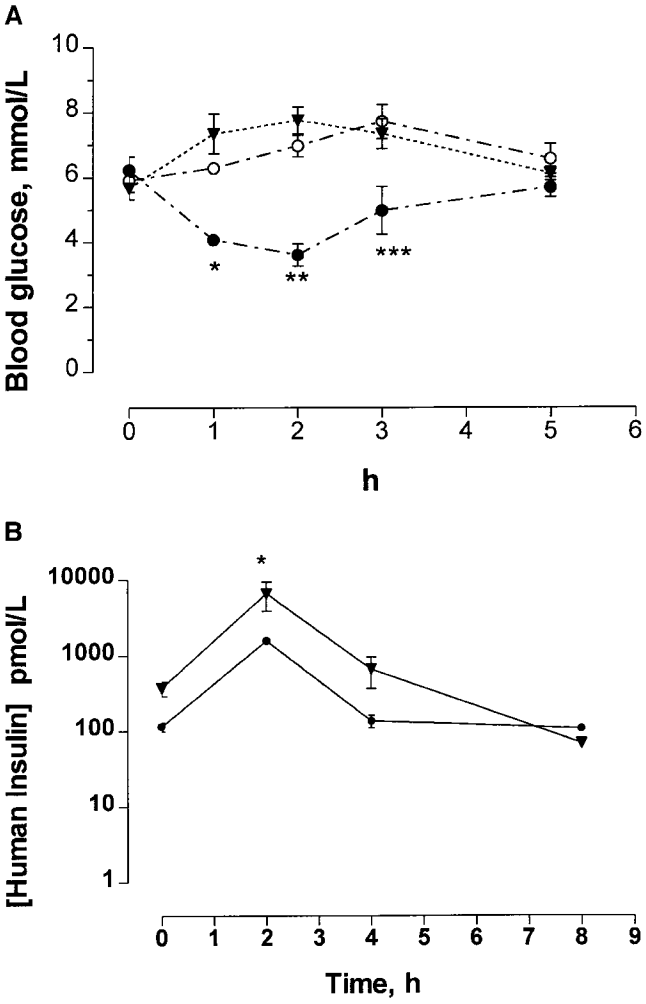
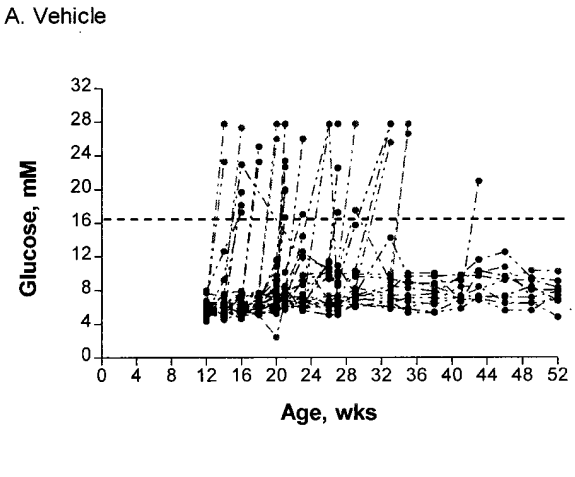
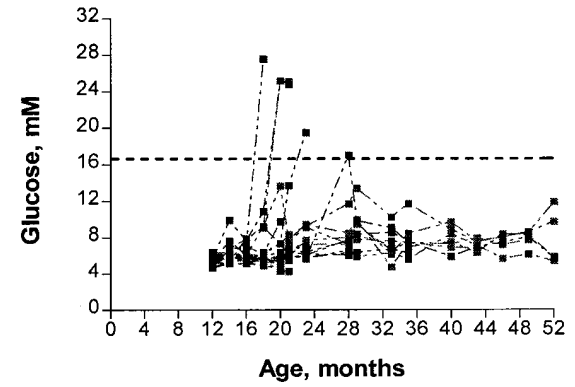


Figure 2. Blood glucose response to insulin analog and insulin. Nondiabetic nonobese diabetic mice were given subcutaneous injection of 5 μg of human insulin analog in NPH diluent (*closed upside down triangles*), intact human NPH insulin (*closed circles*), or hormone-free diluent (vehicle, *open circles*). (*A*) Blood glucose was monitored every hour after injection (mean±SEM). $*P = 0.029$ (1 h); $**P = < 0.001$ (2 h); $***P = 0.0034$ (3 h). (*B*) Exogenous insulin levels after subcutaneous administration of insulin or insulin analog analyzed using an ultrasensitive radioimmunoassay specific for human insulin (mean±SEM, $P = 0.418$).





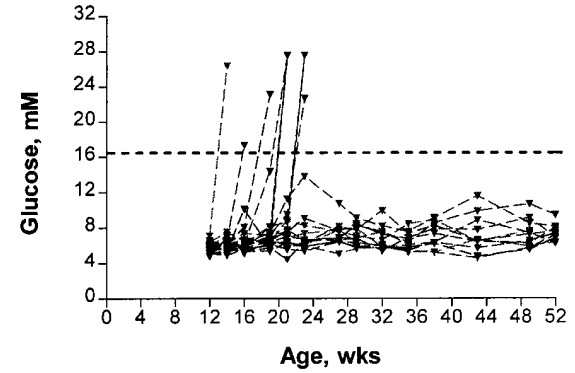


Figure 3. Diabetes onset in vehicle, insulin, or non-glucose-altering insulin analog–treated mice. Female nonobese diabetic mice were given daily injections of 5 μg of insulin analog B25 Asp (*closed upside down triangles*), insulin (*closed squares*), or vehicle (hormone-free diluent, *open circles*), beginning at 12 wk. Diabetes incidence was ascertained by monitoring blood glucose with onset of diabetes when blood glucoses exceeded 16.7 mM/liter (300 mg/dl).

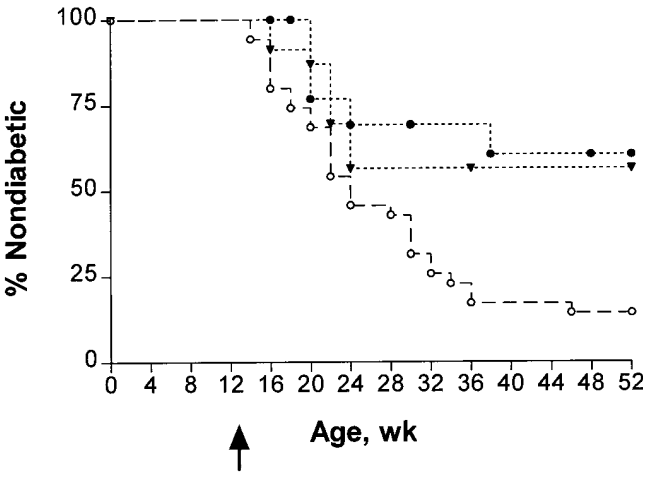


Figure 4. Protection from diabetes by daily subcutaneous injection of non-glucose-altering insulin analog or insulin. Female nonobese diabetic mice were given daily injections of 5 μg of insulin analog B25 Asp (*closed upside down triangles*), insulin (*closed squares*), or vehicle (hormone-free diluent, *open circles*) beginning at 12 wk. Diabetes-free survival was analyzed by the Kaplan-Meier method. Diabetes-free survival was significantly increased in both the analog and insulin-treated mice when compared with the vehicle control group.

analog-treated mice developed diabetes after 24–28 wk (Figs. 3, *B* and *C*, Fig. 4). By 52 wk, diabetes-free survival was significantly increased in both the analog and insulin-treated mice (57 and 61%, respectively) compared to only 14% in the vehicle-treated mice (Fig. 4 and Table I, insulin vs. vehicle, logrank test $P = 0.0073$, hazard ratio 3.39; analog-treated versus vehicle, logrank $P = 0.0066$, hazard ratio 2.59). Moreover, the non-glucose-altering analog was equally effective at preventing diabetes as was insulin (insulin vs. analog logrank test, $P = 0.7441$, hazard ratio 0.84).

We initiated therapy in adult mice with established insulitis. The insulin and analog therapy prevented diabetes, but did not decrease the degree of insulitis. The insulitis score in mice treated with vehicle was 2.1±1.0 (mean±SEM), with insulin, 1.1±0.7, and with analog, 2.1±1.3. There was a decrease in IL-2 receptor expression as assessed by binding of anti-CD25 antibody in insulin and analog-treated mice when compared with vehicle controls, suggesting that the treatments decreased the T cell activation.

Table I. Prevention of Diabetes in Adult NOD Mice

Treatment	Diabetes-free Survival	RR	Logrank Test
	%		*P*
Vehicle	14.3		
Insulin	60.6	3.39	0.0073
Analog	56.5	2.59	0.0066

Adult NOD mice treated with insulin or insulin-analog. Route: daily subcutaneous injection beginning at 12 wk with followup for > 52 wk. Survival proportions were calculated by the Kaplan-Meier method.

Discussion

Administration of insulin or an inactive insulin analog, altered by a single amino acid at position 25 of the B chain, was shown to prevent diabetes in young adult prediabetic mice. The biological properties of this insulin analog have been characterized by a series of in vitro studies by Drejer et al. and Brange et al. (17, 19). Using hepatoma cell–binding assays and free-fat cell bioassays, Drejer at al. demonstrate that the single amino acid alteration of the analog decreases the relative binding affinity to 0.05% for the insulin receptor, and to $< 0.005\%$ for the IGF-I receptor (17). They also show that there was a similar decrease in tyrosine kinase activation (0.2% for the analog vs. 100% for insulin) (17). These in vitro studies demonstrate the importance of the B25 position of the insulin B chain for binding to the insulin receptor. Substitutions of either Leu or Asp at position B25 were shown to dramatically reduce binding to the insulin receptor (16). This report demonstrated that the B25 Asp insulin analog also is inactive in vivo as shown by a lack of a hypoglycemic response after subcutaneous administration. This observation suggests that the hypoglycemic effects of insulin and the binding of insulin to its receptor or the IGF-I receptor are not necessary for diabetes prevention by insulin.

Previous studies in which native insulin was administered by an oral route also demonstrated a reduced incidence of diabetes (8, 20). The hypoglycemic effect of insulin, however, was presumed to be reduced due to partial degradation of insulin in the stomach, and subsequent absorption of insulin peptides (8). In one of these studies, the insulin-treated group continued to develop diabetes, but at a slower rate than the control group. Unfortunately, long-term followup of these animals was not available to ascertain if diabetes onset was only transiently delayed by the oral therapy (20). The advantage of our model is that both insulin and the analog were administered by the subcutaneous route, thus avoiding a second experimental variable of mucosal versus subcutaneous administration of antigen. In our studies, none of the insulin or analog-treated mice developed diabetes after 36 wk of age. We demonstrated that the subcutaneous administration of insulin analog reduced the incidence of diabetes as effectively as insulin without the causing of hypoglycemia. For this reason, this reagent could have therapeutic potential in humans. Immunization of NOD mice with metabolically inactive B chain (21) or insulin peptide B9-23 (7) in incomplete Freund's adjuvant also reduced the incidence of diabetes (7, 21), but these adjuvants are not suitable for use in humans. A potential advantage of the analog used in our studies over the B chain treatments is that the analog is an intact insulin molecule altered by only a single amino acid. Therefore, the analog contains both the A chain and B chain of insulin, which provides the opportunity to modulate responses to known T cell determinants on both the A chain (22, 23) and B chain (24, 25) of insulin.

It is unknown in the current clinical trials whether using subcutaneous insulin in prediabetic children and young adults will have any harmful effects due to repeated transient episodes of hypoglycemia. Our study with B25Asp human insulin analog demonstrated that it is possible to avoid hypoglycemia and still prevent diabetes in young adult prediabetic mice.

The mechanisms for diabetes prevention by insulin are currently unknown, but may include downregulation of islet beta cell antigens as a consequence of metabolic effects of insulin (11, 12), immune modulatory effects of insulin, induction of regulatory T cells, or tolerance by daily exposure to antigenic determinants of insulin, or alteration of lymphocyte function as a direct effect of insulin binding to insulin receptors on lymphocytes (14, 15). Evidence in support of metabolic mechanisms is provided by in vitro studies that demonstrate that islet antigen expression is decreased by lowering glucose concentration (11, 12). Immune modulatory mechanisms, however, are suggested by the demonstration that hyperinsulinemia reduces the number of insulin receptors on lymphocytes (15), and that the initiation of insulin therapy in type I diabetic patients decreases the number of activated T lymphocytes (14). From a mechanistic point of view, our data indicate that insulin prevents diabetes primarily by processes that are independent of the metabolic effects of insulin, and that insulin-specific immune mechanisms may be more important in insulin-induced prevention of diabetes. Finally, it is clear from these animal studies that inactive insulin analogs may prove invaluable agents for the prevention of type I diabetes in humans.

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