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Improved Efficacy of a Tolerizing DNA Vaccine for Reversal of Hyperglycemia through Enhancement of Gene Expression and Localization to Intracellular Sites

Nanette Solvason,† Ya-Ping Lou,† Wendy Peters,* Erica Evans,* Josefina Martinez,* Uriel Ramirez,* Andrea Ocampo,* Rui Yun,* Saira Ahmad,* Edwin Liu,† Liping Yu,† George Eisenbarth,† Michael Leviten,* Lawrence Steinman,‡ and Hideki Garren*  

Insulin is a major target for the autoimmune-mediated destruction of pancreatic β cells during the pathogenesis of type I diabetes. A plasmid DNA vaccine encoding mouse proinsulin II reduced the incidence of diabetes in a mouse model of type I diabetes when administered to hyperglycemic (therapeutic mode) or normoglycemic (prophylactic mode) NOD mice. Therapeutic administration of proinsulin DNA was accompanied by a rapid decrease in the number of insulin-specific IFN-γ-secreting T cells, whereas prophylactic treatment was accompanied by enhanced IFN-γ-secreting cells and a decrease in insulin autoantibodies. Adoptive transfer experiments demonstrated that the protection was not mediated by induction of CD25+CD4+ T regulatory cells. The efficacy of the DNA vaccine was enhanced by increasing the level of expression of the encoded Ag, more frequent dosing, increasing dose level, and localization of the protein product to the intracellular compartment. The efficacy data presented in this study demonstrate that Ag-specific plasmid DNA therapy is a viable strategy for preventing progression of type I diabetes and defines critical parameters of the dosing regimen that influences tolerance induction. The Journal of Immunology, 2008, 181: 8298 – 8307.

Type I diabetes (T1D) results from a T cell-mediated autoimmune destruction of insulin-producing β cells. Both genetic and environmental factors influence disease susceptibility and penetrance. The MHC contains the genetic elements that contribute to the greatest predisposition for susceptibility to T1D; however, certain haplotypes identified in both a murine model and T1D individuals confer a dominant protective effect (1, 2). The powerful influence of MHC on diabetes progression is most likely mediated at the level of thymic selection of autoreactive T cells for the islets, with the high- and low-risk alleles differentially impacting the efficiency of negative selection of autoantigen-specific T cells.

An indirect measure for the commonality of Ags driving T1D in the murine and human systems comes from a comprehensive comparison of crystal structures of class II molecules (3). These studies compared the high- and low-risk MHC alleles between species and reported similarities in the predicted peptide motifs both within and across species between T1D protective and predisposing class II molecules. These results suggest shared class II epitopes in the target autoantigen and support the notion that selection of islet Ag from the murine model has relevance to the human disease.

T lymphocytes recovered from the highly organized lymphocytic infiltrate around NOD islets have been used to identify multiple islet autoantigens. To date, however, insulin is the only islet Ag shown to be required for diabetes progression in NOD mice. A central role for insulin in T1D of NOD mice is demonstrated by an elegant series of targeted deletions of the individual insulin alleles (4–6). Furthermore, recent studies have demonstrated remission of diabetes in NOD mice using insulin-loaded APCs (7). Taken together, these results demonstrate that insulin is a required target in the ontogeny of autoimmune response to islet Ags and that tolerizing the immune response to insulin is sufficient to modify the disease process. These observations are consistent with the fact that an important genetic element contributing to disease susceptibility in T1D individuals is the insulin gene itself (8).

Both CD4 and CD8 reactivity to (pro)insulin have been demonstrated in the NOD mouse and in individuals with T1D (9–13). A well-characterized Ab response to insulin has also been demonstrated in prediabetic NOD mice and has been shown to identify animals within a colony that will develop diabetes most rapidly (14). Abs to insulin are also readily detectable in prediabetic individuals and are part of a constellation of autoantibodies used in prevention trials to identify HLA-susceptible individuals that are most likely to develop T1D in the next 5 years (14).

These observations on the importance of insulin as an autoantigen in animals and the parallels described in humans provided the rationale for several recent clinical trials using an Ag-specific therapeutic approach in which parenteral or oral insulin (15) was administered to at-risk, first-degree relatives of individuals with T1D. Although a delay to diabetes onset was detected in a subset of high-risk individuals in the oral insulin arm, the overall results from these trials failed to show a significant effect of insulin on the time to progression to diabetes onset. However, in the absence of
an assay to measure tolerance to insulin, the question remains whether the lack of efficacy: 1) is the result of insufficient dose escalation, or 2) challenges the pivotal role of insulin in driving the immune response in T1D individuals.

One method for inducing Ag-specific effects is via plasmid DNA (pDNA) technology. The advantages of using pDNA to deliver Ag-specific therapy include the following: 1) its purported ease of manufacture and purification; 2) its relative inexpensive production costs compared with recombinant molecules; 3) its durability for long-term expression of Ag; and 4) its ability to target reactivity to whole protein rather than being limited to specific epitopes. pDNA encoding GAD65 and insulin B chain have been shown to decrease diabetes onset in certain preventative settings when used in mouse models of T1D (16, 17). There has, however, been no systematic evaluation of the dosing and molecular parameters of the pDNA that contribute to efficacy.

In this study, we used NOD mice at various stages of disease to compare plasmids encoding distinct islet Ags implicated in the pathogenesis of T1D and identified pDNA-encoding proinsulin II as the most potent at ameliorating disease pathogenesis. Furthermore, we identified parameters of dosing, expression, and intracellular localization that contribute to efficacy. These studies were performed in prediabetic or hyperglycemic animals and formed the basis for the design of a multicenter phase I clinical trial testing pDNA encoding human proinsulin in individuals with a diagnosis of T1D, which began in October 2006.

Materials and Methods

Generation of proprietary CpG reduced plasmid backbone

To generate a plasmid backbone with decreased immune stimulatory activity, 12 CpG sequences were eliminated from the commercially available pVAX (Invitrogen) plasmid by converting the cytosine to guanosine. This CpG-reduced plasmid was named pBHT1.

Cloning of autoantigens

Autoantigens were all cloned into the mammalian expression vector, pBHT1, and contained the human CMV promoter, bovine growth hormone polyadenylation signal, kanamycin resistance gene, and 3′ UTR origin of replication. To generate the mouse preproinsulin 1 and preproinsulin II expression vectors, the coding sequences of each gene were PCR amplified from mouse pancreas cDNA (BD Clontech catalog 636206). A HindIII-NotI fragment containing the preproinsulin 2 coding sequence and a HindIII-XbaI fragment containing the preproinsulin 1 coding sequence were cloned into the corresponding sites of the pBHT1 vector. These vectors were named preproinsulin I-pBHT1 and preproinsulin II-pBHT1. A nonsecreted version of the preproinsulin II gene was constructed by removing the signal sequence of the gene. The primers Ins2.5.Eco (5′-ATTGAAATTCAAGATGCGTTTGGTCAGCAACACCTTGTG-3′) and Ins2.3.Xho (5′-ATTCTTGAGCAATGTGCAAGTTCTCCACGT-3′) were used to PCR amplify the nonsecreted version and digested for insertion into pBHT1. This new vector was named proinsulin II-pBHT1. A high expression form of preproinsulin II-pBHT1 and the proinsulin II-pBHT1 was made by cloning a HindIII-XbaI proinsulin II fragment into pBHT520, a pBHT1-derived vector containing the chimeric intron of pTarget. The new high expression vectors were called preproinsulin II Hi-pBHT1 and proinsulin II Hi-pBHT1.

Mice

NOD and NOD.scid female mice were purchased from The Jackson Laboratory. All animal procedures were performed in accordance with approved Bayhill Institutional Animal Care and Use Committee protocols. Animals were considered diabetic on the first of two consecutive readings of blood glucose (BG) >250 mg/dl (One Touch Ultra Strip Test) or two consecutive urine glucose (UG) readings of >300 mg/dl (Diastix; Bayer).

Immunizations

Mice were immunized in the footpads with 50 μg of B9-23 peptide emulsified in IFA. Draining lymph nodes (LN) were harvested 7–10 days later and analyzed by ELISPOT.

Antibodies

The 145-2C11 hamster mAb against mouse CD3 was ordered from American Type Culture Collection. The reagent was purified on protein G column, concentrated to 1 mg/ml, and filter sterilized. Endotoxin levels were measured by Limulus amebocyte lysate assay (ELIM Biopharmaceuticals) and were regularly less than 0.09 EU/ml. Insulin B9-23 peptide was synthesized at Princeton Biochemicals and purified by HPLC. Abs used in flow cytometry include rat anti-mouse CD4-PE (BD Biosciences), rat anti-mouse FoxP3 PerCP-CY5.5 (eBiosciences), and rat anti-mouse CD25 FITC (BD Biosciences).

DNA administration

In some experiments, animals received pretreatment with bupivicaine (100 μl of 0.25% in PBS; Sigma-Aldrich) by i.m. injection to quadriiceps 2 days before the first DNA injection to prepare muscle for subsequent DNA uptake. Unless otherwise indicated, animals received a total of 50 μg of DNA in a 200 μl vol (25 μg/100 μl to each quadriceps) at each treatment. DNA was administered by i.m. injection in the vehicle, PBS, with 0.9 mM CaCl₂. Experiments performed to address dosing frequency were performed at weekly (QW), biweekly (Q2W), or monthly (Q4W) dosing intervals.

Anti-CD3 administration

Anti-CD3 was administered at a 5-μg dose in 200 μl vol by i.v. injection for 5 consecutive days. After the fifth injection, no additional anti-CD3 was administered.

Test of DNA in hyperglycemic NOD mice

NOD animals between the ages of 12 and 22 wk were screened twice weekly to monitor BG levels. Animals were considered hyperglycemic when their BG levels were between 190 and 250 mg/dl and treated with bupivicaine, as described. Animals were retested 2 days later, and if the BG levels were greater than 170 mg/dl, but less than 300 mg/dl, the animals were allowed to remain in the study. DNA was administered at the frequencies indicated in the text. Animals were screened weekly for diabetes onset.

Test of DNA in insulin autoantibody (IAA)-positive NOD mice

IAA-positive, 8-wk-old NOD mice were randomized into treatment groups based on index score. Bupivicaine was not used in these experiments. Animals were treated with DNA at doses and frequencies described in the text. Animals were scored weekly for diabetes onset by UG. In one experiment (Fig. 6, D–F), sera were collected biweekly and analyzed for IAA, as described. In a separate experiment (Fig. 8), animals were dosed weekly for 6 mo, then rested for 4 mo. All NOD mice remaining normoglycemic were then immunized with the B9-23 peptide, followed by analysis in ELISPOT assays, as described.

Test of DNA in 5-wk-old NOD mice

Animals were dosed weekly with DNA, as described above, for 6 consecutive wk or with anti-CD3, as described above, for 5 consecutive days (Fig. 4, B and C). At 10 wk of age, animals were rested for 2 wk. At 12 wk of age, sera were harvested and pancreata prepared for histological evaluation, as described.

Adaptive transfer of DNA-treated CD4⁺ CD25⁺ Treg cells

Eight-week-old IAA-positive NOD mice were injected weekly for 8 wk with proinsulin II Hi-pBHT1 (25 μg/100 μl/quadriiceps), pBHT1, or PBS by i.m. administration. One week after the last injection, spleens were harvested, single-cell suspensions were prepared, and T regulatory (Treg) cells were purified using the MACS CD4⁺ CD25⁺ Treg isolation kit and an autoMACS separator, according to the manufacturer’s instructions (both from Miltenyi Biotec). For adaptive transfer, Treg cells (2.5 x 10⁵) were mixed with splenocytes (1 x 10⁶) isolated from a mouse of BG of >250 mg/dl (diabeticogenic splenocytes) and adoptively transferred by orbital vein injection (200 μl in PBS) into 8- to 12-wk-old NOD.scid mice. As positive control, Treg cells from 5-wk-old NOD mice were purified, as described, and coadministered with diabeticogenic splenocytes, as described above. One group received diabeticogenic splenocytes alone (no Treg cells were coadministered).

Insulitis

Pancreata were fixed in situ by gently inflating the peritoneal cavity with 10% buffered formalin. After 5 min, the pancreas was harvested and fixed
overnight in formalin. Tissue was embedded in paraffin and processed for H&E staining. Three levels of the pancreas were evaluated, allowing evaluation of >20 islets per animal. Insulitis was scored in a blinded fashion by a diabetes expert at the Barbara Davis Center for Childhood Diabetes. Insulitis was scored using a morphometric measurement and graded according to the extent of islet infiltration (0%, 1–25%, 26–50%, 51–75%, and >75%).

Statistical analysis

All statistical testing was performed using GraphPad Prism 4.0. For diabetes onset, log rank test was used. For ELISPOT results, Mann-Whitney test was performed.

Insulin autoantibody

Sera were screened in a blinded fashion at the Barbara Davis Center for Childhood Diabetes using a well-described RIA (14). IAA index value of greater than 0.01 was considered positive.

ELISPOT

ELISPOT kits were purchased from BD Biosciences. Experiments were done, according to the manufacturer’s recommendations. Briefly, plates were coated with anti-mouse IFN-γ Abs. One million lymphocytes were plated per well of a 96-well ELISPOT plate with appropriate peptide Ag at concentrations indicated in the text. Cells were incubated overnight (IFN-γ) or for 48 h (IL-10) and developed with appropriate capture Ab. Spots were visualized on an ELISPOT plate reader (Cellular Technology Limited) using Immunospot program.

Cell transfections and insulin ELISA

Cells from the human embryonic kidney cell line HEK293 were seeded in 6-well tissue culture plates (Cell Star Greiner Bio-one catalog no. 82050-842) in DMEM complete medium at a density of 1 × 10^6 cells/well and incubated at 37°C for 24 h. The cells were then transfected with pDNA using the TransIT DNA transfection reagent (Mirus Bio), according to the manufacturer’s protocol. In brief, 3 μl of TransIT reagent was mixed with 200 μl of serum-free DMEM and incubated for 30 min at room temperature. pDNA (0.5 μg) was then added to the Trans-IT-containing medium, and this mixture was incubated for an additional 30 min at room temperature. The DNA-TransIT mixture (200 μl) was then added to the cells, producing a total volume of 2000 μl. After 24 h, the proteasome inhibitor Lactacystin was added to wells at a final concentration of 5 μM, and this mixture was incubated for an additional 30 min at room temperature and then centrifuged in Eppendorf tubes for 2–3 min.

Protein levels in supernatant and cell lysate were analyzed using a commercial ELISA kit from Alpco Diagnostics (catalog 08-10-1149-01), according to the manufacturer’s instructions. ELISA plates were read using a SpectroMax spectrophotometer (Molecular Devices), and standard curve and protein concentration analysis were performed with the SoftMax Pro software.

Results

A noncoding DNA plasmid has no significant effect on TID disease parameters

The NOD mouse model of T1D is sensitive to stimulation through the innate immune system such that CFA, viral infections, and many other general immune stimulants can significantly decrease the incidence of T1D in a colony. To confirm that our proprietary pBHT1 vector alone was incapable of changing the course of T1D in NOD mice, a series of experiments were performed comparing a vehicle control group with animals treated with the pBHT1 empty plasmid vector (no autoantigen insert) (Table I). Diabetes onset was compared in hyperglycemic (BG between 190 and 250 mg/dl) NOD mice and normoglycemic NOD mice. No significant difference was found between vehicle-treated and pBHT1-treated experimental groups. pBHT1 was tested in 5-wk-old NOD mice for the ability to affect IAA development and insulitis, and was shown to have no significant effect on disease pathogenesis when compared with the vehicle-treated control group. Subsequently, pBHT1 was used as the expression vector expressing all autoantigen inserts, and all subsequent results reported in this work compare pDNA-encoding autoantigen with the vehicle control group.

Preproinsulin II, but not preproinsulin I-encoding plasmid provides significant delay in T1D onset in hyperglycemic mice

Identification of animals with BG levels between 190 and 250 mg/dl allows identification of animals immediately before diabetes onset. Indeed, within 1 wk of developing hyperglycemia using this scoring criteria, 50% of animals develop diabetes (see PBS-treated group, Fig. 1, dashed line). However, sufficient islet mass is present in hyperglycemic mice to allow return to normoglycemia if the autoimmune disease is sufficiently ablated, as demonstrated by administration of the well-described anti-CD3 mAb (Fig. 1, solid line). This is a rigorous method for screening novel therapeutics for the ability to delay T1D onset very late in the disease process, whereas animals, nonetheless, have residual β cell mass to restore normoglycemia if the autoimmune disease process is arrested.

Preproinsulin I (Ins 1) and preproinsulin II (Ins 2) vary by only 13/110 aa (Fig. 2A); however, they have distinct roles in the pathogenesis of T1D. Therefore, pDNAs encoding each preproinsulin gene were tested separately in hyperglycemic NOD mice, as described. As seen in Fig. 2, B and C, preproinsulin II, but not preproinsulin I, delayed diabetes onset when administered in a therapeutic setting.

Figure 1. Kinetics of diabetes onset in hyperglycemic NOD mice. BG levels from NOD female mice between the ages of 12 and 22 wk were measured twice weekly. Animals with BG levels between 190 and 250 mg/dl were scored as hyperglycemic and either enrolled in vehicle (PBS, n = 35) or received anti-CD3 mAb (C; n = 20). Animals were scored as diabetic on the first of two consecutive BG readings >250 mg/dl. The LogRank test was used for statistical analysis using Prism GraphPad. Data are pooled from three individual experiments.

Table I. Summary of effect of plasmid backbone on disease parameters

<table>
<thead>
<tr>
<th>T1D Disease Parameter</th>
<th>Vehicle</th>
<th>pBHT1*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes Onset (% diabetic)</td>
<td>88% (n = 25)</td>
<td>77% (n = 20)</td>
</tr>
<tr>
<td>Hyperglycemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-wk-old normoglycemic</td>
<td>71% (n = 20)</td>
<td>60% (n = 20)</td>
</tr>
<tr>
<td>IAA (% IAA positive)</td>
<td>70% (n = 20)</td>
<td>67% (n = 20)</td>
</tr>
<tr>
<td>Insulitis (% infiltration ≥ SEM)</td>
<td>45 ± 4 (n = 9)</td>
<td>42 ± 6 (n = 10)</td>
</tr>
</tbody>
</table>

* Plasmid backbone engineered to minimize CpG motifs (no autoantigen insert).

† Criterion for diabetes onset is two consecutive weekly BG readings >250 mg/dl.

‡ NOD animals enrolled in study when BG level was between 190 and 250 mg/dl independent of age. A total of 50 μg of pBHT1 or vehicle was dosed weekly by i.m. administration for 8 consecutive wk. Results shown are after 8 wk on study.

§ Ten-week-old NOD mice were treated with either 50 μg of pBHT1 or vehicle by i.m. administration biweekly through age 50 wk. Results shown are at 50-wk time point.

‖ IAA levels were determined in a blinded fashion after six weekly injections of either 50 μg of pBHT1 or vehicle by i.m. administration (dosing began at 5 wk of age).

¶ Index values >0.01 were scored as positive.

** Extent of insulitis was measured in a blinded fashion by a trained diabetologist. Morphometric analysis was used to determine the area of islet infiltrated with lymphocytes.
Modification of original preproinsulin II plasmid: subcellular targeting of proinsulin protein expression to the intracellular compartment

Because both CD4 and CD8 T cell reactivity toward insulin have been described, tolerance induction to insulin would almost certainly require silencing both cell types. To this end, a plasmid containing the sequence for proinsulin II was constructed to preferentially deliver the proinsulin protein to the intracellular compartment, thereby targeting the processed peptide fragments to the MHC class I pathway. To target the protein product to the intracellular compartment, the signal sequence was removed from the preproinsulin II sequence, resulting in a new plasmid encoding proinsulin II.

To confirm the distinct intracellular localization of the proinsulin II protein encoded by the preproinsulin II and proinsulin II plasmid, in vitro transfection experiments were performed. Transfection of HEK cells confirmed the distinct localization of the nascent protein products in the cellular compartments as predicted based on the presence or absence of the signal sequence. The protein product of the proinsulin II DNA was detected in both the supernatant (hatched) and lysate (solid), consistent with entry of the protein into the secretory pathway (Fig. 3A). In contrast, the proinsulin protein product of the proinsulin II DNA was undetectable in the supernatant (data not shown), but was present in the cellular lysate if the proteasome inhibitor, Lactacystin (Fig. 3B), was added during the transfection. The protein product of the preproinsulin II DNA is referred to as proinsulin IIsec, whereas the product of the proinsulin II DNA is termed proinsulin IIPsy.

In other systems using pDNA to enhance immunological responses, a key parameter affecting efficacy is expression level of the protein product (18, 19). To evaluate expression level as it relates to suppression of the immune response, high-expressing variants of the preproinsulin and proinsulin DNA plasmids were constructed. The new plasmids contained a chimeric intron upstream of the preproinsulin or proinsulin gene sequence. This chimeric intron is composed of the 5′/H11032 donor splice site from the first intron of the human /H9252-globin gene and the branch and 3′/H11032 acceptor splice site from the intron of an Ig gene H chain V region (20). In other model systems, this intron has been shown to enhance gene expression by 3- to 20-fold (21, 22). Enhanced expression of the proinsulin protein was confirmed in both plasmids (see asterisks). The plasmids containing the chimeric intron enhanced proinsulin protein levels from both plasmids by 2- to 3-fold.

Intracytoplasmic localization and higher expression levels dramatically enhance efficacy

Comparison of the four distinct plasmids, preproinsulin II-pBHT1, proinsulin II-pBHT1, preproinsulin II HI-pBHT1, and proinsulin II HI-pBHT1.
HI-pBHT1, was performed in hyperglycemic NOD mice (Fig. 4A). There was a clear rank order in the potency of the pDNA in protecting from diabetes onset (preproinsulin II / Hi / preproinsulin II / Hi). Targeting of the proinsulin protein product to the cytoplasm proved to be the best strategy for delaying diabetes onset. Furthermore, in both plasmids, the higher expression vector provided more robust protection.

Further testing of these plasmids was done in 5-wk-old normoglycemic NOD mice, with animals being treated weekly with DNA for a total of six injections. Two weeks after the final injection, sera were harvested and pancreata collected for blinded analysis of IAA and insulitis. Treatment with the original proinsulin II DNA significantly decreased the frequency of mice that developed IAA (Fig. 4B). In contrast, neither preproinsulin II plasmids had any effect on IAA development. Histological evaluation of the pancreata using morphometric analysis to determine the extent of islet infiltration also showed a significant decrease in the severity of insulitis (Fig. 4C) following treatment with the proinsulin II pBHT1 (p = 0.04 when compared with vehicle-treated mice), but not the preproinsulin II-encoding plasmids. Therefore, targeting the protein product to the cytoplasmic compartment was essential in impacting multiple disease endpoints in younger mice. The proinsulin II Hi-pBHT1 was not tested in 5-wk-old normoglycemic NOD mice.

Dose-finding studies

To further evaluate the two proinsulin II-encoding plasmids and to understand the impact of higher expression level on potency at distinct dosing frequencies, the proinsulin II-pBHT1 and proinsulin II Hi-pBHT1 were retested in direct comparison in hyperglycemic NOD mice. A dose frequency experiment was performed with each plasmid comparing weekly (QW), biweekly (Q2W), and monthly (Q4W) DNA dosing. For these experiments, anti-CD3 was used for comparison. DNA dosing was stopped after the eighth week in the study, but mice were continuously monitored until week 25. As shown in Fig. 5, A and B, and Table II, treatment with both of the DNAs provided statistically significant delay in T1D onset; however, the proinsulin II Hi-pBHT1 provided the...
most robust protection, confirming that enhanced protein expression correlates with improved efficacy. Importantly, the effect of the DNA on diabetes progression was long lasting, because there was no significant change in percentage of animals with T1D after DNA treatment was stopped at week 8. Furthermore, the insertion of the chimeric intron upstream of the proinsulin II gene and subsequent enhancement of proinsulin protein expression was sufficient to provide protection from T1D onset comparable to anti-CD3 treatment.

Another feature of the protection afforded by the proinsulin II Hi DNA-treated animals was the return to normoglycemia. Therefore, the animals did not stay in a hyperglycemic state, but rather reverted to a normal level of BG. The effect of restoring normoglycemia was seen in the anti-CD3-protected animals as well. Of the proinsulin II Hi-pBHT1 weekly protected mice (Fig. 5D), all returned to normoglycemic levels. Of the proinsulin II Hi-pBHT1 weekly protected mice (Fig. 5E), six of nine mice returned to normoglycemic levels.

Because the proinsulin II Hi-pBHT1 clearly provided the most robust delay in T1D onset, a dose-level experiment was conducted comparing biweekly dosing frequencies at 2, 10, and 50 μg dose levels. A dose-level response was observed in delaying diabetes onset such that the 50 μg dose level provides the most protection ($p < 0.01$), whereas the 10 μg dose is minimally effective ($p = 0.08$). The dose of 2 μg provides no protection ($p = 0.72$) (data not shown).

**Mechanism: immediate effect of proinsulin II DNA is to decrease frequency of insulin B9-23 and B15-23 IFN-γ-producing T cells and IAA levels**

To address the mechanism of action in therapeutic settings, the insulin T cell responses were measured following proinsulin II DNA administration in hyperglycemic NOD mice. As controls, five hyperglycemic and five normoglycemic NOD mice (both at 16 wk of age) received a single i.m. injection of PBS, followed in 1 wk by immunization with a 50 μg preparation of B9-23 peptide in IFA (this sequence also contains the 15-23 epitope). Seven days later, draining LN were harvested and stimulated in vitro with B9-23 peptide or B15-23 peptide, and specific IFN-γ responses were measured. As seen in Fig. 6A, hyperglycemic NOD mice have higher B9-23 responses than normoglycemic NOD mice of the same age ($p = 0.03$; compare open histogram with solid histogram), whereas the B15-23 (Fig. 6B) response is similar between the two groups, albeit significantly lower than the B9-23 response. A third group of age-matched hyperglycemic mice received a single i.m. injection of proinsulin II DNA (Fig. 6A, B, and D, striped histogram), followed by immunization with B9-23, as described. Administration of a single injection of proinsulin II DNA to hyperglycemic mice significantly decreases the number of B9-23-specific, IFN-γ-producing cells compared with the hyperglycemic PBS control mice ($p = 0.02$). Furthermore, the proinsulin DNA-treated mice showed a trend toward lower B15-23-specific

### Table II. Statistical analysis of dose frequency comparison

<table>
<thead>
<tr>
<th>Dosing Frequency</th>
<th>Proinsulin II DNA Value</th>
<th>$n$</th>
<th>No. of Weeks Followed</th>
<th>Proinsulin II Hi DNA Value</th>
<th>$n$</th>
<th>No. of Weeks Followed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weekly</td>
<td>0.004</td>
<td>10</td>
<td>25</td>
<td>&lt;0.0001</td>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td>Every 2 wk</td>
<td>0.018</td>
<td>10</td>
<td>25</td>
<td>&lt;0.0001</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Every 4 wk</td>
<td>0.0026</td>
<td>10</td>
<td>25</td>
<td>0.03</td>
<td>17</td>
<td>25</td>
</tr>
</tbody>
</table>

*Statistical analysis generated by comparing with vehicle control using LogRank test in Prism GraphPad 4.
IFN-γ-producing cells compared with the hyperglycemic control PBS-treated mice (p = 0.07).

To evaluate the effects of proinsulin DNA on the humoral response to insulin, 8-wk-old IAA⁺ NOD mice were treated with proinsulin II DNA and monitored for changes in IAA, as revealed by IAA index scores (Fig. 6, D–F). IAA⁺ NOD mice were randomized between experimental groups based on their IAA index score so that insulin reactivity was comparable at treatment onset. Three groups were compared and included mice treated with the following: 1) 10 µg of proinsulin II pBHT1; 2) 50 µg of proinsulin II Hi-pBHT1; or 3) PBS all administered by weekly i.m. injections. Animals were also monitored weekly for diabetes onset (Fig. 6C). Sera were collected every other week while on study, and the change in IAA was measured in a blinded fashion. As seen in Fig. 6C, only the 50 µg dose level provides significant protection from diabetes onset (○). No protection was observed at the 10 µg dose level (□). The changes in IAA index values are shown for each time point. Results are shown separately for vehicle-treated (D), 10 µg (E), and 50 µg (F) proinsulin Hi-pBHT1-treated mice.

**FIGURE 6.** Proinsulin II DNA effect on IAA and T cell response. A and B, Sixteen-week-old NOD mice were identified that were either normoglycemic (NG; n = 5) or hyperglycemic (HG; n = 10). The normoglycemic NOD mice were treated with PBS. One group of hyperglycemic NOD mice (n = 5) was treated with PBS, and the second group of hyperglycemic NOD mice (n = 5) was treated with a single injection of proinsulin II DNA. Seven days after the DNA administration, all animals were immunized with 50 µg of B9-23 peptide emulsified in IFA. Draining LN were harvested 7 days later and stimulated in vitro with B9-23 or B15-23 peptide in an ELISPOT assay. IFN-γ-secreting peptide-specific cells were quantified using a commercially available ELISPOT kit. Bars indicated the average ± SEM. C–F, Eight-week-old IAA⁺ normoglycemic were randomized between treatment groups based on the IAA index score. Mice were treated weekly with vehicle, proinsulin II Hi DNA at 10 µg/mouse, or proinsulin II Hi DNA at 50 µg/mouse by i.m. administration (n = 22 per group). C, Animals were scored weekly for diabetes onset using UG levels (vehicle, ■; 10 µg of DNA, ○; 50 µg of DNA, □). D–F, Sera were collected every 2 wk and monitored to determine the change in IAA over time. Average IAA index values ± SEM are shown for each time point. Results are shown separately for vehicle-treated (D), 10 µg (E), and 50 µg (F) proinsulin Hi-pBHT1-treated mice.
Mechanism: protection is not associated with induction of CD25+/CD4+ Treg cells

To determine whether Treg cells are induced by proinsulin II Hi-DNA and are responsible for the protection from diabetes progression, CD25+/CD4+ cells (Treg) were purified from proinsulin II Hi-pBHT1-treated NOD mice and adoptively transferred into NOD.scid mice along with diabetogenic splenocytes. IAA+ 8-wk-old NOD mice were administered 50 μg of proinsulin II Hi-pBHT1 weekly by i.m. administration for a total of eight injections. One week after the last injection, splenocytes were harvested and CD25+/CD4+ Treg cells were purified using magnetic beads. Treg cells were coadministered with splenocytes from recently diabetic NOD mice. There was no difference in diabetes onset in mice that received Treg cells from the proinsulin II Hi-pBHT1-treated mice plus diabetogenic cells compared with mice receiving diabetogenic cells alone (Fig. 7A). In contrast, mice that received diabetogenic cells plus Treg cells purified from 5-wk-old NOD mice, a source rich in functionally active Treg activity, were significantly protected from diabetes onset (Fig. 7B).

Mechanism: long-term dosing with proinsulin Hi-DNA induces Ag-specific IFN-γ- and IL-10-producing cells

A second experiment was performed in IAA-positive normoglycemic NOD mice to phenotypically characterize the insulin-specific response in protected animals. Eight-week-old IAA+ mice were dosed weekly with 50 μg of proinsulin Hi-pBHT1, the empty vector, pBHT1 (50 μg), or vehicle (PBS) by i.m. administration. Mice were dosed repeatedly for 6 mo and then left untreated for an additional 4 mo. To complement the adoptive transfer experiments described above, we initially performed FACS analysis on the islet-infiltrating T cells from three normoglycemic NOD mice in the pBHT1 and proinsulin DNA-treated mice to determine whether there was any evidence for an enhancement in the levels of FoxP3 CD4+ T cells associated with proinsulin DNA-mediated protection. On the contrary, the average percentage of islet-infiltrating FoxP3+ CD4+ T cells in the pBHT1-treated mice was higher than that in the proinsulin DNA-treated mice (3.67 ± 0.28% vs 5.03 ± 0.5%, respectively).

To phenotype the insulin-specific immune response after protection from diabetes onset, all animals that remained nondiabetic for the course of the experiment were immunized with B9-23 peptide in IFA. Draining LN were harvested 7 days later and restimulated in vitro with the B9-23 peptide, and the frequency of IL-10- and IFN-γ-secreting cells was quantified. As seen in Fig. 8, there is increase in insulin-specific IL-10- and IFN-γ-secreting cells in the proinsulin Hi DNA-treated mice. It is important to stress that the PBS- and empty vector-treated animals in Fig. 8 were nondiabetic; therefore, the induction of IL-10 and IFN-γ is not simply the consequence of failure to progress to diabetes, but is dependent on the proinsulin Hi DNA treatment.

Discussion

Insulin is a required autoantigen in the ontogeny of the immune response to islets (5). An Ag-specific immunomodulatory therapy that down-regulates insulin autoreactivity could provide a significant step forward in the treatment of T1D. The specific strategy used in this study used cloning both allelic variants of insulin (preproinsulin I and preproinsulin II) into a bacterial plasmid and measuring the impact on multiple parameters of disease after DNA administration. DNA-encoding preproinsulin I had no effect on disease progression, whereas the preproinsulin II DNA delayed diabetes onset consistent with reports from other laboratories using pDNA-encoding elements of the preproinsulin II allele (23, 24). Although the two alleles are 88% identical at the amino acid level, the fact that they elicit different effects in our experiments is not surprising, because they have been clearly shown to have distinct roles in diabetes pathogenesis (3, 4, 10, 25, 26).

A second proinsulin II-encoding plasmid, proinsulin II-pBHT1, was derived from the original preproinsulin II pBHT1 by deleting the leader sequence. This modification encoded a proinsulin II protein (proinsulin<sup>295</sup>) that was sequestered into the intracytoplasmic compartment, but not secreted. This was in contrast to the protein product of the preproinsulin II pBHT1 construct that encoded a proinsulin II protein (proinsulin<sup>295</sup>) that was readily secreted and detected in supernatant after in vitro transfections. Enhanced efficacy was provided by the cytoplasmically targeted proinsulin protein in both hyperglycemic and normoglycemic NOD mice with the proinsulin II pBHT1 decreasing IAA production, the extent of insulitis, and providing the most robust protection from diabetes onset. In our studies, we initially predicted a strict targeting of proinsulin-specific CD8 T cells by the proinsulin II pBHT1 plasmid because of the intracellular localization of the protein product and subsequent Ag presentation mediated by the endogenous or exogenous pathways (cross presentation) (reviewed in Refs. 27 and 28). However, the proinsulin<sup>295</sup> protein clearly modified the B9-23 CD4 T cell response to insulin as well as the Ab response. Direct MHC class II presentation of endogenous peptides has been described (29, 30); therefore, direct transfection of APCs with the proinsulin II plasmid could result in loading of class II with proinsulin peptides. Furthermore, tolerance induction to Ag-specific CD4 T cells can be mediated by cross presentation of the Ag by bone marrow-derived cells (31). In studies not described in this work, expression of proinsulin RNA could be detected at the injection site for 7 days after DNA administration. Therefore, at least conceptually, the normal turnover of muscle and/or skin at the injection site could be a reservoir of proinsulin available for cross
presentation. In the absence of costimulation mediated by the innate immune system, immature APCs that cross present Ag give a strong negative or tolerizing signal to any Ag-specific T effector cell (27, 28), a process speculated as one of the mechanisms of maintaining peripheral tolerance (32).

Appropriate levels of Ag expression are important for efficient priming of an immune response (33) as well as tolerance induction. A chimeric intron placed upstream of the proinsulin II DNA enhanced in vitro protein expression by ~2- to 3-fold and provided a significant enhancement of the in vivo potency of the DNA in blocking T1D onset in hyperglycemic NOD mice. Further evidence for the importance of proper Ag dose were the results from dose level experiments showing that higher amounts of DNA provided more robust protection from disease onset. These results are consistent with the notion that some optimal level of MHC/peptide loading is required for T cells to “see” the Ag, whereas other properties of the Ag, the extent of costimulation, and cytokine milieu determine whether activation or tolerance is the final outcome.

Dosing frequency influences priming and tolerance induction in different ways. In experiments described in this study and in reports from others (34), a persistent expression level of self Ag is required to efficiently blunt an ongoing immune response. Interestingly, in contrast, a key parameter to the induction of a robust Ab response is the inclusion of long intervals between DNA injections (35). This may explain a published report that appears to be in conflict with data shown in this study, in which DNA-encoding proinsulin exacerbated T1D (36). In that report, DNA was dosed infrequently once every several months. Thus, infrequent dosing with long rest intervals or single DNA administrations may cause immunization rather than tolerization and thus enhance the immune response. Indeed, a critical parameter affecting the nature of the immune response to an Ag (activation vs tolerance) is persistence of Ag (37).

In our model system, tolerance induction in response to DNA treatment is not mediated by induction of CD25+/CD4+ Treg cells. This was clearly demonstrated in adoptive transfer experiments in which Treg cells isolated from DNA-treated mice provided no delay in diabetes onset when coadministered with dba1 genetic splenocytes. Furthermore, direct evaluation of islet-infiltrating lymphocytes in NOD mice that had been protected from diabetes onset by repeated proinsulin DNA administrations demonstrated no increase in FoxP3+ CD4 T cells, a marker routinely used to identify CD25+/CD4+ Treg cells. These results are in contrast to other laboratories using autoantigen-encoding pDNA technology to tolerize NOD mice to islet Ags in which protection from diabetes onset could be adoptively transferred to recipient mice (23, 38) or direct visualization of Treg cells was reported (39). Given the differences in the model systems, constructs, and experimental designs, distinct mechanisms may account for tolerance induction in each study.

The protection from diabetes onset in our animals was accomplished by induction of Ag-specific IL-10-secreting T cells. The induction of Ag-specific IL-10-secreting cells is consistent with a body of data describing a Treg cell subset distinct from the Foxp3 CD25+ CD4+ cells (40). These cells, termed Tr1 cells, are induced in response to repeated antigenic stimulation (41, 42). If the B9-23-specific IL-10-secreting cells have any Treg cell activity, we were not able to detect it in our adoptive transfer experiments. Other groups studying Tr1 cell induction in NOD mice after peptide administration using GAD65 as the model system have been successful at adoptively transferring protection; however, the GAD-specific Tr1 cells were purged before transfer using a tetramer specific for this T cell specificity (43). Thus, further enrichment of the B9-23 IL-10-producing cells may be necessary to evaluate the regulatory function of these cells in adoptive transfer experiments.

It remains an open question as to the primary T cell target of autoantigen-encoded pDNA treatment, that is, whether quiescent or activated, naive or memory, or CD4 or CD8 T cells are more heavily influenced by this type of therapy. It may be of significance that DNA treatment administered therapeutically (to hyperglycemic NOD mice) provided more robust protection (p = 0.001) than when dosing started in normoglycemic NOD mice (p = 0.05). One explanation may be that activated effector cells present at the time of diabetes onset are the most sensitive to tolerance induction by this treatment. Proinsulin DNA can modify disease onset when administered early in disease as well, but this may be more heavily dependent on its ability to influence naive Th1/Th2/Tr1 cell differentiation. Indeed, in our own experiments, protection from diabetes onset was accompanied by opposite phenotypes involving IFN-γ. We observed a significant decrease in the number of B9-23-specific IFN-γ-producing cells immediately after proinsulin DNA administration and induction of B9-23 IFN-γ (and IL-10)-producing cells after long-term protection from diabetes onset. Although not directly demonstrated in this study, our current hypothesis is that at least some component of the protection mediated by proinsulin DNA is mediated via the pleiotropic effects of IFN-γ during the pathogenesis of autoimmune disease (44–47). These questions provide the foundation for further investigations on the mechanism of action of tolerizing DNA vaccines for T1D.

Disclosures

Current employees of Bayhill Therapeutics are: Nanette Solvason, Ya-Ping Lou, Erica Evans, Wendy Peters, Josefina Martinez, Uriel Ramirez, Andrea Ocampo, Michael Leviten, and Hideki Garren. Founders of Bayhill Therapeutics are Hideki Garren and Lawrence Steinman. George Eisenbarth is a consultant for Bayhill Therapeutics.

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