Clinical optimization of antigen specific modulation of type 1 diabetes with the plasmid DNA platform

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Abstract Some clinical trials in humans have aimed at modulation of type 1 diabetes (T1D) via alteration of the immune response to putative islet cell antigens, particularly proinsulin and insulin, glutamic acid decarboxylase and the peptide, DiaPep 277, derived from heat shock protein 60. The focus here is on development of a specially engineered DNA plasmid encoding proinsulin to treat T1D. The plasmid is engineered to turn off adaptive immunity to proinsulin. This approach yielded exciting results in a randomized placebo controlled trial in 80 adult patients with T1D (1). The implications of this trial are explored in regards to the potential for sparing inflammation in islets and thus allowing the functioning beta cells to recover and produce more insulin. Strategies to further strengthen the effects seen thus far with the tolerizing DNA plasmid to proinsulin will be elucidated. The DNA platform affords an opportunity for easy modifications. In addition standard exploration of dose levels, route of administration and frequency of dose are practical. Optimization of the effects seen to date on C-peptide and on depletion of proinsulin specific CD8 T cells are feasible, with expected concomitant improvement in other parameters like hemoglobin A1c and reduction in insulin usage. T1D is one of the few autoimmune conditions where antigen specific therapy can be achieved, provided the approach is tested intelligently. Tolerizing DNA vaccines to proinsulin and other islet cell autoantigens is a worthy pursuit to potentially treat, prevent and to perhaps even 'cure' or 'prevent' type 1 diabetes.

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3.1. Optimization strategy 1: Use of plasmids encoding multiple autoantigens or multiple plasmids encoding different autoantigens

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We know that even if type 1 diabetes starts with autoimmunity to one of these islet autoantigens, that there is likely to be epitope spreading, and that it may require a multipronged approach to ‘reverse’ autoimmunity to multiple antigens. When the team at Tolerion, then known as Bayhill Therapeutics, first embarked on antigen specific modulation of autoimmunity with DNA vaccines thirteen years ago, the idea was to personalize the approach. Thus, if an individual had autoimmunity to antigen X and Z, then we would counter with tolerizing plasmids to X and to Z. If another individual had autoimmunity to antigen X and Y, then that individual would receive DNA plasmids to X and Y.

In a paper published in *Nature Biotechnology* in 2003, we showed that we could reduce epitope spreading of the immune response when experimental autoimmune encephalomyelitis (EAE) was induced with different antigens [16]. We first used myelin autoantibody arrays to show the extent of epitope spreading in EAE. We then used four individual DNA plasmids each encoding one of the four major myelin proteins—myelin basic protein, myelin oligodendrocyte glycoprotein, myelin associated glycoprotein and proteolipid protein in this strategy to reduce epitope spreading and reduce the relapse rate seen in EAE.

We demonstrated extensive epitope spreading using autoantibody arrays when EAE was induced with a variety of antigens, either peptides from myelin basic protein p85–99, or peptides from proteolipid protein p139–151, or from a homogenate of mouse spinal cord (Fig. 4).

Using this strategy we were able to reduce the extensive epitope spreading to multiple myelin antigens seen in EAE,
with a cocktail of four plasmids, each encoding one of the major myelin proteins, myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), or myelin associated glycoprotein (MAG) (Fig. 5).

Use of the multiple plasmids reduced epitope spreading and reduced the rate of relapse. Addition of another plasmid encoding IL-4 bolstered the effect (Fig. 6).

In trials in T1D it would be worthwhile to consider adding plasmids to other islet antigens especially if assays for islet cell antibodies reveal that an individual is already responding to antigens other than or in addition to proinsulin. In the trial we reported entry criteria including antibodies to insulin, GAD or IA-2 determined by radioimmunoassay [1]. The opportunity to match tolerizing plasmids to the antibodies actually present in patients would be a logical way to try to induce tolerance to those unwanted adaptive responses to islet antigens.

We review here our previous experience with DNA vaccine to myelin basic protein in relapsing remitting multiple sclerosis. We focus on observations indicating epitope spreading of tolerance, the so called ‘spreading tolerance’. We also comment on the durability of the tolerogenic response. Though our intention was to use multiple plasmids encoding antigens that were targeted by the autoimmune response in MS, the FDA reasonably wanted us to start with one antigen. We chose to engineer a plasmid encoding myelin basic protein. We eliminated stimulatory CpG motifs and replaced them with GpG motifs, then compete with CpG for binding to TLR9 [17]. In phase 1–2 trials in patients with relapsing remitting MS and with secondary progressive MS, we were able to demonstrate reduction of Th1 responses in myelin basic protein reactive T cells using a technique involving flow cytometry and dye dilution. We did not use antigen loaded MHC molecules in this study. We saw that the reduction in gamma interferon responses to myelin basic protein was durable and continued for the remainder of the year after four doses given at two week intervals for nine weeks. There was evidence of spreading tolerance as well, with reductions in antigen specific T cell responses to PLP as well (Fig. 7).

In the phase 2 trial we gave biweekly injections of the DNA plasmid to myelin basic protein on weeks, 0, 2 and 4 and then monthly thereafter for 11 months. There was again evidence of spreading tolerance with reduction of antibody responses in the CSF from week 0 to week 48 across a swathe of autoantigens including antibodies to myelin basic protein, but also to MOG and to PLP [19]. The most pronounced reductions were seen in individuals with the highest titers of anti-myelin basic protein antibodies in their CSF at baseline [19]. The effect was seen at the 0.5 mg dose of plasmid, but not in the placebo group or in the group dosed with the plasmid at 1.5 mg. Such reductions were also noted in the CSF in phase 1–2 trials [18]. In the phase 2 trial antigen specific T cell assays were not undertaken with either multimers or with dye dilution techniques.

One might inquire why we saw the durability of the response after four doses every other week, seen at one year with the DNA plasmid encoding myelin basic protein in the MS trial, while we did not see a response sustained beyond 6 months in the T1 diabetes trial with the plasmid encoding proinsulin. One significant difference of course is that in type 1 diabetes the individual with this disease is injecting insulin repeatedly during the day every day. This is of course not the case in MS, where to be explicit no one is injecting myelin basic protein every day of their life with relapsing remitting MS.

3.2. Optimization strategy 2: Addition of more non-coding GpG motifs either in the plasmid backbone or as free-standing oligomers

One of the features of the tolerizing plasmids is the use of a non-coding immune suppressive motif known as the GpG hexanucleotide. In our plasmids that we have taken into the clinic, we have reduced CpG usage, which is immune stimulatory and replaced the CpGs with GpGs. The GpG motif represents a single base switch from a stimulatory CpG to GpG. The GpG hexanucleotide can effectively inhibit the activation of Th1 T cells associated with autoimmune disease. The GpG motif competes for binding to TLR9 with immunostimulatory CpG motifs [13,17,20].

In experiments with the cocktail of plasmids encoding multiple myelin proteins to treat EAE, addition of GpG
oligonucleotides increased the magnitude of the clinical effect on severity of paralysis and dramatically reduced epitope spreading. This was seen most clearly in experiments where the cocktail of tolerizing plasmids had an additional plasmid encoding IL-4. Combination therapy with the cocktail of myelin DNA plasmids along with a DNA plasmid for IL-4 plasmid DNA vaccine increased efficacy. Addition of GpG-ODN further reduced myelin-specific autoreactive B cell diversity and

Figure 3  Antigen-specific CD8+ T cells were enumerated with Qdot multimer technology using class I HLA multimers loaded with various antigens [21–24]. Antigen and HLA haplotype are shown in each panel. CTL frequencies are defined as percentage of antigen-specific CD8+ T cells. Changes in CTL from baseline to week 15 are shown on the y axis, and percent change in C-peptide from baseline to week 15 is shown on the x axis. Changes in CTL were calculated by subtracting the baseline values from values at week 15 (A and B). Analysis was performed on all treated (0.3 mg: diamonds, 1 mg: triangles, 3 mg: squares, 6 mg: circles) and placebo patients positive for HLA-A2, HLA-A3, and/or HLA-B7 (A) and for control antigens (B). Statistics were performed with linear regression analysis. AUC, area under the curve [1].

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shifted the immune response toward a Th2 isotype (Figs. 8, 9) [23].

3.3. Optimization strategy 3: Addition of genes encoding 'suppressive cytokines' to the DNA vaccine

von Herrath and colleagues first showed that co-immunization with an IL-4-expressing DNA plasmid encoding GAD reduced the risk of augmenting autoaggression and increased the safety margin of DNA based therapy [14]. In von Herrath's work with a DNA plasmid encoding the insulin B chain, the critical role of IL-4 was observed for the Ag-specific protective effect. In female IL-4−/− NOD mice plasmid DNA vaccination to proinsulin did not protect from T1D [21].

Garren and colleagues showed that co-administration of an IL-4 containing plasmid along with a plasmid encoding MOG lead to reversal of EAE. IL-4 expression caused the immune response to be driven toward more Th2-like phenotype. The plasmid expressing IL-4 acts locally on autoreactive T cells via activation of STAT6 [22]. Addition of plasmids encoding suppressive cytokines like IL-4 or IL-10 is worthy of consideration in trials of DNA plasmids encoding islet antigens in T1D.

3.4. Optimization strategy 4: Pulse therapy of anti-CD3 or anti-CD20 followed by tolerizing DNA plasmid, the “knockout punch”

Various immune suppressive monoclonal antibodies have been shown to synergize with antigen specific therapies. Thus, von Herrath's group showed that anti-CD3 and nasal proinsulin enhanced remission from autoimmune diabetes in NOD mice [16].

Figure 4 Extensive intra- and intermolecular spreading of autoreactive B-cell responses, with persistence of reactivity against the inducing encephalitogen, in chronic relapsing EAE. The mice with EAE, induced with either myelin basic protein p85–99, or with PLP p139–151, or with mouse spinal cord homogenate were followed over a 10-week course of relapsing EAE, after which sera were collected and array analysis conducted. Hierarchical clustering was applied to order antigen features identified as having significant differences in array reactivity between the groups of mice. Relapse rates for individual mice are in parentheses and average relapse rates for dendrogram subnodes are indicated along the base.

From Robinson et al. Nature Biotechnology [16].
Thus, it might be possible to pulse an individual with either anti-CD3 or anti-CD20 therapy and then start a tolerizing immune regiment with an approach like the DNA proinsulin plasmid. This combination may be a decisive ‘knockout punch’, combining the ability to target a wide swath of immune cells first, followed by antigen specific tolerance. The approach is worthy of deep exploration.

4. Conclusions

The beauty of the technology underlying the construction of the DNA tolerizing plasmid is that it is relatively easy to modify its backbone and to interchange different coding regions. Thus it is possible to arm the plasmid with constructs for suppressive cytokines like IL-4 and IL-10, or to co-administer plasmids encoding these cytokines. The number of immune suppressive GpG hexanucleotides can be expanded or even given as a separate oligonucleotide construct. Plasmids encoding other islet antigens can be given separately or in tandem constructs can be engineered. The DNA tolerizing plasmids are operative in animal models when given in conjunction with anti-CD20 therapy, thus allowing a two pronged “knockout” punch with anti-CD20 to clear the deck, followed by antigen specific tolerization. Overall, further clinical trials with tolerizing DNA plasmids to proinsulin and other islet cell autoantigens remain a worthy pursuit to potentially treat, prevent and to perhaps even ‘cure’ or ‘prevent’ type 1 diabetes.

Conflict of interest statement

Robinson, Utz, and Steinman are founders of Tolerion, and hold shares. They are inventors on multiple patents for the tolerizing plasmids. The patents have been licensed to Tolerion.

References

Figure 7  Example of decreased T-cell response with BHT-3009. An example of one patient whose myelin basic protein (MBP)- and proteolipid protein (PLP)-specific T-cell proliferative response decreased in response to BHT-3009 is shown. Proliferation was measured using a dye dilution method with the vital dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE). Peripheral blood mononuclear cells were incubated with a variety of antigens and controls, but for simplicity, only the responses to tetanus toxoid (TT), MBP83-99 peptide, and a PLP peptide mix are shown. The upper 3 panels correspond to the baseline response; the middle 3, to the week 9 response; and the bottom 3, to the week 50 response. Proliferating interferon (IFN)-γ-positive CD4 T cells are shown in the upper left quadrant of each fluorescent-activated cell sorter plot. Numbers in red indicate the percentage of cells in each quadrant. A dramatic decrease in IFN-γ-positive cells specific for MBP and PLP is demonstrated by week 9 and persisted to week 50. Importantly, the response to TT is unchanged with dosing, confirming the antigen-specific nature of BHT-3009 [18].


day 56, sera from the mice described in Fig. 1 were collected. autoreactive B cell diversity and shifts toward a Th2 isotype. On DNA vaccine plus GpG-ODN further reduced myelin-specific Combination therapy with myelin/IL-4-tolerizing mouse); peptide abbreviations are as described in the text[20]. species from which each peptide was taken (h, human; r, rat; m, differences identified by the SAM algorithm. Prefixes denote the row, fluorescent reactivity against a myelin peptide or protein represents results from a single animal from each group; each column derived from MBP, MOG, MAG, myelin-associated oligoden-
CpG-ODN or myelin cocktail/IL-4 _ GpG-ODN (C). Ab reactivity was derived from mice treated with either myelin cocktail/IL-4 _ GpG-ODN (B), and two-class SAM analysis of the ratios of IgG2a (yellow) to IgG1 (blue) autoantibody reactivities in samples all four groups (A), two-class SAM analysis of total IgM anti-myelin Abs in serum samples and analyzed using myelin proteome arrays. Multiclass SAM analysis of total IgM anti-myelin Abs in serum samples}

