Immunotherapies for Type 1 Diabetes

Werner Gurr

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1. Introduction

This chapter introduces some of the concepts that underlie the approaches to immunotherapy in type 1 diabetes (T1D) and provides examples of clinical trials that are based on these concepts as well as problems arising in this process.

That immunotherapy is an appropriate approach in T1D is convincingly supported by findings that point to a pathogenesis with close involvement of the immune system. Autoantibodies and T-cells reactive to islet-derived self-antigens in humans and in animal models, HLA alleles that are associated with susceptibility to the disease and partial amelioration after systemic immune suppression all indicate that a therapy for T1D will need to have a component that focuses on the immune system.

The aim of any immunotherapy is to influence or reset pathogenic components or processes in the immune system. Fulfillment of this aim requires targeting of these components and an ideal therapy will minimize the impact on the healthy necessary aspects of the immune system while maximizing the effect on its aberrant aspects. This is a demanding and perhaps not fully achievable goal since we are dealing with a system where intervention directed at any one component will not necessarily remain localized but will have the potential to extend to other components.

Immunological interventions may be divided into two basic groups, namely active and passive. In the latter approach the experimenter or clinician provides the targeting reagents, for example antagonists or monoclonal antibodies binding surface receptors of immune cells. In this case the effects usually last as long as the experimental compound is present to block or dampen the targeted processes. However, there are examples where effects are induced that persist beyond the withdrawal of the targeting compound. In the former approach targeting is achieved by indirect means. Here the experimental compound, for example a potential autoantigen is administered as a vaccine either together with an adjuvant or via a specific
‘tolerogenic’ route. As a consequence the immune system itself generates the targeting response. In contrast to passive approaches effects induced by vaccination take longer to become manifest and it is possible that they persist far beyond the point of vaccine administration because immunological memory may have been generated.

From the definition and characterization of aberrant immune processes to preclinical studies on new therapeutics, the NOD mouse model and its derivatives remain the most important tool for the development of the basic concepts that underlie the understanding of T1D. That human and mouse T1D must differ in important aspects is obvious simply by comparing the lifespan of a human with that of a mouse as well as the fact that NOD mice are inbred while humans are not. The importance of the NOD mouse model lies in its usefulness to generate the essential conceptual understanding that allows comparison and identification of how the disease process in humans differs from that of the mouse. In this regard the NOD mouse model acts as a reference point and guidepost. Where the aim is to understand the pathogenic process that manifests as T1D, insight into the differences between the disease in the human and in the mouse is itself important scientific knowledge. Furthermore, given the relative ease with which mouse models can be genetically manipulated it is not necessary to proceed from mouse to human. The direction can be reversed by introducing human genetic susceptibility elements into mouse models allowing the investigation of how these elements contribute to T1D.

What are the possible targets for an immune intervention? Systemic immune suppression represents a very broad targeting approach and thus causes the most pronounced ‘collateral damage’. Since T1D is survivable without any immunotherapy at all this approach is problematic because of its side effects. Nevertheless studies testing these therapies have been performed. On the next level of specificity therapies exist that do not target the entire immune system but rather its major components. Therapies directed against T- or B-cells have been successfully tested in mouse models and are now in the process of clinical evaluation. Here initial findings indicate that some of these therapies have positive effects without inducing pronounced adverse responses. Increasing specificity further, we reach the area of the so-called antigen specific therapies. Here not all T- or B-cells are targeted but only those that recognize a given islet autoantigen. This is the area where active immunotherapy is applied by vaccinating with an islet autoantigen. It is hoped that exposing the immune system in a ‘tolerogenic’ way to autoantigens induces effects that suppress or re-regulate T-cells with specificity for the appropriate self-antigen. This approach has had some success in the NOD mouse model, however clinical trials in patients with recent onset of T1D conducted so far have had less promising outcomes. The lack of success may have to do with observations coming from the NOD mouse model where the majority of the tested autoantigens and routes of administration are only clinically effective if the antigen is administered well before T1D has become overt. It appears therefore that this type of intervention should be undertaken preventively to fully exploit its potential. There is a further level of specificity where targeting is directed against T-cell receptors of autoreactive T-cells. In this approach the vaccine antigen is the recombinant receptor of the self-reactive T-cell and the resulting immune response blocks activation of, or possibly eliminates, that T-cell. This approach has not been tested in T1D but has proved to be partially successful in models of other autoimmune diseases. Therapies that apply a different
perspective of the immune system include administration of reagents that can block certain cytokine receptors or administration of cytokines presumed to exert dampening effects. Here specificity is defined by the targeted cytokine receptor, which does not need to be restricted to a particular cell type and consequently the question of unwanted side effects becomes relevant. These therapies also represent one approach that allows targeting of the innate component of the immune system, which like the adaptive part plays a role in the pathogenesis of T1D.

These diverse therapeutic approaches demonstrate that it is possible to develop useful means of targeting immune dysfunction in T1D on all levels of specificity. However, it is our view that currently available therapies represent only a small fraction of what is possible because our understanding of the pathogenesis of T1D is still in its infancy, despite the vast increase in knowledge gained over recent decades. The review below focuses on interventions that have been translated -mostly from the NOD mouse model- to humans either at risk of developing T1D or already suffering from the disease. It does not discuss the large number of potential interventions that have shown promise in preclinical studies of T1D performed again mostly in NOD mice.

1.1. The constraints of immunotherapies applied after diagnosis of T1D

It must be kept in mind that pancreatic islets are not simply an aggregation of specialized cells that produce insulin and other endocrine hormones. Rather each islet represents a micro organ with well-developed anatomical structure, innervation and, as an endocrine gland, a sophisticated blood supply. As with any other organ in the body there is a large margin of safety allowing for considerable functional impairment and damage to occur before the onset of overt clinical signs of organ failure. This margin of safety is of course a great benefit for the individual. However, in terms of immune therapeutic intervention this also means that once organ failure has become overt (i.e when diabetes is diagnosed clinically) a large proportion of the organ has been destroyed and the residual mass can restore euglycemia only under ideal conditions - that is the restoration of complete and lasting immune tolerance to islets. In other words an immune therapy that begins after the diagnosis of T1D is unlikely to be sufficient on its own to restore euglycemia. If permanent restoration of euglycemia is the aim then therapies that are initiated after diagnosis of T1D will need to contain a component that addresses regeneration/re-growth of pancreatic islets. If the view of pancreatic islets as (micro) organs is adopted then the chances of successful re-growth of human islets may be limited. This also means that for immune therapies that are initiated after diagnosis of T1D the aims need to be set lower than full restoration of euglycemia and permanent insulin independence. These therapies therefore aim at maintaining residual islet mass that still exists after diagnosis of T1D and that can persist for years or even decades after disease onset. Parameters such as improved glycemic control, a decrease in the dose of exogenous insulin needed and maintenance or slower decrease of C-peptide levels measure success of these therapies. Although these aims are less glorious than independence from exogenous insulin they are nevertheless worthy of pursuit and can mean significant improvement in the quality of life of the treated patients.
2. T or B-cell targeting

2.1. Therapy with anti-CD3 antibodies

That treatment with antibodies directed against CD3, a component of T-cell receptor complex, might have potential in the treatment of autoimmune disease was evident since the discovery in vitro that immobilized anti CD3 antibody could render T-cells non-responsive or anergic to subsequent stimulation by antigen [1]. Studies in NOD mice revealed that treatment with a hamster antibody against CD3 could reverse diabetes even when it was given at a stage when the mice were already hyperglycemic. This was an important feature in light of the constraints of immunotherapy applied in human patients as outlined above. It was also in contrast to many other promising therapy approaches tested in the NOD mouse that needed to be administered preventively before the onset of overt clinical signs of T1D in order to be effective. Adding to the attractiveness of this approach was the observation that a single course of treatment lasting for 5 days was sufficient to reverse hyperglycemia for several months and that treated NOD mice were resistant to the induction of disease by spleen cells from diabetic donors [2; 3]. This indicated that rather than a passive mechanism such as a blockade of the CD3 receptor some kind of active mechanism of regulation must have been involved in the generation of the clinical effect. Over the years this mechanism has been studied in great detail in mouse models and it has become clear that rather than induction of non-responsiveness in the T-cells a complex cascade of effector mechanisms is initiated by the anti CD3 treatment. It was discovered that binding of the antibody to the CD3 receptor transmits a strong activation signal to the T-cell, which triggers a ‘cytokine storm’ and then death of T-cells. The ensuing cellular debris is ‘cleaned up’ by macrophages, which release IL-6. The increased levels of IL-6 together with TGFβ create an environment that favors the development of a subset of T-cells characterized by the secretion of the cytokine IL-17. As these T-cells circulate through the body they pass the small intestine. Epithelial cells at this site respond to the presence of IL-17 with the increased expression of the chemokine CCL20. Since the IL-17 releasing T-cells have a receptor for this chemokine they accumulate in the small intestine. It appears that once these IL-17 releasing T-cells have reached the small intestine they assume a phenotype that allows them to suppress the proliferation of other T-cells and consequently to also attenuate self-reactive T-cells [4]. Thus the impact of a short treatment with anti CD3 antibodies can induce immune suppression that lasts much longer than the presence of the antibody in the system.

Among immune therapies with broader specificity applied to patients with T1D the treatment with anti CD3 antibodies has been tested most extensively despite side effects such as ‘cytokine storm’ and a selection of recently evaluated trials is summarized below.

All anti CD3 treatments have been given to patients with recent onset of T1D and in accordance with the constraints outlined above, statistically significant increases in the reversal of disease i.e. full independence from exogenous insulin within the study time frame are not observed. However, differences between treatment and placebo groups have been observed and most prominently where results of patient subpopulations are analyzed. This was shown in a trial that observed treated patients and the placebo group for up to four years after treatment with a humanized anti CD3 antibody which was given over a period of six consecutive days.
Statistically significant reduction in daily insulin needs vs. the corresponding placebo group were reported for patients whose residual beta cell function at baseline was above the median of all patients as well as for patients whose age was below the median age. These patients also had a slower decrease in C-peptide levels than the placebo group i.e. their residual beta cell function was maintained for longer than that of the placebo group. Levels of HbA1c (glycated hemoglobin) were also positively affected by the treatment but this occurred again only in younger patients [5; 6]. The dependency of treatment efficacy on age and on the residual beta cell mass (correlated to the time interval between diagnosis of T1D and treatment start) was also observed in a recent published study that compared different dose regimens of an anti CD3 antibody [7]. The fact that anti CD3 treatment was more effective in younger patients was explained by the age-dependency of the insulinis process. Islet inflammation was detected in children but rarely in adolescents and adults. Furthermore, late onset T1D patients are presumed to suffer from a less severe form of the disease. Therefore a more pronounced loss of residual beta cell function was observed in the younger placebo subgroup compared to the older placebo subgroup and the effect of the anti CD3 treatment consequently became more clearly visible in younger patients[6].

Side effects induced by the treatment with anti CD3 antibody occurring in most patients are transient and are in accordance with the mechanism of action of this approach. Fever, which might be explained by the ‘cytokine storm’ triggered by the antibody treatment, a syndrome similar to acute mononucleosis correlating with an increase in EBV copies which may be a result of the activation-induced T-cell death upon anti CD3 administration are some of the adverse events reported for this treatment. Lymphomas as consequence of the anti CD3 treatment have not been observed.

2.2. B-lymphocyte depletion in patients with recent onset of T1D

While it has been established in the mouse that T-cells are necessary and sufficient to cause the disease the role of B-cells in the pathogenesis of T1D is more indirect. Islet antigen self-reactive T-cells both of the helper and cytotoxic type from diabetic NOD mice can transfer the disease to NOD-SCID recipients whereas B-cells are unable to do so. The contribution of B-cells to the pathogenesis of human T1D is likely also to be more indirect. This is suggested by a report of a child with X-linked agammaglobulinemia who developed T1D [8]. Nevertheless, B-cells must participate in the pathogenesis of T1D because it is possible to prevent the disease by B-cell depletion and it has been shown that B-cells are necessary for the initiation of insulitis in the NOD mouse [9; 10]. B-cells are very efficient antigen presenting cells particularly after they have been activated, which could occur in the accumulation of inflammatory cells in the islets during the pathogenesis of T1D. The rationale for the use of B-cell depletion in humans would therefore be a reduction of antigen presentation, which would result in less T-cell activation and an attenuated inflammatory process. It could also include the elimination of cytokines produced by B-cells that might be damaging to the islets and reduce further recruitment of immune cells to the islets. It is also possible that depletion of B-cells with an antibody initiates complex mechanisms similar to those thought to underlie the effects of treatment with anti CD3 antibody.
Targeting of B-cells is achieved by an antibody against the CD20 molecule, a cell surface phosphoprotein that is expressed during the mid-stages of B-cell development but which does not occur on hematopoietic stem cells or normal plasma cells [11]. Anti CD20 antibody is approved for the treatment of B-cell lymphomas and was used in a study of patients with recent onset of T1D (median time interval between diagnosis of T1D and first infusion 81 days). Four consecutive infusions were given over an interval of 22 days. The results of this trial assessed 12 month after study begin resemble those obtained by anti CD3 treatment- slower decrease of C-peptide levels, lower levels of glycated hemoglobin and lower requirement for exogenous insulin in the treated vs. the placebo group. Although not statistically significant, subgroup analysis again tended to suggest a better response in children and adolescents. Side effects included fever, rash and pruritus as consequence of the ‘cytokine storm’ (or cytokine release syndrome) triggered by the first injection of the antibody. Again these effects were transient and did not reappear when subsequent doses of the anti CD20 antibody were administered [12].

It is not known whether the effects of T- or B-cell targeting with antibodies can be prolonged or increased if the treatment is given repeatedly or if T-and B-cell targeting are combined. The guess here is that an increased risk of adverse side effects might counterbalance positive effects gained by repeated or combined administration of T or B-cell depleting antibodies and/or that repeated administration become less efficient because the immune system activates counteracting mechanisms.

3. General immunosuppression

3.1. Autologous nonmyeloablative stem cell transplantation and treatment with cyclosporin

Before reviewing antigen specific immunotherapies two studies with very broad targeting of the immune system shall be mentioned. In one study peripheral hematopoietic stem cells of patients with recent onset of T1D were mobilized, harvested and frozen before immune ablation was achieved by administration of high dose cyclophosphamide and anti thymocyte globulin. The previously harvested hematopoietic stem cells were then infused. During the time needed for the immune system to regenerate extensive supportive care including antibacterial, antiviral and antifungal prophylaxis as well as patient isolation in rooms equipped with air filters was required. This approach resulted in reversal of T1D in the majority of the patients. 12 of the 23 patients participating in this trial became independent from exogenous insulin and this state lasted for 14 to 53 months while 8 patients relapsed and resumed insulin use at low doses [13]. In the insulin-independent group C-peptide levels at 24 and 36 months post transplantation of stem cells had increased while values of glycated hemoglobin had decreased significantly compared to pre transplantation values. The rationale for this study was the possible reconstitution of immune tolerance after an ‘immunologic reset’ by high dose immunosuppression followed by autologous hematopoietic stem cell transplantation [14]. However, it is known from the NOD mouse that the self-reactive tendency of the immune system cannot be eliminated permanently by this approach. Once the immune system
has regenerated autoimmune responses are eventually re-established and islet destruction resumes. This process is reflected by the prolonged but not permanent state of independence from exogenous insulin experienced by the majority of the treated patients in this trial. The results of this study raise the questions whether the increase in C-peptide levels and independence from exogenous insulin was due to a process of regeneration of islets or due to an attenuation of the inflammatory environment the islets were exposed to. Since the majority of the insulin-free patients discontinued insulin use between 3 days before stem cell transplantation (i.e. during the process of immune ablation) and 34 days after transplantation of stem cells it is reasonable to assume that the latter mechanism was dominant at least initially because this time span would appear to be too short to allow extensive regeneration of islets. There was however one patient who achieved insulin independence 610 days after stem cell transplantation and in this case regeneration of islets may have played a role and it is possible that this process also contributed during later stages to the increased C-peptide levels observed in the patients with long term independence from exogenous insulin. If attenuation of the inflammatory environment that islets are exposed to is an important early effector mechanism and if insulitis in humans is predominantly found in children but seldom in adolescents and adults then this approach would be expected to work best in children with recent onset of T1D. However none of the patients in this study was younger than 14 years and therefore the best possible effect might not have been achieved.

The above-mentioned trial could not have been designed as a controlled and blinded study and it is possible that that some of the remissions observed were not related to the treatment but represent spontaneous remissions. However, the close correlation of the observed remissions with the immune ablative treatment and their duration argued for a genuine effect of the treatment. There are results from a double blind and placebo-controlled study applying broad targeting of the immune system with cyclosporine. They show a statistically significant increase in complete as well as complete and partial remissions at the 9th month in the treated vs. placebo group with the effects being more pronounced in the subgroup with whole blood cyclosporine levels of ≥300ng/ml [15].

Certainly immune ablation followed by autologous stem cell transplantation even if it has to be performed only every 3-4 years is not something that could be considered suitable for repeat administration. This also holds in regards to the continued administration of cyclosporine to patients with T1D especially since even a short lasting course of the drug (12.5 +/- 4 months) accelerated the rate of progression of the urinary albumin excretion rate and tended to induce a loss in kidney function [16].

4. Antigen specific therapies

4.1. Insulin

Although it appears from the approaches presented above that the more severe the therapeutic intervention the better its success antigen specific therapies remain attractive conceptually because they allow for an intervention that is more precisely targeted. Rather than targeting
all T-cells (or the immune system in its entirety) the idea is to apply an approach that controls only those T-cells that are self-reactive. Here an important question concerns the specificity of the self-reactive T-cells that ‘merit’ control. It is obvious that insulin is considered a major self-antigen as it is the defining protein of the beta cells that are impaired and destroyed during the pathogenesis of T1D. There are many experimental findings that confirm this view such as the presence of anti-insulin autoantibodies as a proven prediction tool for the assessment of diabetes risk in the pre-clinical state. Furthermore, among the group of beta cell proteins that have been studied to date as potential self-antigens insulin is one of the few that fulfills a formal requirement for a beta cell protein to be considered a self antigen: insulin specific T-cell clones and lines derived from NOD mice can reliably transfer diabetes to NOD-SCID recipients. Another question concerns how these self-reactive T-cells can be targeted and here the mechanisms that mediate oral or nasal tolerance offer a possible approach. Oral or nasal tolerance is defined as the specific suppression of cellular and/or humoral immune responses to an antigen by prior administration of the antigen via the oral or nasal route. The mechanisms of oral tolerance are thought to have evolved in order to generate peripheral tolerance to external agents that gain access to the body via a natural route (the digestive or respiratory tract). As consequence these external agents are ‘seen’ by the immune system as internal components that become part of self. Two different but not mutually exclusive mechanisms have been defined that can mediate oral tolerance, depending on the amount of antigen administered orally: Induction/activation of regulatory T-cells has been reported to occur when low doses are given whereas induction of anergy or deletion of T-cells appears to be the main mechanism involved when higher doses are administered [17]. According to this schematic, if insulin specific self-reactive or autoaggressive T-cells were to be targeted, feeding of insulin would result - upon presentation of insulin by specialized gut-associated antigen presenting cells - in the activation of insulin-specific regulatory T-cells in the gut. These T-cells then migrate to the pancreatic lymph nodes where they encounter epitopes derived from endogenous insulin and become reactivated. This leads to the secretion of IL-10 and TGFβ-cytokines, which can attenuate the ongoing inflammatory process. Because it is mediated by cytokines this mechanism would not only target insulin reactive T-cells but would suppress T-cells with other specificities as well. A degree of specificity would be generated because both types of T-cells - autoaggressive and regulatory - would become activated in the same location (pancreatic lymph nodes or islet infiltrates) but not in other sites. This is one reason why antigen specific therapies thought to rely on T-regulatory cells might be better applied before the onset of T1D. Once islets have been destroyed the pancreatic lymph nodes can no longer activate T regulatory cells because beta cell antigens are no longer presented.

Anergy or deletion of insulin reactive T-cells might also be achieved by oral administration of insulin with the latter mechanism potentially leading - through the presence of debris from apoptotic insulin specific T-cells - to the generation of T regulatory cells according to the process discovered to be activated by i.v. administration of anti CD3 antibodies. It should be mentioned in advance, that in the studies administering oral or nasal insulin presented below, parameters that would indicate which, if any, of the proposed mechanism (tolerance/anergy/activation of T-regulatory cells) had been triggered were not acquired.
Oral administration of insulin has been tested as intervention in patients with recent onset of T1D [18] and oral as well as nasal insulin have been given to persons at risk of developing T1D in order to assess the potential of this approach to prevent or delay the onset of the disease. The ‘Diabetes Prevention Trial-Type 1’ (DPT1) screened first and second-degree relatives of patients with T1D for the presence of islet cell antibodies. Relatives who had anti islet cell and anti insulin antibodies but a normal glucose tolerance and first phase response to intravenous insulin were projected to have a 5-year risk of 26-50%. 372 of these individuals were randomized in the oral insulin study. (DPT1 also included a group of individuals classified as having a risk of greater than 50% who received intravenous instead of oral insulin [19]). The follow-up in the oral insulin study was 4.3 years. During this time there appeared no differences between placebo and control groups. The average proportion of subjects who progressed to diabetes was 6.4% per year in the oral insulin group and 8.2% per year in the placebo group. However, upon subgroup analysis there appeared to be a beneficial effect in those individuals who had a higher anti insulin autoantibody titer (≥80nU/ml, n=263). In this group the proportion who developed diabetes was 6.2% per year in the oral insulin group and 10.4% in the placebo group [20]. This effect became even more pronounced if analysis was confined to those with an anti insulin autoantibody titer of >300nU/ml (n=132) with a projected delay of the disease of almost 10 years [21]. These findings were encouraging but since the subgroup analyses had not been prespecified they could not be considered a positive outcome. Another important result this trial yielded was the confirmation that the parameters used to predict development of T1D in relatives of individuals with the disease were sufficient and accurate. Risk was projected to be 26-50% whereas the actual observed value was 35% over 5 years. Accurate risk prediction is essential for the design of further prevention trials, one of which is currently ongoing and builds on the hypotheses generated by the evaluation of the oral insulin DPT1 trial (better efficiency of the treatment in individuals with higher anti insulin autoantibody titers).

A second prevention trial used nasal instead of oral insulin and a screening and staging approach different from the DPT1. In this case cord blood samples of infants were tissue typed for the presence of the T1D susceptibility allele HLA-DQB1. Carriers of this allele and an additional cohort consisting of their siblings were repeatedly tested for the presence of T1D-associated autoantibodies. Individuals of the two cohorts who were positive for two or more autoantibodies but free of clinical diabetes were invited to participate in the prevention trial. Individuals enrolled in this trial were younger than those in the DPT1 (1.6-5.2 years vs. 7-14 years in the DPT1). 224 individuals of the HLA-DQB1⁺ cohort and 40 individuals of the sibling cohort were randomized to receive intranasal insulin or placebo with a median duration of the intervention of 1.8 years. This trial failed to demonstrate a positive effect of intranasal insulin in all analyzed groups. The annual rate of progression to diabetes in the HLA-DQB1⁺ cohort was 16.8% for the group receiving intranasal insulin vs. 15.3% for the placebo group. In the sibling cohort these values were 10.8% vs. 6.0% respectively. In contrast to DPT1 a subgroup analysis of individuals with high anti insulin autoantibody titers did not show any benefit of intranasal administration of insulin. Although this trial failed to demonstrate positive effects of intranasal insulin it showed that by screening for HLA risk alleles a cohort with a disease risk similar to that of first-degree relatives could be identified from the general population [22].
Thus even if these two trials largely failed in their primary aim they nevertheless clearly demonstrated the ability to accurately predict disease risk, which is essential to optimize the timing of preventative therapies.

4.2. Glutamic acid decarboxylase

Besides oral or nasal administration of an autoantigen there is in T1D models another approach to induce tolerance. In this case a candidate autoantigen is injected subcutaneously together with the adjuvant alum. Although using an islet autoantigen as a vaccine to prevent or ameliorate disease might appear strange it has nevertheless been shown in the mouse model of T1D that this approach can be effective. The idea is that such a vaccination either activates regulatory T-cells or that it converts autoaggressive T-cells to a non-destructive phenotype. This approach has been tested in humans with GAD65, which is the 65 kd isoform of the autoantigen glutamic acid decarboxylase. In contrast to the prevention studies with oral and nasal insulin the trials with GAD65 have been conducted in individuals with recent onset of T1D. A phase II trial tested the safety and efficacy of vaccination with human GAD65 in alum (two subcutaneous vaccinations with 20µg GAD one month apart) in patients with recent onset of T1D (n=70). Results of this study were reported 30 months and again 4 years after treatment. Of the subgroups prespecified in the protocol (HLA classification, age, sex, baseline GAD autoantibody levels) only duration of T1D had a significant influence on the efficacy of the vaccination. In the patients vaccinated less than 6 month after diagnosis of T1D both fasting and stimulated C-peptide secretion decreased significantly less in the GAD-alum group than in the placebo group by month 30 and this positive effect was retained at 4 years after treatment. There was no significant difference between the GAD-alum and the placebo group for patients treated 6 month or more after diagnosis. As expected, vaccination with GAD-alum lead to strong increase of the GAD autoantibody titers, which was sustained to month 30 and a neurological assessment was performed because of concerns that this might lead to stiff-man syndrome. However, there were no notable neurological differences between treatment and placebo group. In accordance with the B-cell responses anti GAD cytokine responses assessed in PBMCs of treatment and control groups at 15 months showed a significantly increased release of most of the tested cytokines (IL-5, 10, 13, 17, IFN-γ and TNFα) in the GAD-alum group. Furthermore increased GAD-induced levels of FOXP3, a transcription factor associated with T regulatory cells, was found in the GAD-alum group [23] [24]. Given the findings of this trial a second study (phase III) was conducted, which enrolled patients within 3 month of the diagnosis of T1D. Patients were randomly assigned to receive one of three study treatments: either two (n=108) or four vaccinations with GAD-alum (n=111) or a vaccination with the adjuvant alum alone (placebo group, n=115). This trial with a follow up time of 15 months failed to show improvements in stimulated C-peptide levels after either the two or the four-dose vaccination when compared to the placebo group. Pooling of data from both groups with GAD vaccinations failed to show a significant effect on stimulated C-peptide levels compared to the control group [25].
4.3. 60kDa heat shock protein (DiaPep277)

A third antigen tested for its therapeutic value in human T1D, is the 60kDa heat shock protein (hsp60). While GAD and especially insulin are specifically expressed in pancreatic islets this is not the case for hsp60, which is expressed throughout the body. Although anti hsp60 autoantibodies can be detected in patients at the onset of T1D they are not useful as predictive markers for disease beyond what can be achieved by measuring anti insulin or anti GAD titers. If hsp60 is an autoantigen in T1D and is widely expressed throughout the body one would expect to find inflammation driven by hsp60-reactive T-cells in other organs as well. However this is not the case and raises the question as to whether there are beta cell/islet intrinsic factors that set this site apart immunologically from other parts of the body.

In therapeutic applications hsp60 is not given as a whole protein but as a peptide derived from the native sequence of human heat shock protein 60. The sequence of this peptide was first identified in the NOD mouse with the help of diabetogenic T-cell clones responding to the *M. tuberculosis* hsp60. Heat shock proteins are highly conserved proteins and it was discovered that these T-cell clones cross-reacted with the human - and presumably with the mouse form - of hsp60 and specifically recognized an epitope in the C-terminal part of hsp60, which was termed peptide277. Vaccination of NOD mice with peptide277 in mineral oil delayed T1D [26]. Since the sequence of this peptide contained two cysteine residues a more stable form was subsequently generated in which the cysteine residues were replaced by valine. The more stable form of peptide277 was also effective in delaying T1D in NOD mice and was termed DiaPep277 [27]. These studies suggested that the mechanisms mediating the effects of vaccination with DiaPep277 might be similar to the ones proposed for vaccination with GAD (e.g. induction of T regulatory cells). It has become evident however that DiaPep277 (and hsp60) can also exert direct effects on the immune system. Hsp60 can activate B-cells via the Toll like receptor 4 (TLR4), which respond by producing IL-10 [28]. Furthermore, TLR4 activation by hsp60 also occurs in macrophages and dendritic cells promoting pro-inflammatory effectors. At the same time hsp60 can also induce anti-inflammatory effects promoted through TLR2. It is reported DiaPep277 does not engage TLR 4 but only TLR2, which leads to the generation of a T-cell mediated anti-inflammatory environment [29].

Several phase II trials have been conducted with DiaPep277 in patients with T1D. In one of these trials that focused on the changes in immunological parameters after treatment, DiaPep277 was administered subcutaneously in a 10% lipid preparation with the placebo group receiving mannitol in 10% lipid preparation. Three different doses of DiaPep277 were tested (0.2mg, 1mg and 2.5mg). Four injections of the drug or the placebo were given over a timeframe of 12 months and a total of 48 patients were enrolled with onset of T1D between 200 and 800 days before start of treatment. Glucagon-stimulated C-peptide production significantly decreased over 12 months in all groups except the group receiving Diapep277 at 2.5mg. The decrease in C-peptide production over 12 months was significantly less in the 2.5mg than in the placebo group. Absolute daily insulin dosage did not decrease over time in any of the groups [30]. These results are in accordance with an earlier trial that found a significantly higher stimulated C-peptide concentration in the treatment vs. placebo group at 6 and 10 months after start of treatment. This earlier trial also found a significantly reduced insulin
requirement at 10 months and observed that individuals with higher C-peptide concentration at the time of initiation of treatment showed better preservation of C-peptide concentrations 10 months later [31]. Therefore the rule that the earlier treatment is started the more efficient it tends to be also applies to this approach. The former study was accompanied by an extensive evaluation of immunological parameters before, during and after treatment. As expected, it was observed that immunological responses were quantitatively and qualitatively highly diverse among the subjects. Nevertheless, after development of new methods to evaluate the results obtained from proliferation and cytokine release experiments, some interesting information could be derived. An IL-10 response but not a proliferative response to DiaPep277 before initiation of treatment, and a decrease or loss of proliferative response subsequent to treatment, appeared to provide a correlate for clinical efficiency. These biomarkers might reflect some kind of tolerance to DiaPep277 (hsp60) and appear to be associated with improved clinical outcome. These findings imply that the status of the immune response prior to therapy may be predictive for treatment outcome. Proliferative responses after treatment with DiaPep277 were frequently specific for hsp60 in that responses to GAD or tetanus toxoid were not or only weakly altered [32]. Treatment with DiaPep277 therefore appeared immunologically effective and specific. One phase III trial with DiaPep277 was recently concluded and awaits publication of the results and another phase III trial is currently underway.

What could be reasons for the limited success of the antigen specific therapies presented above? From a conceptual point of view there is a concern that in these therapies there is always a risk that administration of the candidate autoantigen does not lead to attenuation of the autoimmune reaction but rather leads to its exacerbation. This is especially the case when autoantigens are administered with an adjuvant such as was done in the GAD-alum trials. We have observed while studying the Reg proteins as potential autoantigens in T1D that vaccination of NOD mice with an N-terminal fragment of RegII in alum leads to acceleration of T1D instead of prevention [33]. A similar observation was made in BB rats, which like the NOD mice spontaneously develop T1D. Here insulin given orally with an E.coli-derived endotoxin-free bacterial adjuvant containing acidic glycolipoproteins lead to an acceleration of the disease compared to the group receiving oral insulin alone [34]. Although the GAD-alum studies did not show any acceleration of T1D in the treated groups, it is noteworthy that in the T1D prevention trial with nasal insulin the subgroup of children who presented with three or four types of autoantibodies before the start of the treatment had an unadjusted hazard ratio of insulin vs. placebo of 1.50. This hazard ratio implied a possible risk of an accelerated effect on the onset of T1D in this cohort. It should also be noted that mechanisms involving the activation of regulatory T-cells such as suggested by the findings of the GAD-alum study and considered to be an important factor in oral tolerance generation may not necessarily have only beneficial effects on T1D. Regulatory T-cells are thought to exert their effects via cytokines (e.g. IL-10 or TGF-β), which might on the one hand attenuate self-reactive effector T-cells. But on the other hand these cytokines might also negatively impact beta cell biology and accelerate beta cell destruction by enhancing insulitis through modulation of the release of other cytokines and the islet microvasculature [35]. Cytokines are molecules with a broad range of effects that may differ depending on the target cells. Therefore a therapy that relies on the alteration of cytokine profiles as important effector mechanism carries the risk that these alterations although
beneficial to some systems (e.g. T-cells) might be detrimental to other affected cells (e.g. beta cells, endothelia). The clinical outcome might thus depend on the sum of all these effects and might not be predictable.

5. Conclusion

The analysis of the trials presented here suggests that treatment efficacy can differ from subgroup to subgroup. This indicates that there might not be a single therapeutic approach that fits all. Rather the observations suggest that it may be necessary to establish an individual profile that goes beyond the standard parameters such as sex, age, family history, time of diagnosis of T1D, HLA type, and autoantibody profile for each person intending to undergo an immune therapeutic intervention. These parameters might include the spectrum of T-cell responses to beta cell autoantigens (in terms of proliferation as well as of cytokine release), characterization of the gut flora [36; 37], imaging of islet inflammation [38] type and time of prior vaccinations and infections, season [25], and might even include psychological parameters such as familial stress levels [39]. As new approaches are translated from the pre clinical stage to individuals at risk of developing T1D or to patients already suffering from the disease the palette of possible interventions will grow more diverse. Obtaining highly differentiated profiles may refine the process of matching the time point and the type of immune intervention to an individual and thus optimize outcome.

Author details

Werner Gurr

Address all correspondence to: Werner.gurr@yale.edu

Yale University, Dept. of Internal Medicine/Endocrinology, New Haven, USA

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