CLINICAL
INVESTIGATION

Diabetes vaccines: review of the clinical evidence

As preservation of residual β-cell function is clinically important, such an effect constitutes clinical evidence. Traditional vaccination strengthening the immune reaction against an antigen/microbe may well be relevant for Type 1 diabetes (T1D) but progress takes time. Use of probiotics is another way of influencing the immune system at the border of 'vaccination'. Methods of reducing a pathological-specific immune response, 'inverse' vaccination, are being developed. Use of insulin or its relatives (proinsulin and B-chain of insulin) is still experimental. Diapep277®, a heat shock protein, may modulate the immune system in a favorable way, and subcutaneous 'vaccination' with GAD-alum has shown encouraging results in T1D with recent onset. Perhaps autoantigens should be administered via DNA vaccines. It is possible that these will soon be part of clinical practice for the treatment of diabetes.

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What does 'clinical evidence' mean in Type 1 diabetes?

Type 1 diabetes (T1D) is the most common serious, life-threatening disease in children in Western countries, with a rapid increase of incidence all over the world [1]. It causes substantial morbidity and mortality [2,3]. This is despite intensive treatment with multiple daily injections of insulin, adapted to regular meals with suitable content based on self-monitoring of blood glucose. When discussing clinical evidence or the clinical relevance of interventions to preserve residual insulin secretion questions regarding reduction of insulin dose often arise. For the patient it would of course be extremely relevant if insulin injections were no longer needed. However, if exogenous insulin is needed then it is of very limited interest for the patient if an injection contains 6 U or 3 U. In this situation it is more interesting to consider whether blood glucose control is good or not. However, as the treatment goal of T1D should always be to achieve as close to normal blood glucose and hemoglobin A1c (HbA1c) as possible, most studies are designed whereby a difference in HbA1c between the treatment and control groups has to be regarded as a failure to treat all patients well enough. Thus, there should hopefully be no difference in HbA1c between the treatment and control groups. However, it is not uncommon that patients with residual insulin secretion do show lower HbA1c, as residual insulin secretion facilitates metabolic control, decreases the risk for serious hypoglycemia and also decreases the risk of keto-acidosis [4]. Very modest β-cell function, with peak stimulated C-peptide levels above 0.2 pmol/ml, has already been reported to reduce long-term complications [5]. Furthermore, C-peptide itself has been proposed to decrease the risk of complications and there is increasing evidence that C-peptide is not just a peptide connecting the two insulin chains, but an active peptide, perhaps a hormone, with several important effects [6]. The relevance of saving β cells and

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improving their function has become even more clinically relevant, as some studies indicate that the β cells may regenerate [7,8]. If so, there is new hope regarding what an end of the destructive process could mean.

Taken together, the values of preserving insulin secretion and C-peptide means that if a diabetes vaccine can be shown to preserve residual insulin secretion/C-peptide, this would constitute 'clinical evidence' [9].

The β-cell destructive process

To understand the role of vaccines in T1D it is relevant to give a short background on the etiology and pathogenesis of the disease. The accepted view is that most β cells of the islets of Langerhans are lost at the diagnosis of T1D. The β cells are believed to be killed by a gradual autoimmune process precipitated and promoted by genetic and environmental factors. In recent years the dogma of complete death of the β cells have been questioned, and regeneration of the β cells, which I proposed in 1981 **(Figure 1)**, seems plausible. In fact, many β cells may still be present in the pancreas although they do not secrete insulin. It is not known what precipitates or stimulates the self-destructive process, but viral infections could be important (e.g., coxsackie virus [CVB], cytomegalovirus [CMV], Epstein–Bar virus [EBV] and rota virus) as well as nutritional agents from cow's milk proteins or gluten. Another hypothesis suggests that a heavy burden on the β cells leading to increased demand for insulin because of, for example, increased weight, reduced physical exercise, increased psychological stress, among other factors, causes the presentation of autoantigens, and perhaps also heat shock protein, which may precipitate an autoimmune reaction in genetically predisposed individuals whose immune system has for other reasons lost balance. Reasons for a less well-balanced immune system could include increased hygiene and/or abnormal gut flora. The autoimmune process leads to insulitis. Mononuclear cells, mainly T cells, are thought to kill the β cells. Auto-antibodies are usually found, but regarded more as a marker of the process, rather than playing a causal role. The auto-antibodies react either against the islet cells (islet cell antibodies [ICA]) [10] or against specific autoantigens such as insulin auto-antibodies against insulin (IAA) [11], against glutamic acid decarboxylase (GADA) [12], against tyrosin phosphatase (IA-2A) [13] or against zinc transport antigen (ZnTA) [14]. Thus, these antigens are attacked by their own immune system. Insufficient immune regulation is thought to allow a self-destructive process. Several immune interventions have been performed with the aim of preserving residual β-cell function, but so far with insufficient efficacy and/or with unacceptable adverse effects [15–23]. In recent years, interventions with monoclonal antibodies against CD-3 or CD-20 have been more encouraging [24–27]. However, these types of treatments cause rather common and occasionally serious adverse events, and are therefore not the treatment of choice, not least for preventive interventions in healthy children with increased risk of developing T1D. Therefore, there is a great need for other, more specific types of intervention that do not suppress the immune system but either modulate and rebalance the system, or in the best case scenario create tolerance against the autoantigens involved in the autoimmune process.

Vaccines to prevent infections via an antigen-specific increase in the immune response

The relationship between vaccines and T1D has been discussed for decades, either in the context that vaccines could contribute to the development of T1D, or that T1D could be eliminated by some kind of vaccination. In the 1920s precipitation of T1D after mumps infections had already been described [28]. If mumps do play an important role, a general vaccination against mumps might either prevent T1D, or vaccination with living virus might initiate an autoimmune process leading to an increased incidence of T1D. None of these associations seem to be true [29,30]. Neither have there been any associations between vaccinations against other microbes and the development of diabetes [31]. The hygiene hypothesis suggests that the immune system would deviate less often towards an autoimmune process if the immune system was occupied by an ongoing defense against a serious enemy. In accordance with this hypothesis, there have been studies on the relationship between Calmette vaccination and T1D. Experiments in animals gave some support to this hypothesis, but no
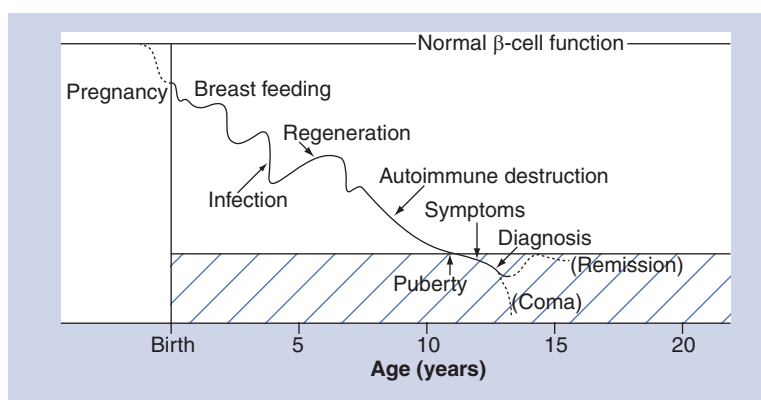

Figure 1. Slide displayed at a Nordic Symposium in connection with the Annual Meeting of the Scandinavian Society for the Study of Diabetes, Linköping, 1981. Type 1 diabetes was proposed to develop after a long autoimmune process destroying the β cells. Events during pregnancy and the importance of breast-feeding was suggested, and later shown to be relevant, and regeneration of β cells was proposed as a possibility.

clinical effect on β-cell preservation has been seen [32] nor any effect on incidence of T1D [33] by vaccination against tuberculosis in humans.

Certain virus infections have been suspected to cause T1D. Epidemiological studies have provided evidence that CVB frequently occurs in subjects who later develop T1D [34]. CVB4 is the most common serotype detected in prediabetic individuals and patients with recent-onset T1D. In fact, the CVB4 strain E2 was once isolated from the pancreas of a diabetic child who died, and the virus was then passed into islet cells and found to cause diabetes in mice [35]. This strain is able to induce a persistent infection in β cells with disturbance of β-cell function [36]. CVB4 may also infect the thymus, leading to immunological tolerance to CVB4, which might play a role in the breakdown of central self-tolerance to β cells [37].

However other viruses in addition to enteroviruses are also suspected. Rotavirus [38], mumps, as mentioned above, as well as CMV, EBV and Varicella-Zoster virus [39]. Irrespective of the mechanism of possible diabetogenic action, such as molecular mimicry, direct toxic effect or 'bystander' activation of T cells against islet antigens, one way to protect the β cells and prevent T1D would be vaccination against these types of infections (e.g., vaccination against CVB). However, to date this way of preventing T1D has been disappointing.

'Vaccination' with probiotics for the prevention of T1D

Several facts suggest that the gut is involved in the development of the autoimmune process leading to T1D [40]. The intestinal barrier may be disturbed, which might facilitate passage of proteins that may contribute to the autoimmune process. Such proteins may come from cow's milk [41], and bovine insulin in cows milk has been identified as a possible cause of an autoimmune reaction against insulin [42]. Furthermore, depending on the gut flora, the maturation of the immune system may become influenced, as well as mucosal immunity later on. Colonization of the gastrointestinal tract starts at birth [43]. Microbes come from the mother and facultative aerobic Gram-positive cocci (staphylococci, streptococci and enterococci) and enterobacteria are the first bacteria to colonize the intestine [44]. Maternal vaginal lactobacilli can also transiently colonize the infant. Bifidobacteria subsequently become the predominant species in breast-fed infants [45], while formula-fed infants receive other intestinal microflora, including enterobacteria, lactobacilli, bacteroides, clostridia, bifidobacteria and streptococci.

Probiotics are defined as living organisms that exert health benefits beyond inherent general nutrition [46]. There is some evidence that probiotics can influence immune function, probably through effects on antigen-presenting cells, regulatory T cells and effector T and B cells [47]. Thus, some studies suggest that probiotics might decrease the occurrence and severity of allergic diseases, atopic eczema in particular [48] and some experimental studies have shown that probiotics may prevent autoimmune diabetes in nonobese diabetic (NOD) mice [48,49]. However, although an interesting hypothesis, there are no clinical studies showing that inoculation or 'vaccination' with probiotics prevents T1D or influences the natural course of the disease.

Vaccinations with heat shock protein

Two decades ago Cohen *et al.* used heat shock proteins in order to modulate the autoimmune process in T1D [50]. Although it is unclear whether heat shock protein can be regarded as an autoantigen in diabetes, a β-cell target antigen in NOD/Lt mice was found to be cross-reactive with the 65-kDa heat shock protein (hsp65) of *Mycobacterium tuberculosis* [50] and on ingestion in certain numbers the onset of β-cell destruction was associated with the spontaneous development of anti-hsp65 T lymphocytes and autoimmune diabetes could be induced but also treated by the use of this hsp65. Later, the same group showed that a specific peptide, DiaPep277®, was an active component and this peptide has been utilized with intriguing results [51]. Female NOD mice of various ages up to 17 weeks were treated with a single inoculation of DiaPep277 given before or after the onset of overt hyperglycemia. This therapy was accompanied by regression of insulitis and the reappearance of histologically normal islets. Successful peptide therapy was associated with downregulation of T-cell immunity to the DiaPep277 [51].

Later studies in humans with newly diagnosed T1D have shown that subcutaneous administration of DiaPep277, causing no adverse events, leads to β-cell preservation in adults [52]. Thus, 35 patients with T1D and basal C-peptide above 0.1 nmol/l were assigned subcutaneous injections of DiaPep277 1 mg and mannitol 40 mg in vegetable oil (DiaPep277; n = 18) at entry, 1 and 6 months, or three placebo injections (mannitol in vehicle; placebo; n = 17). The primary end point was glucagon-stimulated C-peptide production. A total of 31 patients completed 10 months of follow-up and were included in the intention-to-treat analysis. At 10 months, mean C-peptide concentrations had fallen in the placebo group (n = 16) but were maintained in the DiaPep277 group (n = 15; 0.26 [SD: 0.11] vs 0.93 [0.35] nmol/l; p = 0.039). Need for exogenous insulin was higher in the placebo than in the DiaPep277 group. HbA1c concentrations were rather low (~7%) in both groups. T-cell reactivity to hsp60 and p277 in the DiaPep277 group showed an enhanced T-helper-2 cytokine phenotype. No adverse events were seen. Thus, treatment of newly diagnosed

T1D adults with DiaPep277 seemed to preserve residual insulin secretion, perhaps through the induction of a shift from Thr-1 to Thr-2 cytokines. However, the study was small, and the result could depend on sporadic outliers, and in children the effect has been less evident. There seems to be T cells and auto-antibodies against human hsp70 in children with recent-onset T1Ds according to a study looking at T-cell proliferative responses (stimulation index [SI]) and auto-antibodies to human hsp60, hsp70 and hsp90 proteins in 25 children (mean age 10.1 ± 3.8 years) with newly diagnosed T1D [53].

However, the preservation of residual insulin secretion seen in adults, could not be confirmed in diabetic children and adolescents [54,55]. Yet the immunological effects are interesting [56]. Thus, the immunological efficacy of therapy with DiaPep277 seems to be correlated with clinical outcome. A total of 48 C-peptide-positive patients were assigned subcutaneous injections of DiaPep277 0.2, 1.0 or 2.5 mg (n = 12 per dosage) at entry, and 1, 6 and 12 months, or four placebo injections (n = 12). T-cell autoimmunity to hsp60, DiaPep277, GAD and tetanus toxoid (recall response control) were assayed by proliferation and cytokine secretion assays (ELISPOT) at regular intervals until 18 months after the first injection. All treated patients at each dose of peptide demonstrated an altered immune response to DiaPep277, while the majority of placebo-treated patients did not respond (p = 0.00001). Cytokine secretion in response to therapy was dominated by IL-10. IL-10 concentration before therapy and decreasing autoantigen-specific T-cell proliferation were associated with β-cell preservation. These results are encouraging. It may well be that 'vaccination' with DiaPep277 may be one way of down-regulating the autoimmune process in T1D, although the clinical evidence so far is weak.

'Inverse vaccination': efforts to achieve antigen-specific reduction of the immune response

Traditional vaccination is a way of strengthening the immune reaction against an antigen, usually an infectious microbe. Methods of reducing a pathological-specific immune response can be regarded as a sort of 'inverse' vaccination, which would be of great value in the treatment of autoimmune diseases such as T1D, as well as in allergy. Allergy is a different form of immunological attack against external antigens. In allergy one way of treating the disease has been to create tolerance against the allergens by presenting the antigen/allergen(s) in a suitable dose and way to the immune system. Gradually this treatment has become very efficacious [57] and the adverse events, for example because of abnormal response to the allergen (e.g., anaphylactic reaction) has become rare.

It would be natural to try to try to reduce or stop an autoimmune process in a similar way, by presenting the autoantigen. Thus, instead of trying to suppress the immune system, one way of modulating the immune response may be to present antigen(s) in a way that causes the immune system to change from an aggressive to a tolerant reaction [58]. However, we still do not understand the mechanisms by which this might work, and why a boost of immunity might protect against an autoimmune disease. Studies involving experimental animals may give some insights, although as always information from animal studies cannot automatically be transferred into the human situation.

We still know little about the mechanisms involved in achieving this goal. If self-reactive T cells directed against autoantigens cause diabetes, a major question is why such self-reactive T cells occur. The thymus has a central role in the development of immunological self-tolerance where naive and competent T lymphocytes are generated. They are educated to recognize and tolerate proteins [59,60]. The growth hormone/IgF axis is important for the maintenance of thymus function beyond childhood [61]. Two mechanisms seem to be necessary for self-tolerance: clonal deletion of self-reactive T cells issued from the random recombination of TCR genes (negative selection), and the generation of self-antigen-specific natural regulatory T cells (Tregs) that can inactivate self-reactive T cells in the periphery when they have escaped intra-thymic negative selection [62]. In T1D auto-reactivity against insulin is a common and early phenomenon. The important role of thymic insulin for development of self-tolerance has been demonstrated in transgenic mice [63]. Genes from the insulin family are all expressed in the thymus, mostly IgF-2, and then IgF-1, and thereafter insulin. IGF-2, a major factor in fetal development, might need to be more protected than insulin. Because of the strong homology there may well be cross-tolerance to members of the insulin family. As IgF-2 seems to a be a very important and early part of the insulin family it is possible that presentation of IgF-2 could also create tolerance to insulin. To my knowledge, there are no clinical studies in humans that try to present IgF-2.

Administration of intact autoantigen proteins
■ Vaccination with insulin

Proinsulin and insulin and its different chains are the only autoantigens that are specific for the β cells. Insulin has been used in trials to prevent diabetes among first-degree relatives with increased risk of developing T1D. In the Diabetes Prevention Trial – Type 1 Diabetes (DPT-1), a randomized, controlled trial of low-dose parenteral insulin, human ultralente insulin at 0.25 units/kg/day was given to subjects with a greater than 50% 5-year risk

of developing T1D. To give such large doses of insulin subcutaneously every day should perhaps not be regarded as immunotherapy, or 'vaccination' but rather as β-cell support. In any case, this treatment had no effect [64].

Oral insulin is not supposed to be absorbed in such an amount that it could affect glucose homeostasis or support remaining β cells, but this administration can instead be seen as an immune intervention, an autoantigen 'vaccination'. The DPT-1 trial randomized 372 relatives of subjects with T1D, positive for IAA and with normal intravenous and oral glucose tolerance test (IVGTTs and OGTTs), to oral insulin 7.5 mg/day or placebo, and although the result was negative when comparing the groups with the prespecified inclusion criteria, subanalyses suggested that T1D was significantly delayed in those individuals who did react against the autoantigen insulin more strongly with high concentrations of IAA [65]. This indicates that autoantigen therapy may have its best effect in patients whose immune system recognizes the antigen.

Insulin has also been used to prevent T1D in infants. The first auto-antibodies seen in young children are usually IAA and therefore it was reasonable to try to prevent diabetes in high-risk individuals followed from birth with insulin administration. Administration of intranasal proinsulin had shown effect in experimental animals [66] and there are arguments for the presentation of the antigens on the mucosa. However intranasal administration had no effect [67]. This does not prove that the idea was wrong, but rather that this method of administration with the dose in question in that population was not efficacious.

Administration of the insulin B-chain has been shown to prevent diabetes in experimental animals [68]. A combination of the insulin B-chain fragment, which contains an epitope recognized by the immune system, has been combined with Freunds adjuvant and then also tried in humans. A total of 12 newly diagnosed T1D adults were randomized to either a single dose of this 'vaccine' or placebo in a double-blind pilot study [69]. There was no significant effect on C-peptide, but quite interesting effects on T-regulatory cells were observed, which is encouraging for further studies. It will be important to learn how to use different autoantigens to modulate the auto-immune reaction.

The administration of proinsulin to prevent diabetes or preserve residual insulin secretion has been reported. There are, however, several studies presenting these autoantigens to the immune system by administering plasmid DNA, which will be discussed later in this review.

■ Vaccination with glutamic acid decarboxylase

During my own studies with plasmapheresis used as an immune intervention in newly diagnosed T1D children [16], we discovered a new diabetes-related antigen,

with an estimated weight of 64 kD [70], which later on was shown to be GAD [71]. In the CNS gamma-aminobutyric acid (GABA), formed when glutamic acid, or glutamate, is decarboxylated by GAD. It has been suggested that GABA regulates hormone release in the pancreas and/or functions as a paracrine signaling molecule that is of importance for communication between the β cells and other endocrine cells in the islets. But the specific function of GAD in pancreatic islets is unknown, as well as its role in the pathogenesis of diabetes. Thus, the reason GAD is a major autoantigen in autoimmune diabetes is unknown.

Nevertheless, auto-antibodies to GAD are common in T1D and there are convincing data from the NOD mouse model of T1D that administration of the isoform GAD65 can prevent autoimmune destruction of pancreatic β cells and the subsequent need for exogenous insulin replacement [72,73]. These and many other studies have justified studies in humans.

An adjuvanted formulation, based on Alhydrogel®, was developed to provide the drug product (Diamyd®) used for evaluation in clinical trials. Alhydrogel is the product of aluminum hydroxide (alum), which is a conventional adjuvant in vaccines for children that contain aluminum adjuvants including diphtheria/tetanus/pertussis (DTP), pneumococcal conjugate, hepatitis B, hepatitis A, anthrax and rabies. Aluminum salts are well recognized as preferentially inducing a humoral (Th2) rather than cellular immune response. As subjects with ongoing autoimmunity resulting in T1D are likely to be biased towards a Th1 (or cellular) immune response to autoantigens, alum is used to overcome this bias and 'steer' the response induced by GAD away from a cellular towards a humoral response in order to minimize the likelihood of exacerbating cell-mediated β-cell destruction. Inclusion of adjuvant was also rationalized to minimize the quantity of antigen required for treatment by maximizing its immunogenicity.

A preclinical safety evaluation program has been conducted to support the progression of Diamyd into clinical development. Evaluation of all preclinical safety studies performed to date has not provided clinical safety concerns. Likewise, evaluation of the effects of Diamyd in several different animal models of autoimmune disease did not indicate any potential for undesirable effects on the immune system, and Phase I studies in humans were conducted 1999. A Phase IIa study in 47 latent autoimmune diabetes in adults (LADA) subjects extended the experience to Diamyd. This randomized, double-blind and placebo-controlled dose-finding Phase IIa study also demonstrated efficacy in preventing β-cell destruction in the 20-μg group [74]. There were no serious adverse events (SAEs) during the 6-month-long main study period. A minority of injections resulted in injection-site

reactions that were mild and most, in particular 'tenderness', occurred mainly on the day of the injections. Fasting C-peptide levels at 24 weeks were increased compared with placebo (p = 0.0015) in the 20-μg group but not in the other dose groups. In addition, both fasting (p = 0.0081) and stimulated (p = 0.0236) C-peptide levels increased from baseline to 24 weeks in the 20-μg dose group [61] and even though the number of patients was very small, this result was encouraging. Follow-up after 5 years completed in 2008 still show a significantly beneficial effect of the 20-μg dose of Diamyd. There have been very few adverse events and none of these are considered to be treatment related [75].

A Phase IIb, randomized, double-blind, placebo-controlled multicenter Diamyd study in 160 LADA subjects was then performed in Sweden. Subjects received GAD65 20 μg of or placebo on two occasions 4 weeks apart. The trial had a main study period of 18 months and was scheduled for unblinding in June 2007. Unfortunately, the efficacy variables of study had to be invalidated due to concerns regarding the labeling process of the investigational product. It was impossible to guarantee with certainty whether the patient had received drug or placebo. No safety concerns have been raised in this study. No SAEs have been observed 30 months after the first injection.

Phase IIb studies in children & adolescents

To investigate the safety and efficacy of Diamyd in T1D, a Phase II clinical trial in 70 recently diagnosed T1D children and adolescents has been conducted [76].

The study was a randomized, double-blind, placebo-controlled multicenter study using the same dose regimen as in the successful group of the previous LADA trial. The main study period of 15 months was completed and the trial partly unblinded for sponsor and statistician, in August 2006, but continued blinded for all other investigators for another 15 months of follow-up. Outcomes from this study have provided strong support for the clinical safety and efficacy of Diamyd. Thus, the treatment was very well tolerated and no treatment-related adverse events have been reported after more than 4 years follow-up. Regarding efficacy, both treatment groups showed a progressive decrease from baseline in both fasting and stimulated C-peptide secretion. There was no significant effect of treatment on change in fasting C-peptide after 15 months (primary end point, chosen based on the previous LADA trial). However, there was a significant effect of treatment on change in fasting C-peptide seen after 30 months (p = 0.045), which was also seen when change in C-peptide/plasma glucose ratio was taken into account (p = 0.02). Stimulated C-peptide secretion, as measured by area under the curve (AUC), decreased significantly less in the GAD-alum-treated group than in the placebo

group, both after 15 (p = 0.01) and 30 months (p = 0.04). The statistically significant effect of treatment on change in fasting and stimulated C-peptide at month 30 remained after adjusting for differences in duration of diabetes, age, gender and baseline GADA levels.

Insulin requirement in both treatment groups increased in the course of the study, and HbA1c and plasma glucose levels increased during the study. HbA1c did not differ between the groups, given the therapeutic target used by physicians.

Duration of diabetes had a significant influence on the efficacy of treatment (p = 0.05 for fasting at month 30 and p = 0.03 for stimulated C-peptide AUC at months 15 and 30). In patients treated within 6 months of diagnosis, both fasting and stimulated C-peptide secretion (AUC) decreased significantly less in the GAD-alum-treated group as compared with the placebo group over 30 months (fasting, p = 0.03; and stimulated, p = 0.04) while no such difference was observed in patients with a duration of diabetes of 6 months or more (Figure 2). The observed treatment effect in the short-duration group cannot be attributed to a few outliers, and this effect on C-peptide preservation is still seen after more than 4 years follow-up [Ludvigsson J et al., Unpublished Data].

Effect of Diamyd on the immune system

In the Phase IIb trial, mechanistic studies were performed. In the group treated with GAD-alum, GADA levels increased rapidly, reached a maximum at 3 months and then decreased, but remained significantly higher than in the placebo group. There was no change of epitopes, but a shift in isotypes with a reduced percentage of IgG1 and increased IgG3/IgG4 detected in GAD-alum-treated patients [77]. Thus, the treatment was able to induce long-lasting GAD-specific immune responses, still detectable 30 months after the first injection, and it in fact remains after 48 months [77]. B cells are known as antibody-producing cells, but the B cell function is much more complex. They also have an important function as antigen-presenting cells in T-cell differentiation, mediated by cytokines. B cells also produce IL-10 and TGF-β, and could be involved in the development of regulatory T cells and their recruitment to sites of inflammation. It has been suggested that 'regulatory' B cells, arising from nonregulatory autoreactive B cells could suppress pathogenic T-cell responses.

Spontaneous and phytohemagglutinin-induced secretion of all cytokines was similar in samples from children receiving GAD-alum and placebo, both before and 15 months after the first injection. Cytokine secretion of IL-5, IL-10, IL-13, IL-17, IFN-γ and TNF-α, but not of IL-6 and IL-12, in response to *in vitro* stimulation with GAD65 increased in GAD-alum-treated patients from baseline to month 15, and this GAD-specific response
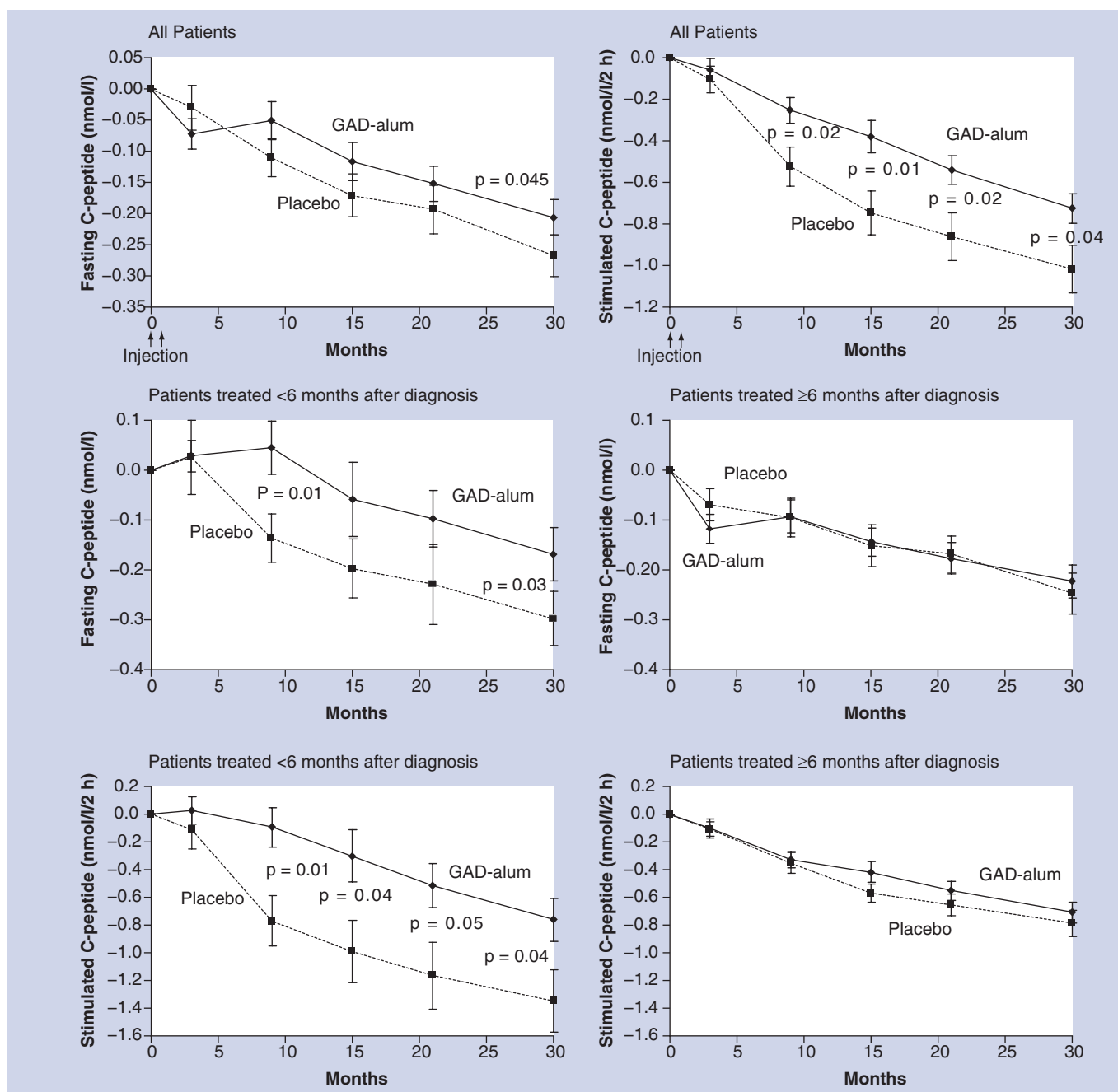
Figure 2. Treatment with GAD-alum has shown significant preservation of residual β-cell function in patients with recent onset of Type 1 diabetes.
Reproduced with permission from [76].

continues after up to 48 months. GAD-induced cytokine secretion, except for IL-12, was significantly higher in samples from patients treated with GAD-alum than in samples from the placebo group 15 months after the immune intervention.

Increased GAD65-induced expression of FOXP3 and TGF-β was observed at month 15 in cells from GAD-alum-treated patients compared with placebo, and their expression correlated in the GAD-alum group but not in the placebo group. Whether an increased expression of FOXP3 is a sign of increased T-cell regulation and/or T-cell activation cannot be answered yet.

As mentioned above, after 48 months there are clear GAD-specific effects on the immune system suggesting both a Th2 deviation, a decrease of activated T cells and increase of T-regulatory cells [Ludvigsson J *et al.*, Unpublished

Data]. It appears difficult to understand how short-term autoantigen treatment leads to a long-standing effect on parameters such as cytokine production and FoxP3-positive cells. Continuous secretion of GAD may perhaps contribute to this long-term effect. Irrespective of the explanation, the interpretation could be that Diamyd treatment has deviated the immune system towards tolerance against the autoantigen GAD. It may seem surprising that GAD-alum treatment may have an effect, but treatment with the β-cell-specific insulin has not been shown to so far, but there may be several explanations for this, such as different administration, doses and formulations. Furthermore, bystander suppression may be involved when even a nonautoantigen like Diapep277 may have an effect.

Ongoing trials with GAD vaccination
Two Phase III trials with Diamyd in T1D have started, one in Europe (EU Phase IIII ClinicalTrials.gov identifier: NCT00723411; Johnny Ludvigsson, PI) and one in the USA (US Phase III ClinicalTrials.gov identifier: NCT00751842; Jerry Palmer, PI), both with the same design. In each trial 320 patients aged 10–20 years with T1D for at most 3 months, a fasting C-peptide over 0.1 pmol/ml and GADA positive will be randomized in a double-blind controlled trial into three arms. In one arm the patients are given GAD65-alum (Diamyd) 20 μg subcutaneous at day 1, 30, 90 and 270, while the patients in the second arm are given GAD65-alum (Diamyd) 20 μg at day 1 and 30 followed by placebo at day 90 and 270, while the patients allocated to the third arm will get placebo at all four time-points. The patients will be followed for 30 months. The primary end point is the change from baseline (visit 2) to month 15 (visit 6) in C-peptide ($AUC_{mean\ 0-120\ min}$) during a mixed meal tolerance test. Secondary end points include:

- HbA1c, change between baseline and subsequent visits;

- Exogenous insulin dose per kg body weight and 24 h, change between baseline and subsequent visits.

Recruitment has been completed in the European trial (November 2009) with 334 patients included. In approximately 140 of the European patients mechanistic studies of both humoral and cell-mediated immunity have been performed. The American trial has had a slower recruitment, partly because only patients aged over 16 years were accepted for inclusion during the first year. The recruitment is now going very well.

No results can of course be reported from these ongoing Phase III trials, but it appears that the treatment is easy to administrate, well tolerated, and so far no clear treatment-related SAEs have been reported.

In addition to the above-mentioned studies, an intervention trial in newly diagnosed T1D patients aged 3–45 years is being performed by TrialNet (TrialNet Intervention ClinicalTrials.gov identifier: NCT00529399). According to the preliminary protocol, patients will be randomized in a double-blind, controlled study into three arms, one with subcutaneous injections of GAD65-alum (Diamyd) 20 μg at day 1, 30 and 90, a second arm with subcutaneous injections of GAD65-alum (Diamyd) 20 μg at day 1, 30 and placebo at day 90, and a third arm with placebo at all time points. The main aim of this trial is to study the effect of GAD vaccination on the immune system.

A third trial is ongoing in the USA (NIDDK Combination study ClinicalTrials.gov identifier: NCT00837759) where Diamyd is combined with sitagliptin and lansoprazole, which will hopefully stimulate β-cell regeneration. However, the potential of β-cell regeneration in diabetic patients is still controversial; experimental data have been reported but no definite proof exists in the clinical setting.

In addition to interventional trials at the onset of T1D prevention trials using GAD-alum treatment of high-risk individuals are underway both in Europe and the USA. Thus, a pilot prevention trial has started in southern Sweden (Swedish pilot prevention study: not yet registered [in process]), where high-risk children are identified as part of the so-called DiPiS (Diabetes Prevention in Skåne) study, in which newborn children in the general population have been screened for autoantibodies. Thus, children with GADA plus at least one more diabetes-related autoantibody are treated with either GAD65-alum (Diamyd) 20 μg or placebo subcutaneous at day 1 and 30. The main aim is to study safety. Larger studies of the efficacy of preventive treatment are being planned both in Europe and the USA. It should be clearly stated that the efficacy of autoantigen prevention remains to be proven.

DNA vaccines
To get a T-cell response, antigens have to be presented by the antigen presenting cells (APCs). However, instead of delivering intact proteins, DNA vaccines can be used. A protein encoded by plasmid DNA can either be produced outside the APCs if the plasmid DNA is administered into a muscle, or the plasmid DNA may be taken up by the APCs where the encoded protein is presented [78]. Proteins encoded by DNA vaccines can induce different types of antigen-specific immune responses, and perhaps also some nonspecific reactions.

The most common route of administration is intramuscularly, which is thought to favor Th1 responses, or intradermally, which is thought to favor a Th2 response. Promoters from viruses, such as CMV,

can be used. Certain sequences seem to stimulate a Th1 response and should therefore be avoided in the treatment of T1D. One way of skewing the response towards Th2 may be to co-administer plasmids encoding Th2 cytokines.

DNA vaccines have been used for a long time in the treatment both of infectious diseases [79], and of cancer [80] and in transplantations [81]. In autoimmune diseases we do not want an increased antigenic response but tolerance through a decreased response. Self-tolerance is normally achieved in the thymus through negative selection when lymphocytes with high affinity for the autoantigen are deleted. However, low-affinity lymphocytes may escape, and later be involved in an autoimmune reaction. DNA vaccination might lead to the production of memory cells, which then should lead to a long-term immune response, but to get tolerance we would prefer hyporesponsiveness, and it is possible to induce apoptosis of autoreactive lymphocytes. However, this tends to be transient [82]. A more effective method would be to induce T-regulatory cells, and there are numerous publications showing such results [78,83].

DNA vaccines to create tolerance in autoimmune disease are mainly administered to experimental animals, where there have been several encouraging results. Prevention of diabetes in experimental animals is possible by giving plasmid DNA encoding for proinsulin [84,85] as well as for the insulin B chain [86]. Injection of plasmid DNA encoding for GAD has been shown to be effective in preventing diabetes in NOD mice [87], while others have achieved a similar effect by combining plasmid DNA encoding for a fusion protein consisting of both GAD, IgG and IL4 [88]. In mice, induction of GAD65-specific Tregs by the combination of GAD65 DNA vaccine and anti-CD3 antibody is enhanced in a genetic background favoring higher numbers of GAD65 precursor T cells [89], and in mice, the expression of GAD65 in conjunction with the pro-apoptotic protein BAX leads to better protection [90]. Treatment with a recombinant vaccinia virus expressing GAD (rVV-GAD65) has also been shown to be effective in the prevention of autoimmune diabetes in NOD mice by induction of the active suppression of effector T cells [91]. IgG1 antibodies increased while the IgG2 subtype was unchanged, and IL-4 increased, also suggesting a Th2 deviation. The increase of IgG1 antibodies may be positive as it has been shown that administration of anti-GAD antibodies can prevent the development of diabetes in NOD mice [92]. The preventive effect was almost complete when the treatment was given before development of insulitis, whereas immunization after the development of insulitis had no effect. Enough large dose was critical for the effect.

Before clinical use there are several unresolved problems. We do not know the mechanisms by which DNA vaccines may work in autoimmune disease, and correct dosing is crucial, as a wrong dose can lead to an increased immune response and a more aggressive disease process [92–94]. Furthermore, it is important to be sure that the DNA is not integrated into the host chromosome. Another problem might be production of antibodies against that DNA.

β-cell regeneration

The common view has been that when somebody develops diabetes there is no longer any capacity for the β cells to regenerate. However, in addition to the evidence for loss of β cells in the human pancreas being weak, there has been a lack of studies on β-cell regeneration in the human pancreas. In recent years some studies have suggested that the old paradigm may be wrong and that β cells can regenerate [7,8]. One substance that might stimulate β cell regeneration is glucagon-like peptide (GLP)-1 [95]. In a clinical trial the authors tried a GLP-1 agonist (exenatide) in combination with monoclonal antibodies interfering with IL-2 (daclizumab) given to patients with longstanding T1D, but with some residual insulin secretion, to see whether the treatment could improve C-peptide as a sign of more functioning β cells. The study was scientifically well done, but the result was negative [96].

A peptide called islet neogeneis associated protein (INGAP), has been found to have pancreatic regenerative capacity. Administration of INGAP in animals has caused increased β-cell mass and reversal of hyperglycemia, and it is hoped that INGAP may have regenerating potential in humans. Daily introduction of INGAP or placebo has been tried in a double-blind, randomized trial in both Type 1 and Type 2 diabetic patients [97]. A per-protocol analysis showed increased arginine-stimulated C-peptide during the treatment period, but this effect was not seen 30 days after the end of treatment, which is surprising if the results are interpreted as a sign of increased β-cell mass. Furthermore, the drop-out rate was high, mainly because of adverse effects on the injection site in the actively treated group, and intention-to-treat analyses showed no effect on C-peptide. Nonetheless, the results are interesting.

Future perspective

Vaccines in diabetes will perhaps soon be part of clinical practice. To date, GAD-alum treatment is the only autoantigen administration that has shown efficacy in clinical Phase II studies of young T1D patients with recent disease onset, while treatment with DiaPep277, from the heat shock proteins, has shown encouraging results, mainly in adults. Both these treatments are

extremely simple, very well tolerated by the patients and have so far shown no treatment-related adverse events. However, this is no guarantee for long-term safety. We cannot exclude that GAD treatment will lead to adverse events in the long run, related to its role in the CNS. There are ongoing Phase III trials on GAD in both Europe and the USA in patients with recent onset of T1D, as well as other studies that are being performed (e.g., by TrialNet), and a Phase III trial also using DiaPep277. If the efficacy of GAD-alum treatment can be confirmed this is proof of concept that administration of an autoantigen may be a way of creating tolerance and decrease an autoimmune process. This will then be the first step in a process testing dosing, intervals, duration of treatment, combinations with different autoantigens and perhaps also combinations with other treatment modalities. This could result in not only a milder disease, but perhaps that some patents go into remission. Treatment in high-risk individuals may perhaps prevent some cases of the disease. DNA vaccines may be found to be another effective way of creating tolerance against different autoantigens and might be combined with plasmid DNA encoding for Tregs. Finally, we must not give up our efforts to identify etiological factors behind the disease process, which may finally lead to primary prevention with, for example, vaccines against diabetogenic virus strains.

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