Review

Restoration of self-tolerance is a feasible approach to control ongoing beta-cell specific autoreactivity: its relevance for treatment in established diabetes and islet transplantation

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Abstract

Major progress has been made over the last 10 years in understanding the immune mechanisms underlying autoimmune Type I (insulin-dependent) diabetes mellitus. It seems that immunotherapy is the best approach for preventing and/or arresting beta cell destruction. For immunotherapeutic approaches to be clinically applicable to Type I diabetes, they cannot rely on the use of non-specific immunosuppressants. Their relative ineffectiveness over the long term and the risk of recurrence, once the drug is withdrawn, requires permanent drug treatment which augments

problems of infection and drug toxicity. One solution is to selectively tackle the pathogenic immune response. Operationally, this requires establishing immunological tolerance, i.e. a state of durable antigen-specific unresponsiveness in the absence of generalised immunosuppression. This review aims to present the rationale and clinical applicability of possible immunointervention strategies in Type I diabetes aimed at restoring self-tolerance to beta cells. [Diabetologia (2001) 44: 521–536]

Keywords Autoimmunity, immunotherapy, self tolerance, CD3 antibodies, autoantigens.

Type I (insulin-dependent) diabetes mellitus is an autoimmune disease that is genetically driven and leads to selective destruction of insulin secreting beta cells [1–4]. Major insights into the immunopathological mechanisms have been found through studies of animals with spontaneous diabetes such as the bio breeding (BB) rat and the non obese diabetic (NOD) mouse which share many similarities with the disease in humans [5, 6]. Compelling evidence indicates the central pathogenic role of CD4⁺ and CD8⁺ T lymphocytes that are specific for beta-cell antigens

[2, 3, 5, 7–10]. Several autoantigens have been identified, that play a part in the pathogenic autoimmune response i.e., insulin and proinsulin [11–17], glutamic acid decarboxylase [GAD] [18–21], IA-2 [22–26], the heat shock protein hsp 60 [27–31]. Major susceptibility genes are located within the class II major histocompatibility loci in rodents and humans. These include RT1 Class II(u) in the rat, I-A^{g7} in the NOD mouse and HLA DR3/ DR4 in humans [1–4]. In the NOD mouse it has been suggested that intrinsic structural and biochemical features of the I-A^{g7} molecule are implicated in the high degree of spontaneous T-cell autoreactivity [32–34].

Current treatment of Type I diabetes essentially involves the use of exogenous insulin. The modalities of this palliative replacement therapy have improved, progressively leading to a much lower incidence and severity of long-term complications, but not abolishing them. In this context immunotherapy could be considered as the most satisfactory solution to the prevention and cure of Type I diabetes. The spectacular results obtained in the NOD mouse and the BB

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Abbreviations: NOD, Non Obese Diabetic; BB, Bio Breeding; GAD, Glutamic acid decarboxylase; Hsp, Heat shock protein; LCMV, Lymphochoriomeningitis virus; MHC, Major histocompatibility complex; TCR, T cell receptor; EAE, Experimental allergic encephalomyelitis; ALS, Antilymphocyte serum; IL-Interleukin; IFN, Interferon; GM-CSF, Granulocyte macrophage colony stimulating factor.

rat as well as the proof of principle shown in humans by cyclosporine trials have supported the attempts to develop this strategy [1–3].

Problems that have, however, dampened the initial enthusiasm and slowed the progression of clinical programs, include side effects. In a clinical condition like Type I diabetes, it is impossible to accept any life-threatening or even organ-threatening hazard. Conventional agents available for immunotherapy have been essentially small chemicals (corticosteroids, azathioprine, methotrexate, cyclophosphamide, cyclosporin), which have non-specific activities (i.e., unrelated to the antigens involved in the pathogenic responses) and affect a very broad range of immune responses. A major drawback of these agents is their relative ineffectiveness over the long-term and the likely risk of the pathogenic immune process recurring after the drug is withdrawn. This requires indefinite drug treatment with the dangers of drug toxicity and recurrent infections. In a discussion of risks and benefits, these side effects, which have always been considered acceptable in the field of transplantation, are totally unacceptable in Type I diabetes. This is supported by the results of cyclosporin trials carried out in patients with recent onset diabetes [35, 36]. The potential risks associated with the chronic use of cyclosporin, which mainly include drug toxicity (nephrotoxicity) and overimmunosuppression, were kept under control for relatively short periods of treatment (about one year) [35, 36]. But Type I diabetes is a chronic disease and the general finding was that stopping non-specific immunosuppression inevitably led, in the vast majority of patients, to prompt relapses with no possibility for a lifelong treatment.

We believe that the best method to circumvent these difficulties is to give treatment over short periods of time yet affording long-term antigen-specific therapeutic effects. From an operational point of view this implies restoring in the autoimmune host the lost self-tolerance to beta-cell autoantigens. The central questions are then whether this is feasible in a reproducible way once overt disease is established (i.e., once the aggressive autoimmune response has already destroyed a great proportion of target beta cells) and whether there are safe clinically applicable therapeutic means to restore self-tolerance in adult diabetic subjects.

Another difficulty is linked to the very narrow window of time that elapses between the onset of clinically overt diabetes and the final destruction of most or all beta cells. This peculiarity of Type I diabetes, compared with other autoimmune diseases, leads to considering prediabetic patients, whose diagnosis has now become reliable thanks to specific serologic and genetic markers, as a population of choice for immunointervention. This approach is complicated and demanding due to the logistics of the high scale

screening needed, i.e. firstly, thousands of diabetics' relatives need to be recruited to enroll the adequate number of subjects and secondly, a long period of time is required to complete the trial (subjects in the control group show the disease in sufficient numbers only after an average of 4 to 6 years).

We shall discuss here the various strategies that can be used to induce tolerance to beta-cell autoantigens. Before doing so we shall briefly review present knowledge on the mechanisms of self-tolerance considered a basic feature of the immune system.

Mechanisms sustaining self-tolerance in healthy subjects

Physiological tolerance is the state in healthy subjects, where the immune system does not increase pathogenic self-destructive immune responses against the organism harbouring it. That autoimmune diseases do not develop in the healthy host was long interpreted as the result of simple elimination of all autoreactive B and T lymphocytes at the time of their differentiation in the central lymphoid organs, namely the bone marrow and the thymus (central clonal deletion) [37]. There is now definite proof, especially through the study of transgenic mice, that negative selection of developing B and T lymphocytes is one major mechanism eliminating autoreactive cells. It also has become evident, however, that such selection is far from complete because it involves lymphocytes bearing receptors having high affinity for the autoantigens expressed within central lymphoid organs. Thus with T cells, the main actors in Type I diabetes, although the array of autoantigens expressed in the thymus seems wider than initially thought (thymic expression of various beta-cell autoantigens such as proinsulin [38, 39], I-A2, and GAD was shown not only as mRNA but also at the protein level), islet autoantigen reactive T cells, presumably of low affinity, are found in high proportions at the periphery in healthy subjects [40]. These potentially harmful T cells that had escaped thymic selection are held in check by various immune mechanisms, grouped under the term "peripheral tolerance". These mechanisms include deletional and non-deletional processes that probably operate in variable proportions depending on the situation. Non-deletional mechanisms mainly include indifference, anergy, and T-cell mediated immunoregulation.

Indifference applies to the situation in which autoreactive T cells do not "see" the autoantigen due to its nonprofessional presentation (in the absence of costimulatory signals). This has been experimentally proven using double transgenic mice expressing a protein of the lymphochoriomeningitis (LCMV) virus in their beta cells (which in this case could be considered as an autoantigen) and a high proportion of

peripheral CD8⁺ cells expressing the T-cell receptor specific for the LCMV protein [41]. These animals were completely disease free and as such reflect the situation found in most healthy subjects in whom beta-cell autoantigen reactive T lymphocytes “peacefully” coexist with the target organ with no evidence of ongoing immune activation [41]. This study proposed that beta cells, that do not express class II MHC molecules or costimulatory molecules, were poor at presenting the autoantigen to autoreactive T cells and thus could not elicit their activation. This was further supported by the fact that destructive insulitis and diabetes were very effectively triggered in these mice upon LCMV infection, a situation in which the viral antigens are presented by professional dendritic cells [41].

T cell anergy differs from ‘indifference’ in that the cells are only partially activated and as a result of this deficient signalling are not able to respond to the specific antigen by proliferating or producing cytokines, while remaining fully responsive to polyclonal stimulation (i.e., a stimulant that activates all T lymphocytes in a non-antigen-specific manner). According to the classic definition, *in vitro* anergized T cells are elicited upon encounter of the antigen in the absence of co-stimulation and because the partially activated cells express the IL-2 receptor, the phenomenon becomes reversible upon addition of exogenous IL-2 [37]. So far, with the exception of some TCR transgenic models or in the situation occurring after superantigen treatment (i.e., bacterial or endogenous retrovirus products which activate CD4⁺ T cells by cross-linking the beta chain of the TCR to MHC class II molecules), it has been difficult to prove that anergy is operational in models of spontaneous self-tolerance.

T-cell mediated immunoregulation is a mechanism of peripheral tolerance particularly relevant to our discussion because it represents a privileged target for immunointervention strategies that have been successfully applied in experimental models of Type I diabetes which are being clinically tested. The major role of specialized subsets of immunoregulatory T cells in controlling, down modulating or suppressing the pathogenic potential of autoreactive T lymphocytes has been established in a number of models [42–44] including spontaneous and induced Type I diabetes.

In the case of NOD mice there is compelling evidence that prediabetic mice harbour immunoregulatory T cells and that a decrease in their numbers or their functional capacity, or both, is a major event contributing to the progression to overt disease. Co-transfer experiments have shown that CD4⁺ T splenocytes from prediabetic mice fully prevent disease transfer by diabetogenic cells (from the spleen of diabetic NOD) into immunoincompetent recipients [45, 46]. The presence of these immunoregulatory

T cells explains why diabetes transfer is only obtained in NOD immunodeficient recipients, i.e., NOD neonates, NOD scid [congenic NOD mice that are lymphocyte deficient due to the expression of the severe combined immunodeficiency (scid) mutation] or young adult NODs that have been either sublethally irradiated [2, 9, 45, 47] or thymectomized (as adults) and treated with depleting antibodies to CD4, a co-receptor for helper T cells [48]. Lastly, cyclophosphamide (an alkylating agent that in different models, including delayed type hypersensitivity [49] and experimental allergic encephalomyelitis (EAE) [50–53]) has been shown to selectively affect T-cell dependent regulation and triggers acute diabetes within 2 weeks of injection into young prediabetic NOD mice [2, 47, 54]. A mere toxic effect on beta cells has been excluded by a study showing that cyclophosphamide-induced Type I diabetes is prevented by the transfusion of mononuclear cells from non-diabetic NOD mice [54]. Further characterisation of these immunoregulatory CD4⁺ T cells that protect against diabetes transfer showed that they also express L-Selectin (CD62L) and more recent data also pointed to their expression of CD25 (the α chain of the IL-2 receptor). Immunoregulatory T cells concentrate in the thymus of 3 week old NODs, explaining why thymectomy of female NOD at this young age accelerates disease onset [55]. In the thymus these T cells concentrate within the mature mainstream CD4⁺CD62L⁺ compartment. These phenotypic characteristics clearly distinguish them from NK T cells, which express an activated phenotype (CD62L⁻) and are deficient in NOD mice [56, 57]. The data available indicate that the regulatory effect of these CD4⁺CD62L⁺CD25⁺ is not mediated through the production of T helper (Th) 2 type cytokines such as IL-4 or IL-10, or both, which when given systemically [58, 59] or overexpressed in beta cells (in the case of IL-4 but not of IL-10) [60] were shown to inhibit Type I diabetes.

The BB diabetes prone rat (BB-DP) is congenitally lymphopenic and this lymphopenia particularly concerns a subset of immunoregulatory T lymphocytes expressing the RT6 alloantigen [61, 62]. Administration of depleting antibodies to RT6 in BB resistant (BB-DR) rats, derived from BB-DP, that are not lymphopenic, exhibit normal proportions of RT6⁺ T cells and are protected from disease, triggers acute diabetes [63]. Moreover, diabetes in BB diabetes prone rats is prevented by the transfer of syngeneic RT6⁺ T cells [64].

Similarly, T-cell mediated Type I diabetes could be induced in susceptible rats (RTu haplotype) following thymectomy and γ irradiation (a treatment that promotes lymphopenia). In these animals Type I diabetes is effectively prevented by transfer of CD4⁺RT6⁺ cells, also staining TCR $\alpha\beta$ ⁺Thy⁺OX40⁺CD45RC^{low} from syngeneic untreated animals [65].

Proposed strategies to restore beta-cell tolerance in animal models of diabetes

A high number of therapies have been applied in NOD mice which successfully prevented the onset of diabetes [66]. The variety of approaches applied are quite vast (Table 1). Among them we shall concentrate on the administration of candidate autoantigens, the use of monoclonal antibodies to selected T-cell receptors and two transplant situations, conventional allogeneic bone marrow transplantation and inoculation of syngeneic islets directly into the thymus.

Although among all the potential candidates, it has been impossible to identify one beta-cell autoantigen that fulfills the criteria of the triggering antigen, the impressive finding was that all of them were highly effective in preventing NOD disease. This has been shown to be the case for GAD65 given either intravenously or intrathymically and for GAD67 [21, 67, 68]. Initial studies were done using the whole antigen molecules but comparable results were obtained with some of the GAD peptides. Protection was also obtained by insulin treatment via the intravenous, subcutaneous, oral or nasal route [14, 15, 69–76]. Of note, administration of the purified insulin beta chain resulted in the same therapeutic effect as that of the native hormone, thus supporting the role of an immune-mediated rather than a metabolically-mediated effect. [15, 69, 70, 73, 74]. Finally, heat shock protein 60, the autoantigen initially described and extensively characterized by the group of I. Cohen, and particularly one of its constituent peptides termed p277, also exhibited a clearcut capacity to prevent disease in NOD [28, 29].

The *in vivo* restoration of self-tolerance induced by the autoantigens was associated with a decreased responsiveness to the tolerogen, as assessed by proliferation, that also spread to the other locally expressed candidate autoantigens [21, 67, 77]. A similar phenomenon was initially described in the EAE model and was termed bystander suppression [78]. In several situations the protection observed upon administration of autoantigens (either whole molecules or protective peptides) was associated with a Th2 immune deviation. Thus specific T cells preferentially produced IL-4, IL-5 and IL-10 upon *in vitro* stimulation [31, 79, 80] and the specific beta cell response preferentially included immunoglobulins expressing Th2-dependent isotypes such as IgG1 [31, 77]. In some cases the antigen-induced protection was also shown to be transferable to naive recipients, by the infusion of CD4⁺ T cells from tolerant hosts, thus confirming active tolerance as a major mechanism of action [71].

Therapeutic monoclonal antibodies to T-cell surface receptors, produced by hybridomas, were initially developed in the early 1980's as an alternative to

Table 1. Antibody-based immunotherapy of spontaneous Type I diabetes

Antibody	Prevention	Effective Treatment of established disease	Reference
NOD Mouse			
Polyclonal ALS	ND	+	[126]
Monoclonal Antibodies			
CD3	+	+	[125, 145]
TCR $\alpha\beta$	+	–	[106]
CD4	+	–	[103]
CD8	+	–	[189]
CD40Ligand	+	–	[107]
CD28	+	–	[190]
CD25 (IL-2Rec.)	+	ND	[191]
MHC ClassII	+	ND	[102]
anti-Interleukin 6	+	ND	[192]
anti-IFN γ	+	ND	[193]
ICAM-1/LFA-1	+	ND	[194, 195]
VLA4	+	ND	[196]
BB Rat			
Polyclonal ALS	+	+	[197]
Monoclonal Antibodies			
CD25 (IL-2Rec.)	+	ND	[198]
anti-IFN γ	+	ND	[199]
MHC ClassII	+	ND	[200]

polyclonal antilymphocyte serum (ALS) [81, 82]. They included homogeneous sets of antibodies with predefined specificity for a single antigenic determinant or epitope. The very restricted specificity of these antibody preparations ensures a lack of toxicity which has been confirmed in monkeys for several antibodies [83–85]. Of note, like ALS, some monoclonal antibodies showed tolerogenic properties in various transplantation and autoimmunity models. In the transplantation setting the tolerogenic properties of both polyclonal and monoclonal anti-T cell antibodies was shown not only in rodents but also in valid preclinical models such as non-human primates [86–90, 90–100].

Prevention of diabetes onset has been reported for a number of monoclonal antibodies given early in the development of the spontaneous disease, *i.e.* in neonates [101] and in young prediabetic NOD mice [102, 103]. In some cases monoclonal antibodies have also been given to prevent accelerated forms of the disease such as those induced upon diabetogenic cell transfer [104] or cyclophosphamide treatment [105]. The antibodies that showed the highest efficacy were those targeting receptors involved in antigen recognition such as major histocompatibility molecules (MHC), the CD3/T cell receptor (TCR) complex and its co-receptors as well as the receptors involved in the transduction of costimulatory signals. Thus, major effects were reported with antibodies to MHC class II [45], TCR $\alpha\beta$ [106], CD3 [101], CD4, CD8 [103–105] and CD40L [107].

Two interesting transplant procedures have also been shown to effectively prevent NOD disease. The first concerns the replacement of the hematopoietic system of the autoimmune recipients by whole body irradiation and conventional bone marrow transplantation [108]. NOD mice (6 months old) irradiated and reconstituted with bone marrow cells from young BALB/c mice did not have insulinitis or overt diabetes [108]. NOD mice, reconstituted with BALB/c bone marrow cells showed normal T-cell and B-cell functions.

These results imply that the basic defect driving the disease is already present at the stem-cell level. Recent data have pointed out that B cells could play an important part in autoantigen presentation in diabetes. In fact, NOD mice that are totally deprived of B cells upon inactivation of the immunoglobulin genes after homologous recombination do not have insulinitis or diabetes [109, 110].

The suitability of the thymus as a “privileged” transplant site was initially described in conventional allogeneic transplant models [111]. The authors reported durable survival of allogeneic islets implanted in the thymus of young (8 week old) adult rats (rendered diabetic after streptozotocin treatment) under the cover of a single injection of polyclonal ALS [111]. The ALS treatment alone did not assure the survival of an identical number of allogeneic islets implanted into an extrathymic site [111]. Alloantigen-specific tolerance was instituted in recipients of the intrathymic grafts because they accepted long-term secondary islet grafts from the same original donor implanted extra-thymically while they rejected those from a third party [111]. The effect was at least partly ascribed to the central deletion of alloreactive T-cell effectors. Further data suggested that the antigen presenting cells within the islet allografts were the mediators of the tolerogenic effect. Thus *in vitro* cultured islets (deprived of antigen presenting cells) survived indefinitely within the thymus of allogeneic recipients, probably because of their lower immunogenicity, but could not induce tolerance as shown by the rapid rejection of second donor islet grafts implanted extra-thymically [112].

This strategy was then applied to the autoimmune situation where it was shown that prevention of insulinitis and diabetes in BB diabetes-prone rats followed the intrathymic implantation of syngeneic islets from BB diabetes-resistant rats [113]. Similar findings were reported in NOD mice when syngeneic islets were intrathymically transplanted at birth or within the first few weeks of life [114–116]. This effect is quite remarkable in confirming the “immune privilege” of the thymus. In fact in experimental and human diabetes, extrathymic implantation of syngeneic islets into autoimmune recipients was associated with their rapid destruction from the recurrence of the autoimmune disease. This is even more striking

considering that the effect is obtained in the complete absence of immunosuppression. Concerning the mechanisms, it is plausible that, as discussed for the allogeneic situation, at least part of the effect is mediated through central deletion of autoreactive T cells. This, however, does not exclude that peripheral tolerance mechanisms such as immune deviation which have been implicated in the protection induced by the intrathymic inoculation of well characterized beta-cell autoantigens, could also operate in this model.

As we discussed previously, these results are of importance because they have improved our knowledge of the mechanisms underlying the disease. Their potential clinical application, however, raises major issues.

How is it possible to realistically choose among so many therapies which are mostly applied at a very early stage of the disease? Which ones could be the most appropriate for immunotherapy in humans?

It is quite evident that despite its effectiveness, allogeneic bone marrow transplantation is, at present, a completely unrealistic approach because of the life threatening risks associated with graft-versus-host disease. The situation could evolve in the future if recent data showing that effective bone marrow replacement can be obtained through the association of less myeloablative conditioning regimens [117], the regular use of T-cell depleted bone marrow (to avoid GVHD) [118] or the infusion of high stem cell doses [119] are confirmed.

The intrathymic implantation of beta-cell autoantigen(s), either in the form of intact islets or of better characterised antigenic molecules, are problematic because of the well known progressive involution of the thymus gland after puberty. It remains to be shown that the observations in very young rodents (even neonates in some situations) are reproducible in older and larger animals (such as pigs or monkeys) presenting with more or less involuted thymuses. Although the attempts to promote transplant tolerance in dogs through the intrathymic inoculation of donor splenocytes mostly failed [120, 121], there are reports of prolonged allogeneic microchimerism in non-human primates upon intrathymic inoculation of CD34⁺ donor bone marrow cells in the absence of associated immunosuppressive treatment [122].

Approaches based on either autoantigen treatment or the use of selected monoclonal antibodies are then left to be considered. The major problem is again that a good deal of skepticism was generated because the data was obtained mostly in very young prediabetic mice, posing the question of their therapeutic relevance to the human disease. In other words, which of these agents might still be efficient in the clinical context of patients who have already destroyed a great proportion of their insulin secreting beta cells or, even worse, who are overtly diabetic?

One tentative answer to these questions is that the preclinical interest of the NOD model is closely related to the age of the animal. Although many antigen or antibody therapies are effective in very young mice (or what misleadingly some authors define as "overtly autoimmune animals" in referring to NOD mice with established insulinitis but no diabetes), only few strategies were shown to have a durable effect, when applied short-term, in more advanced disease, i.e., NOD mice older than 14 to 15 weeks or overtly diabetic mice. Before going into the details of these results on the use of some beta-cell autoantigens, CD3 antibody and ALS, the main issues that support the feasibility of treating patients presenting with overt Type I diabetes will be discussed.

The treatment of immunologically advanced disease and of recently diagnosed Type I diabetes

Ideally one would prefer to use immunotherapy in prediabetic patients who still have sufficient beta-cell mass. These patients can be identified with good reliability using islet specific antibody markers which could even be improved when the meaning of autoantibody fine specificity and isotype pattern (Th1 versus Th2) is better known. In fact, immunotherapy of prediabetics is rendered difficult by the logistic problems linked to patient screening (very large cohorts needed) and by the unacceptable risk of accelerating the disease. Hence the interest in treating recently diagnosed patients. The central question is to determine whether there is a sufficient insulin secreting beta-cell mass left at the time of diabetes onset to allow, in case the immunological assault is efficiently controlled, recovery of an adequate metabolic balance over the long-term.

There are few data on Type I diabetic patients to answer this question precisely. In NOD mice recent data provided a very detailed and interesting scenario [123]. The beta-cell mass and rates of beta cell proliferation and insulin secretion were measured in NOD from 8 to 18 weeks of age (when a high proportion of the animals were diabetic). Beta-cell mass in 8-week-old NOD mice was 69% of control mice (NOD scid) and despite compensatory proliferation (as assessed by 5-bromo-2-deoxyuridine uptake) was reduced to 58% and 27% of control mice at 13 and 18 weeks of age, respectively. Thus, at the time of overt diabetes the residual beta-cell mass represented about 30% of control values [123]. Moreover, this study also showed that in 18-week-old diabetic NOD mice, insulin secretion was decreased to a greater degree than beta-cell mass, suggesting the presence of beta-cell dysfunction in addition to reduced mass.

This is in keeping with the notion that the physical destruction of beta cells is to a large extent preceded

by a phase of reversible T-cell-mediated inflammation which impairs the capacity to release insulin in response to conventional stimulations. Thus, heavily infiltrated pancreatic islets from non-diabetic 13-week-old female NOD failed to respond with insulin secretion to high glucose concentrations when examined immediately after the isolation. This inhibition of beta-cell function was, however, fully reversible upon clearing of the immune cell infiltrate after 7 days of *in vitro* culture [124].

Similarly, *in vivo*, it is sufficient to administer agents that clear the insulinitis rapidly and completely, within the first 2 to 7 days of overt diabetes onset, to induce complete normalisation of glycaemia within no more than 24 to 48 hours. This was the case with antibodies to TCR [106], CD3 [125], and with ALS [126] and of course independently from the more durable tolerogenic effect that some of these treatments might have. Thus, at variance with CD3 antibody and ALS, antibodies to TCR $\alpha\beta$ do rapidly reverse hyperglycaemia despite that they do not restore self-tolerance, as shown by the disease relapse observed within a few days of the end of treatment [106]. As a whole these results could also correlate with data showing that *in vitro* T lymphocytes from patients with Type I diabetes suppressed glucose-stimulated insulin release by normal isolated islet preparations [127]. In humans, autoantibodies have been reported as being, at least in part, responsible for inhibition of the first phase of glucose-induced insulin release [128, 129].

Thus, these data implying a stage during development of Type I diabetes when beta cell destruction can be counteracted and beta cell function restored, provided the immune aggression is arrested, constitute a solid rationale for immunointervention in overt Type I diabetes. This conclusion was fully supported clinically by the steady and durable increase in endogenous insulin production observed after cyclosporin treatment in recently diagnosed Type I diabetic patients [35, 36]. Adult patients were enrolled to receive cyclosporin or placebo within approximately 1 to 2 months of the appearance of the first clinical symptoms. The two trials, one conducted in France and the other in Canada and Europe [35, 36], disclosed a proportion of disease remission, as assessed by stimulated C-peptide production and decrease of the insulin need, that was higher in patients receiving the active drug as compared with patients given placebo. In placebo-treated patients the transient restoration of C-peptide production was probably due to the correction of hyperglycaemia by insulin treatment and subsequent reduction in beta-cell glucotoxicity and beta-cell rest [130]. The state of insulin independence that was regained in a large percentage of cases can be increased (up to 70%) if treatment is started earlier [131]. Even if cyclosporin was not active in all patients the results obtained suggest that stopping the progression of T cell-mediated aggres-

sion of beta cells could have a real clinical benefit as assessed by improved control of diabetes (HbA_{1c}) and decreased insulin-related constraints and side effects. Cyclosporin, however, was not the ideal drug due to its side effects, namely short-term [132, 133] and long-term nephrotoxicity [134, 135]. In fact the treatment was reduced and discontinued in all patients after 12 to 18 months. This was associated with disease relapse since no tolerance had been induced.

Treatment of advanced disease with beta-cell autoantigens

Most of the data have been obtained through the administration of the whole antigens or the antigenic peptides, very early in the development of NOD disease. However, the administration of antigenic peptides at a later phase still exerted a therapeutic effect. This has been the case in particular for a GAD peptide that prevented the recurrence of disease on syngeneic islet grafts in diabetic mice for up to 2 months [79]. Similarly, administration of the hsp p277 peptide up to 17 weeks of age, when most mice are already overtly diabetic, preserved the beta-cell mass in the absence of exogenous insulin treatment [29].

Clinical Application. On the basis of these experimental results, various clinical trials have been initiated. In prediabetic subjects i.e. at-risk relatives of patients with Type I diabetes, promising phase I studies tested the therapeutic efficacy of subcutaneous or oral insulin [136, 137]. In addition, a large-scale preventive study (DPT1) has been started and its results should be available in approximately 3 to 4 years.

A phase I trial using the whole GAD 65 human molecule in prediabetic subjects has also been recently started.

Phase I/II trials in recently diagnosed diabetic subjects using the p277 peptide of hsp 60 have been completed or are in progress, with promising preliminary results in terms of safety and pharmacodynamics.

Potential risks associated with this form of therapy are those of immunomodulating the disease toward aggravation rather than the expected protection. This would be particularly troublesome for prediabetic subjects. Although this possibility is experimentally shown by the acceleration of the disease observed upon autoantigen treatment in extreme conditions using a transgenic mouse model [138], it has not been observed in patients, on the basis of presently available data.

In the case of the trials using the p277 peptide in patients presenting with recent onset diabetes, the therapeutic effect could be insufficient or need a relatively long time to be established and thus inefficient in controlling aggressive disease.

Reversal of established disease with CD3 antibody

Antibodies to CD3 are potent immunosuppressive agents that act by inducing partial depletion and by reversibly clearing CD3/TCR complexes from the T-cell membrane by antigenic modulation [139–141]. Antibodies to human CD3 (OKT3) have been widely used in clinical transplantation for both the prevention and treatment of organ allograft rejection [142–144]. Apart from their immunosuppressive properties, CD3 antibodies can also express tolerogenic properties. In the rat, a CD3 antibody induced permanent engraftment of histoincompatible vascularized heart grafts and permanent tolerance as assessed through the survival of donor skin grafts whereas third party grafts were normally rejected [86]. This strategy was recently extended to non-human primates. A CD3 immunotoxin administered to Rhesus monkeys, associated with donor bone marrow cells or 15-deoxyspergualin, induced long-term survival of completely mismatched renal allografts [98, 99]. The same authors implanted three monkeys presenting with spontaneous Type I diabetes with xenogeneic pancreatic islets under the cover of two injections of CD3 immunotoxin supplemented with cyclosporine and steroids administered on days 0 to 4. No additional immunosuppression was given thereafter. All three islet recipients remained euglycaemic at 410, 255, and 100 days of follow-up despite recovery of peripheral T cells to normal proportions [97].

Data from our laboratory showed that in NOD mice CD3 antibodies could reverse recent onset disease and restore tolerance to beta-cell antigens [125, 145]. In NOD mice presenting with full-blown diabetes, as assessed by the presence of hyperglycaemia (≥ 4 g/l) and glycosuria, a consecutive 5 day treatment with low doses (5 to 20 μg) of the hamster anti-CD3 monoclonal antibody 145 2C11 [146] induced complete disease remission as assessed by progressive (within 2 to 4 weeks) return to normoglycaemia in the absence of insulin treatment [125, 145]. The remission was durable (mice were followed up for 8 to 10 months) and not related to long standing immunosuppression since, by 8 weeks from the last injection, CD3 antibody-treated mice were fully immunocompetent as assessed by their capacity to reject histoincompatible skin grafts [125]. We also showed that the effect observed was antigen specific because syngeneic islet grafts were not destroyed when implanted in CD3 antibody-treated animals, at variance with what was observed in untreated, overtly diabetic control NOD female mice [125]. Syngeneic islets led to full metabolic reconstitution of NOD mice which were still diabetic 4 to 5 weeks after CD3 antibody treatment. This stresses that mice showing CD3 antibody-mediated arrest of the autoimmune process could still be hyperglycaemic if the beta-cell mass left at the time the treatment is instituted was insuffi-

cient to guarantee the return to a normal metabolic balance. In our experience the treatment had to be started within 7 days from the detection of the first signs of overt diabetes [125].

Similar data (remission of disease and restoration of self tolerance in overtly diabetic NOD mice) have also been reported using ALS [126]. At variance with CD3 antibody, however, profound and quite prolonged T-cell depletion was required to achieve long-term remission of established diabetes [126].

The immune mechanisms that could account for the therapeutic effect of CD3 antibody in Type I diabetes are only partially understood. Our present data favor both the partial physical removal of the beta-cell specific autoreactive lymphocytes (particularly those infiltrating the islets at the time of treatment institution) and the restoration of T-cell mediated immunoregulation. Thus, within the first 24 to 48 hours of treatment the insulinitis is completely cleared, which probably explains the rapid reversal of hyperglycaemia. Within two weeks of the last injection the insulinitis, including CD3⁺αβ⁺CD4⁺ and CD8⁺ cells, progressively reappears but remains confined to the periphery of the islets with no clear signs of active beta-cell destruction [125, 145]. This is distinct from what is observed in untreated age-matched NOD mice that regularly have an invasive and destructive type of insulinitis. Protected animals, however, harbour high numbers of pathogenic T cells since effective Type I diabetes transfer is obtained through the infusion of their spleen cells into adult irradiated syngeneic recipients [145]. This observation also argues for the existence of immunoregulatory T cells controlling the pathogenic potential of diabetogenic effectors in protected mice. This is further supported by the recurrence of the disease upon cyclophosphamide injection [145] and the fact that CD4⁺CD62L⁺ spleen cells from CD3 antibody protected mice inhibit diabetes transfer afforded by diabetogenic cells (our unpublished results). The active nature of this tolerance and the possible involvement of cytokines is indirectly suggested by the abrogation of the CD3 antibody therapeutic effect when cyclosporin is administered concomitantly [145].

Conventional CD3 antibodies are mitogenic and when injected in vivo induce, in the experimental and clinical context, a massive though transient (lasting a few hours after only the first antibody injection) systemic release of several cytokines including TNF, IFNγ, IL-2, IL-3, IL-4, IL-6, IL-10 and GM-CSF [147, 148, 149, 155]. Peripheral T cells also transiently express some activation markers such as the α chain of the IL-2 receptor (CD25) and CD44 [156]. Although transient, this cytokine release is responsible for an acute "flu-like" syndrome, lasting 48 to 72 h after the first injection and in which the most frequent symptoms are high fever, chills, headache and repeated episodes of vomiting and diarrhea

[147–149]. This explains why despite the interesting experimental results described in NOD mice, conventional monoclonal antibodies to human CD3, such as OKT3, have not been used to treat patients with recent onset Type I diabetes.

This major problem could be overcome through molecular engineering design of humanised monoclonal antibodies. In fact, the mitogenic capacity of CD3 antibodies is monocyte-dependent and closely correlated with the capacity of the Fc antibody portion to interact with Fc receptors on monocyte and macrophages, probably facilitating cross-linking. This is why CD3 antibody F[ab']₂ fragments, which lack the Fc portion, are not mitogenic [157, 158]. Moreover, the mitogenic (proliferative) response of mouse monoclonal antibodies to human CD3 correlates with the affinity of the murine isotype for human monocyte Fc receptors (IgG2a >> IgG1 >> IgG2b >> IgA) [159]. This is in keeping with the pattern observed in vivo because, in mice, CD3 F[ab']₂ fragments of 145 2C11 are well tolerated and do not promote massive cytokine release [125, 145, 158] and, in transplanted patients, a murine IgA antibody to human CD3 did not promote the acute syndrome [160].

Of note, the experimental and the more recent clinical data confirmed that non-mitogenic CD3 antibodies fully retain their therapeutic activity [145, 158, 161–166].

These observations led to the design of humanised CDR-grafted CD3 antibodies engineered to include specific mutations within the constant Fc regions that abrogate their capacity to bind to Fc receptors and to elicit their mitogenic potential [167–169].

At present two humanised non-mitogenic CD3 antibodies are under study in pilot clinical trials in recent onset Type I diabetes.

One has been characterised and is an engineered version of OKT3 [169]. This humanised γOKT3–5 antibody has two mutations in its human Fc. It is not mitogenic in vitro and did not induce in vivo release of cytokines in an experimental model in which human splenocytes from human donors were inoculated into severe combined immunodeficient mice (hu-SPL-SCID mice) [169].

The other antibody, CAMPATH3, is derived from the rat YTH 12.5 [168, 170]. The CAMPATH3 antibody is an aglycosylated human IgG1. Both in vitro and in vivo studies confirmed its lack of mitogenicity [168]. In particular, when injected into transgenic mice expressing the human CD3-ε chain, CAMPATH3 did not induce the release of TNF, at variance with the original parental antibody [168].

Pilot clinical trials using both of these antibodies for the treatment of established renal allograft rejection episodes confirmed their good acute tolerance [165, 166]. Moreover, in both cases the therapeutic effectiveness as assessed by acute rejection reversal

[165, 166] was fully comparable to that described for OKT3 in the context of the preliminary pilot trials conducted in the early 1980's [142].

Two clinical trials have been started to evaluate the effect of these humanized CD3 antibodies in recently diagnosed diabetic subjects. The primary endpoint is the maintenance of endogenous insulin production (C peptide).

The specific problem of islet transplantation

Implantation of a sufficient amount of healthy islets through islet transplantation constitutes a radical cure for established Type I diabetes because it can restore normally regulated insulin secreting capacity and good metabolic control, thus limiting the incidence and severity of the degenerative complications commonly seen in diabetic patients who are on insulin treatment [171–173]. Islet transplantation also represents a safer alternative to whole pancreas transplantation [171–175]. The implantation technique for islet grafts is much simpler and studies in animals have shown that pretreatment of rodent islets can reduce their immunogenicity so that successful engraftment occurs in the absence of chronic immunosuppression. A similar achievement, however, has not yet been made in humans because of logistic and biologic difficulties.

Transplantation of islets or of beta cells in Type I diabetic patients remains a major challenge because, at variance with organ transplants into non-autoimmune hosts, this treatment faces the additional problem of reactivation of the memory anti-beta cell autoimmune aggressive response. The first indication of susceptibility of beta cells to recurrent autoimmunity originated from experiments on islet transplantation in the BB rat [176]. These initial observations were subsequently confirmed by other investigators who worked in the BB rat and also in the NOD model. Implantation of syngeneic islets under the kidney capsule of overtly diabetic BB rats and NOD mice is followed by total destruction of the islets within a few days [125, 177–180]. In the clinical setting this reactivation can be fast and vigorous, even if remaining dormant for several years, as indicated by the rapid destruction of a pancreas transplant between HLA identical twins [181–183].

So far, as in other organ transplantation, therapeutic approaches used in islet transplantation in humans have been based on a combination of immunosuppressive agents, most frequently chemicals such as cyclosporin or other immunophilin binding drugs, corticosteroids and azathioprine. These compounds act through non-specific mechanisms and therefore not only suppress the reactivity to transplant antigens but also to many other antigens not related to the

transplant. Their major drawback is the risk of over-immunosuppression.

Over the past years, progress has been made in the isolation of human tissue and the development of new immunosuppressive agents, leading to an increase in the success of islet transplantation [171–175]. This is shown by the encouraging results recently reported by Shapiro et al. (Personal Communication). Patients received allogeneic islets under the cover of corticosteroid-free triple immunosuppression including a humanized monoclonal antibody to the α chain of the IL-2 receptor, FK506 and mycophenolate. At 11 months of follow-up all patients were insulin-free while on immunosuppression maintenance.

The ideal therapeutic strategy would be that in which the immune system of the host can be selectively immunosuppressed to the alloantigens and autoantigens unique to the islet beta-cell targets, thus avoiding the hazards of long-term non-specific immunosuppression. Such a strategy would simultaneously avoid the destruction of the islet implant by the alloimmune response to donor specific antigens, the relapse of the autoimmune attack to beta-cell specific antigens and the need for continuous immunosuppressive treatment. If the therapies discussed above aimed at restoring self tolerance to beta cell antigens prove to be effective, they could represent a major tool to “condition” the recipients of allogeneic islet transplants. In this particular setting, where the islet transplant presents both the autoantigen and the alloantigen, restoring immune tolerance to the autoantigen could help in establishing tolerance to the alloantigen. In mice, tolerance to skin allografts can be induced by a short treatment with depleting or non-depleting antibodies to CD4 and CD8 [87, 88, 100, 184]. The underlying mechanisms for this immune tolerance are usually non-deletional, not based on physical elimination of the alloreactive effector T cells. Instead, the tolerant hosts harbour potent immunoregulatory T cells that effectively control the pathogenic activity of alloreactive effectors [87, 88, 100, 184]. Tolerance, once induced, is dominant or “infectious”, i.e., it can be acquired by non-tolerant T cells in the absence of further immunosuppression [100, 184] and it can also spread to additional alloantigenic determinants if all determinants are presented together on the same antigen presenting cell (APC), a phenomenon termed “linked suppression” [185]. When the determinants are presented by the same APC, tolerance to one antigen in one tissue allograft will facilitate tolerance to other alloantigens on other transplants if the different grafts are sharing some of their alloantigens [100, 184, 185]. There is no reason why linked suppression could not be exploited to damp T-cell responses to both autologous and allogeneic islet-specific antigens that are both shared by the implanted islet transplants.

The question arises as to whether there is any evidence for linked suppression as an operational mechanism in the islet transplant setting.

There is some interesting clinical evidence that could provide a positive answer to this question [172]. These authors conducted a trial using pools of in vitro cultured islet cells (and not of fresh intact islets) from different donors implanted into patients with Type I diabetes at variable times after a kidney transplantation. The immunosuppression applied at the time of the kidney transplant consisted of an association of conventional chemical agents such as cyclosporin, azathioprine and steroids, to which a prophylactic course of antithymocyte globulin (ATG) was added in some of the patients. In contrast to most clinical studies, in this protocol the islet implantation was done without any increase in the maintenance immunosuppressive regimen [172]. One major result was that all the patients who received ATG at the time of the kidney transplant exhibited long-term survival of the islet beta cell graft that allowed progressive tapering of insulin [172]. In contrast, patients who did not receive ATG at the time of the first kidney allograft rapidly rejected the beta cell transplants [172]. Moreover, in vitro T-cell analysis done before the islet implantation and at regular intervals thereafter disclosed that peripheral lymphocytes from patients harbouring the long-term surviving grafts showed a decreased frequency of both alloantigen and autoantigen specific T-cell precursors [186]. Conversely, in patients showing destruction of the islet implants, either one or both T-cell specific responses were detected at high frequency [186]. These data suggest that the long-term survival observed in a subset of the patients could be related to a state of operational tolerance.

Based on the knowledge of the field, three elements can be identified in this protocol which together could have had an impact on the long-term islet allograft survival observed:

Firstly, cultured islet beta cells are used instead of fresh islets. There is considerable evidence, at least in the experimental field, showing the decreased immunogenic capacity of cultured islets compared with freshly isolated islets due to the elimination of passenger leukocytes.

Secondly, a biological agent (ATG) is used at the time of the first transplant. Polyclonal antilymphocyte antibodies have been shown since the early 1970's to exert immunosuppressive and tolerogenic properties. Of note, pioneering work initially done using rodents [187, 188], was successfully extrapolated to relevant preclinical models in non-human primates [93–96].

Thirdly, sequential implantations of two allografts sharing HLA haplotypes were carried out which through linked suppression could have favored the "spreading" of tolerance from the kidney specific set

Table 2. Immunointervention strategies to induce tolerance in autoimmune diabetes

Strategy	Experimental models	Ongoing clinical trials
Administration of beta-cell autoantigens	+	+
Polyclonal anti-T cell antibodies	+	–
Monoclonal anti-T cell antibodies	+	+
Systemic delivery of immunosuppressive cytokine	+	–

of alloantigens to the islet specific set of alloantigens and autoantigens.

It will be of extreme importance to assess whether linked suppression could also operate in the kind of clinical situation we are discussing.

Conclusion

Experimental data indicate that tolerance to beta-cell antigens could be induced in prediabetic subjects and also in recently diagnosed diabetic subjects using distinct approaches (Table 2). Two of these strategies led to phase I and II clinical trials, namely the administration of soluble beta-cell antigens and of CD3 monoclonal antibodies. These approaches elicit much hope but might not be effective in the short-term or in the long-term unless both approaches are used together.

Other critical clinical problems are the difficulties in quantifying the remnant beta-cell mass at the time of diabetes onset as well as the risk of chronic beta-cell mass erosion and insulin resistance. The approach to these problems involves earlier treatment in prediabetics or in recent onset diabetics.

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