Review

The differentiation of the immune system towards anti-islet autoimmunity. Clinical prospects*

C. Boitard

Service d'Immunologie Clinique, INSERM 25, Hôpital Necker, Paris, France

More than one hundred years have elapsed since Oscar Minkowski and Joseph von Mehring first showed that total pancreatectomy induced diabetes mellitus in the dog [1]. Although Minkowski was actually studying the role of non-esterified fatty acids in fat absorption, he was performing the right experiment to establish the first link between the pancreas and Type 1 (insulin-dependent) diabetes mellitus. The link between the pancreas and insulin was established when Banting and Best characterized pancreatic extracts. We now have a good understanding of the mechanisms involved in the selective destruction of islet Beta cells in Type 1 diabetes, the most common form of insulin-dependent diabetes in Europe. Insulinitis was first discovered in 1965, when the analysis of pancreata from recent-onset Type 1 diabetic patients revealed an infiltration of the islets of Langerhans by mononuclear cells [2–4], most of which were found to be T lymphocytes expressing activation markers such as class II major histocompatibility complex (MHC) antigens or high-affinity interleukin-2 receptors [5]. CD8⁺ T cells are predominant in man, although they may reflect a late phase of the disease process [5, 6]. In the non-obese diabetic (NOD) mouse, which can be used for sequential analysis of insulinitis prior to the development of hyperglycaemia, an initial predominance of CD4⁺ T cells has now been reported [7, 8]. The next step was the discovery of islet cell antibodies in 1974 [9, 10]; whatever their pathophysiological relevance [11], autoantibodies provide the most reliable diagnostic marker for ongoing anti-islet autoimmunity in normoglycaemic subjects, with a predictive value which reaches 100% in first-degree relatives of Type 1 diabetic patients with high autoantibody titres [12–14]. Other autoantibodies have since been identified [15–17] which also have predictive value [18–20].

The third step was the identification of genetic markers for Type 1 diabetes. A genetic background to Type 1 diabetes was strongly suggested by the existence of clinical clusters within families and in twins, while an auto-

immune mechanism was indicated by the frequent association of Type 1 diabetes with known autoimmune diseases. The conjunction of underlying immune phenomena and the availability of markers for polymorphic structures essential for the cellular interactions involved in immune responses [21], led to the discovery of an association and linkage between insulin-dependent diabetes and defined MHC alleles [22, 23]. The association which was first noted with class I MHC alleles (B8, B18, B15) was later shown to be stronger with serological markers for DR-encoded (DR3, DR4) class II MHC alleles [24, 25]. Class II genes were characterized in the early 1970s as “immune-response genes” controlling reactions to synthetic antigens in the mouse [26] and were thus candidates for the control of autoimmune responses to organ-specific autoantigens [27, 28].

The fourth, logical step involved an immunological approach to the treatment of Type 1 diabetes [29]. Randomized trials were conducted using cyclosporin A [30, 31], azathioprine or steroids or both [32, 33]. Immunosuppression-induced remissions of insulin-dependence in terms of insulin requirement or C-peptide secretion, thus provided the best evidence so far for the role of autoimmunity in human Type 1 diabetes. But the efficacy of immunosuppression was transient, with relapses occurring within 6–40 months, thus precluding the use of conventional immunosuppression except in controlled trials. Both the efficacy and secondary failure of this approach probably result from an oversimplified view of the nature of the immunosuppression and the immune reaction to Beta cells, however, new directions for immunointervention derive from this work.

Anti-Beta cell immunity

The role of T lymphocytes in the development of anti-Beta cell autoimmunity is supported by the predominance of T cells within insulinitis, the association of diabetes with defined HLA antigens and the efficacy of T-cell-directed immunosuppression. Studies of human Type 1 diabetes

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come up against the absence of a known autoantigen on Beta cells and the lack of straightforward assays for self-reactive T cells in vitro. As a result, T cells have mostly been studied by enumerating total T-cell subsets or evaluating non-specific T-cell functions. The reported decrease in the CD4⁺/CD8⁺ T-cell ratio, the increase in circulating T cells expressing activation markers [34, 35] and the increase in CD4⁺ T cells expressing the CD45 R marker [36, 37] (which presumably mediates delayed-type hypersensitivity and suppressor/inducer functions), all show largely overlapping values with those found in healthy control subjects. This is also the case for normoglycaemic subjects at risk for Type 1 diabetes, precluding the use of these characteristics as diagnostic markers. The study of antigen-specific T cells by means of rosette formation with insulinoma cells [38, 39], cells blocking Beta-cell function in vitro [40, 41], cytotoxic cells and cells proliferating in the presence of human or xenogeneic islet cells or cell extracts [42] has not been conclusive, given the absence of defined MHC restriction of Beta-cell antigen presentation. The possible role of antigen-presenting cells present in the human cell preparations used, or lymphokines secreted in vitro, has not been thoroughly investigated. The recent demonstration that MHC-restricted autoreactive T cells specific for a 38-kDa granule protein or glutamate decarboxylase can be detected in Type 1 diabetic patients [43, 44] and cloned [45, 46] is fundamental. It will nevertheless be difficult to determine whether such clones play a primary role or are rather simply a secondary activation event in human diabetes. Most of what has been learnt about T-cell regulation of anti-islet immunity has come from animal models. Although a fine T-cell regulatory balance controlling BB rat disease has been described [47, 48], this review will refer mostly to the NOD mouse model.

The NOD mouse

The NOD mouse was first developed in Japan from a cross of JCR × JCL mice and brother-sister intercrosses. Most colonies show a female predominance of clinical diabetes. The pancreas remains free of islet infiltration up to 3–4 weeks of age, but an infiltrate mainly composed of CD4⁺ T cells then gradually develops. Macrophages are seen at an early stage of the insulinitis. Clinical diabetes develops after 10 weeks of age when lymphocytes penetrate the islets and CD8⁺ T cells are recruited. Controversial observations of early B-lymphocyte infiltration have been reported. The NOD mouse shows no significant changes in the CD4/CD8 T-cell ratio [49, 50]. Decreased natural killer cell activity, resistance to herpes virus, increased antibody production to thymo-dependent antigens, deficient activation of suppressor T cells in syngeneic mixed lymphocyte reactions (MLR) or of cytotoxic T cells in allogeneic or syngeneic MLR, and deficits in cytokine production have all been reported [51–54] but have made no clear contribution to our understanding of the disease process. Salivary and lacrymal gland infiltration, as well as thyroiditis, have been reported in the NOD mouse [55–

57], and autoantibodies have been detected against insulin and islet cell antigens [52, 58–62].

The NOD mouse MHC encodes a unique Aα^d/Aβ^{nod} class II antigen which differs from I-A^d by five amino acid substitutions in the first external domain of the β chain, including a serine in position 57 instead of the aspartic acid in conventional mouse strains [63–66]. Crosses between NOD and C57BL/10 mice, which do not express I-E molecules, identified a resistance MHC gene which is not related to I-E [67–69]. The role of I-E antigens in resistance to diabetes is supported by NOC crosses with transgenic Eα C57BL/6 mice and Eα^d transgenic NOD mice [70, 71]. However, following crosses of NON/Lt with the NOD strain, insulinitis (although not clinical diabetes) was seen in first-backcross animals expressing I-E antigens [63]. The transfer of Aβ transgenes carrying an aspartic acid at position 57 or a proline at position 56 (instead of histidine) into the NOD genetic background prevents both diabetes and insulinitis [72, 73]. The resistance conferred by I-A antigens carrying aspartic acid at position 57 is, however, questionable since (NOD × C57 BL/10) F1 chimeras grafted with NOD T-cell-depleted bone marrow show a high incidence of diabetes despite the expression of the I-Aβ^b chain within the recipient thymus [68]. The role of the MHC background in providing recessive or dominant genes with low penetrance is also controversial. Occasional heterozygotes for the MHC region develop clinical diabetes. Determining whether diabetes-related genes are susceptibility or resistance genes is likely to require the full characterization of non-MHC genes and their relationship with immune or islet functions. Genetic susceptibility regions have been defined outside the MHC, on chromosomes 1, 3 and 10, by studying crosses/backcrosses of NOD with NON/Lt C57BL/6J, or C57BL/10 mice [74–76] and further genes have been identified in NOD × spretus crosses [77]. This shows the existence of polygenic susceptibility which will have to be taken into account in predictive human genetic tests.

The effect of the NOD phenotype on bone marrow cells has been shown by the transfer of diabetes by NOD marrow stem cells into resistant (NOD × NON) F1 [78] or C3H/HeN [79] chimeras. Similarly, no diabetes or insulinitis was observed in Balb/c nu/nu bone marrow-reconstituted NOD [80, 81], or CBA/J bone marrow-reconstituted (NOD × CBA/J) F1 chimeras grafted with NOD islets [82]. In allophenic NOD-C57BL/6 chimeras, the development of insulinitis correlates with the NOD phenotype on lymphoid cells and the thymic cortex [83]. The protective role of allogeneic bone marrow antigen-presenting cells was finally evidenced in (NOD × NOD.H-2^{non}) F1 animals reconstituted with a mixture of NOD and NOD.H-2^{non} congenic bone marrow [84].

All immune reactions go through successive steps. The first is antigen presentation, involving both direct interactions between antigen-presenting cells and specific T-cell clones, and indirect interactions mediated by cytokines. Class II MHC antigens are the key structures involved in antigen presentation. The final stage involves effector T cells which directly interact with target cells, and B lymphocytes which produce antibodies. Successive regulatory steps take place between these initial and final phases.

Fig. 1. Characteristic features of diabetes transfer in the NOD mouse

Evidence from cloning experiments that autoreactive T cells are present in autoimmune conditions is fundamental to this scheme. The successive regulatory steps which take place in the development of diabetes in the NOD mouse need to be addressed both in the diabetic and non-diabetic states to understand why self-tolerance breaks down and to design strategies to restore tolerance.

The direct mechanism of Beta-cell destruction

Transfer models have been developed with which to study effector mechanisms of Beta-cell destruction. Efficient transfer of diabetes has been obtained following the injection of spleen cells from diabetic NOD donors into both 8-week-old male NOD recipients (which have a low incidence of diabetes [85]), and naive neonates (as spontaneous diabetes only develops after 10–12 weeks of age [86]). In adult recipients, pre-irradiation was required to achieve efficient transfer; spontaneous insulinitis due to recruitment of radioresistant host lymphoid cells may have interfered with transfer. In recipient neonates, the first event was the expression of class II MHC antigens on endothelial cells. Insulinitis was characterized by CD4⁺ T-cell infiltration followed by recruitment of CD8⁺ T cells [87, 88]. In both recipients, purified T cells from diabetic NOD mice provided efficient transfer. The recruitment of autoreactive B lymphocytes and autoantibody production was not required, since the treatment of recipients with a monoclonal antibody directed against the μ chain of IgM prior to and after birth did not reduce transfer efficiency despite Beta-cell depletion [89].

In human Type 1 diabetes the role of blocking or cytotoxic autoantibodies remains elusive, as they are not detected in all patients. The lack of strict Beta-cell specificity of islet cell antibodies rules out their participation in Beta-cell damage. No correlation has been found between the induction of remission upon treatment with cyclosporin A and the presence or pattern of islet cell antibodies [90, 91]. In the NOD mouse, islets exhibit an impaired glucose metabolism and insulin secretion which are reversed in vitro as islets become depleted of infiltrating cells [92].

Both CD4 and CD8⁺ T-cell subsets are required to transfer diabetes to neonate and adult NOD recipients [86, 93], as confirmed by the prevention of transfer by treatment of pre-irradiated adult recipients with anti-CD4, anti-CD8 [93–95] or anti-CD3 monoclonal antibodies. We used CD4- or CD8-depleted recipients (thymectomy at 3 weeks) treated with depleting anti-CD4 or anti-CD8 monoclonal antibody to determine the nature of the effector cells. No transfer was achieved with purified CD8⁺ T cells into CD4-depleted recipients, whereas a low efficiency of transfer was obtained by injecting purified CD4⁺ T cells into CD8-depleted recipients. However, CD8 depletion, although complete in the periphery as assessed by flow cytometry, was ineffective in islets, where 2–10% of CD8⁺ T cells were still present in histological sections.

Beyond the involvement of T cells, other effector mechanisms have been thought to mediate Beta-cell destruction. Allogeneic islet grafts devoid of class II antigen-expressing (passenger) leucocytes are not rejected through an allogeneic immune response, even in diabetic NOD mice which ultimately show recurrent CD4⁺ T-cell infiltration and diabetes across an MHC barrier [96, 97]. The role of non-MHC-restricted natural killer cells in the BB rat has also been suggested [98–100]. In vitro studies have shown that interleukin-1 exerts selective Beta-cell toxicity in vitro and inhibits insulin secretion by the isolated perfused pancreas [101, 102]. However, exposure to cytokines of syngeneic islets in contact with allogeneic islets grafted under the kidney capsule does not lead to their destruction in vivo [103] and treatment with tumour necrosis factor or interleukin 1 prevents diabetes in NOD mice [104–107]. The demonstration that K^d-restricted CD8⁺ T cells from diabetic NOD mice stimulated for 24 h by interleukin 2 in vitro lyse ⁵¹Cr-labelled islet cells [108] and the expression of perforin by islet-infiltrating CD8⁺ T cells on tissue sections [109], both suggest that CD8⁺ T cells are the main effector cells in the disease process, but they require activation by helper CD4⁺ T cells in transfer models [88, 110]. The need for a final activation step of effector T cells in the NOD mouse has been confirmed by the prevention of transfer by in vivo treatment of recipients with monoclonal antibodies directed against NOD class II MHC (I-A^{NOD}) antigens [111], anti-interferon γ [112, 113] and anti-macrophage monoclonal antibodies [114] (Fig. 1).

The primary activation step in the immunoregulatory cascade

The second issue we have addressed in diabetic mice is the level at which the primary activation of the anti-Beta cell immune reaction occurs in the T-cell regulatory cascade. We first investigated at which level of the immune cascade we could prevent the development of diabetes by hitting cell-interaction structures with monoclonal antibodies in vivo. The T-cell receptor is composed for two chains: α and β or γ and δ . Both include a monomorphic domain anchored to the cell membrane and a variable domain, the antigen recognition unit. Class II MHC antigens are also

Fig.2. The T cell regulatory cascade. Defining the primary activation step of the anti-Beta cell autoimmune reaction is a prerequisite of any logical strategy to induce tolerance. Any immune intervention beyond this step may prove useless in inducing long-term protection in susceptible individuals

composed of two chains, each with two extracellular domains which carry antigenic peptides on antigen-presenting cells. Anti-CD4 monoclonal antibody treatment prevents diabetes in the NOD mouse [115, 116] and other models [117, 118]. We similarly found that spontaneous Type 1 diabetes is prevented long term by treating female NOD animals with a monoclonal antibody directed against either the monomorphic domain of the T-cell receptor β chain [119] or private epitopes on NOD class II MHC antigens [111]. Thus, the interaction between antigen-presenting cells and CD4+ T cells is central to the autoimmune activation process.

The role of antigen expression in the primary activation of the anti-islet immune response was addressed by evaluating autoreactive T cells in Beta-cell deprived NOD mice in comparison with naive NOD animals. The capacity of T cells from diabetic NOD mice, but also from ageing non-diabetic animals, to transfer diabetes into pre-irradiated 8-week-old recipients was used as a marker of autoimmune activation. Beta-cell-deprived animals were obtained by a single injection of a toxic dose of alloxan at 3 weeks of age and were maintained on insulin treatment for up to 6 months. The activation of autoreactive T cells, evaluated at 6 months by the capacity of spleen T cells to transfer diabetes, was not seen in alloxan-treated animals. Alloxan or insulin treatment and sustained hyperglycaemia in spontaneously diabetic animals did not alter their capacity to transfer diabetes. Autoreactive T cells are thus not activated in autoantigen-deprived animals. The primary activation step traces back to an antigen-presenting cell-CD4 interaction and Beta-cell antigen expression (Fig. 2).

The role of regulatory T cells in the NOD mouse

There is indirect evidence that suppressor mechanisms may control the development of diabetes in non-diabetic animals. First, an acute form of diabetes is induced by cyclophosphamide in young NOD mice [120]; second, thymectomy at 3 weeks accelerates the development of diabetes in female animals [121]; finally, irradiation is required to achieve successful transfer of diabetes [85], showing a protection state in adult recipient animals. To evaluate this protection state, we reconstituted irradiated

adult recipients with lymphoid cells from non-diabetic animals and evaluated their capacity to protect against the transfer of diabetes. Reconstitution with spleen cells from non-diabetic 8-week-old NOD females was able to protect against the transfer of diabetes by spleen cells from diabetic animals. Protective spleen cells were shown to be CD4+ T cells and were not detected in mice thymectomized at 3 weeks, or in NOD neonates, indicating either that the T cells were short-lived or that they colonized the periphery after 3 weeks of age [122]. In this model CD4-mediated suppression was shown to be specific for the islet immune reaction. The role of suppressor CD4+ T cells was also demonstrated by the successful transfer of diabetes in non-irradiated thymectomized 8-week-old NOD recipients, as long as prior depletion had been performed with anti-CD4 monoclonal antibody. The role of the thymus in the NOD model has been strengthened by evidence of a maturation defect of thymic T cells [123] and an intrinsic defect of the T-cell response to concanavalin A and anti-CD3 antibodies [124]. The significance of these defects with regard to morphological abnormalities of thymic cortex epithelial cell architecture is unknown [125, 126] (Fig. 3).

Two functional types of CD4+ T cells have been found in the NOD mouse. One activates the transfer of diabetes, while the second shows suppressor activity. Interestingly, CD4+ T cells have been subdivided, on the basis of different lymphokine secretion patterns, into TH1 (interleukin 2 and interferon γ) and TH2 subsets (interleukin 4 and 6). Data assigning definite functions to cells showing the two patterns of lymphokine secretion remain controversial. Phenotypic markers can also be used to discriminate between CD4 subsets. On the basis of CD45 expression, the expression of low-molecular-weight (CD45 RO) and high-molecular-weight (CD45 RA, B or C) antigens depends on alternative splicing of exons 4, 5 and 6 of the CD45 gene. We used monoclonal antibodies which recognize high-molecular-weight isoforms in the mouse to study the distribution of CD45 RA+ CD4+ T cells in the NOD model. CD45 RA expression has been associated with the TH1 subset in man, and the distribution of CD45 RA+ cells was modified in mature NOD thymic T-cell populations. A considerable increase in CD45 RA CD4+ T cells was found in NOD lymph nodes between 1 and 3 months of age. Moreover, most infiltrating CD4+

Fig. 3. The role of regulatory T cells in NOD mouse diabetes. Evidence for suppressor CD4+ T cells relies on functional experiments in the NOD mouse. A molecular characterization of these regulatory T cells (i.e. antigen specificity, T-cell receptor) will require the isolation of T-cell clones with suppressor functions

T cells in the pancreas were CD45 RA+. The treatment of NOD mice with an anti-CD45 RA monoclonal antibody from 12 to 32 weeks of age was as efficient as anti-CD4 monoclonal antibody treatment in preventing the development of Type 1 diabetes and insulinitis. By contrast, the transfer of diabetes was not influenced by CD4-CD45 depletion of diabetic spleen cells or treatment of recipients with the same anti-CD45 RA monoclonal antibody (P.Sempé, unpublished results). The NOD mouse disease thus involves complex regulatory pathways in which CD4+ CD45RA+ T cells may be involved in early immune activation but not in late activation steps involved in disease transfer. Preliminary evidence also suggests that suppressor CD4+ T cells co-express CD45 RA.

Molecular characterization of CD4+ T cell-antigen presenting cell interactions

Experiments have so far been based on functional parameters. The results indicate key interaction between CD4+ T cells and antigen-presenting cells in the NOD model, and the need for the molecular characterization of interaction structures on autoreactive CD4 and antigen-presenting cells. Initial support for this idea has come from the identification of biochemical constraints in the amino acid sequence of the external domain of the β chain of the class II antigen-presenting structure [127]. Position 57 (located at the end of the α helix), may affect the insertion of the autoantigenic peptide into the antigen-presenting cleft on the dimeric class II molecule.

Characterization of the T-cell receptor for antigen on autoreactive T cells raises the major issue of restricted variable-region (V) gene usage, with both theoretical and therapeutic implications. Different V β gene families have been identified by preventing diabetes with anti-V β monoclonal antibodies in vivo and by studying autoreactive T-cell clones, ruling out V β -specific immunotherapy in this model [128–130]. The study of (NOD $\times$ SWR) intercross-backcross animals which show a deletion of V β 5, 8, 9, 11–13 genes confirmed that receptors using these genes are not mandatory for the development of diabetes [131, 132].

The third structure to be characterized is the peptide inserted within the class II cleft on antigen-presenting cells. Two approaches have so far been developed to define putative target autoantigens. One is the study of T-cell clones which proliferate specifically in response to islet-cell-derived antigens [133–140]. The second is the study of the antigenic specificity of polyclonal sera with islet cell reactivity. In the NOD mouse, we identified circulating autoantibodies against a 58-kDa antigen by Western blot analysis of Rin5F cell extracts. Autoantibodies were detected in all diabetic NOD mice tested, and in non-diabetic mice from the first weeks of the onset of insulinitis. A monoclonal antibody against the 58-kDa antigen was used to test a large panel of non-islet-related cell lines, and revealed the expression of the 58-kDa antigen on a murine neuroblastoma cell line. This allowed us to identify the 58-kDa antigen as peripherin, an intermediate filament protein which is expressed by peripheral neuronal cells. The sequence of the neuronal protein has been determined. None of the other cell lines tested (including thymic, fibroblast, and pituitary cells) express peripherin [62, 141–143].

Interestingly, other putative autoantigens have been evidenced in the NOD mouse, including insulin [59, 60], heat-shock protein 65 [137, 138] and a 64-kDa antigen [144] probably corresponding to that characterized in human Type 1 diabetes. These are part of an increasing list of autoantigens against which autoantibodies or autoreactive T cells have been identified in man and which may have therapeutic implications in Type 1 diabetes. Extensive characterization of T-cell reactivity to these antigens, including autoreactive epitope mapping, and their role in initiating or relaying the autoimmunization process will be required: this is an easier task in animal models (in which tolerance induction can be tested as a strategy to prevent the activation of autoreactive T cells) than in human Type 1 diabetes.

Immunotherapy: clinical prospects

The goal of immunotherapy in Type 1 diabetes is to induce long-term protection following transient therapeutic intervention. Cyclosporin studies indicate that suppres-

Table 1. Target autoantigens in Type 1 (insulin-dependent) diabetes mellitus

Insulin, proinsulin
Glycolipids, ganglioside GT3
64 kDa antigen, glutamic acid decarboxylase
Carboxypeptidase H, PM-1, polar antigen
38-kDa secretory granule membrane component
Peripherin
Heat-shock protein 65
Insulin receptor
Endocrine cell antigens
Cytoskeleton proteins (tubulin, actin) Reticulin
Nuclear antigens (ssDNA, ssRNA)

sion of T-cell activation blocks the anti-islet immune reaction in man. However, the transient effect observed is likely to be a major drawback with all conventional immunosuppressive agents. Immunological efficacy is hampered by side-effects due to toxicity to non-lymphoid tissues. The effect on CD4+ T cells, whatever their antigenic specificity, results in broad untargeted immunosuppression. Other limits arise directly from the mechanisms of action of cyclosporin. Cyclosporin binds to a cytosolic receptor with peptidylprolyl isomerase activity, resulting in the blockade of interleukin-2 gene transcription and T-cell proliferation [145, 146]. The action of cyclosporin is thus restricted to resting T cells which is a major limitation since most islet infiltrating cells are activated; it is also a likely explanation for the delayed clinical action of cyclosporin in Type 1 diabetes. Finally, the treatment has a very narrow safety margin since at the time of clinical hyperglycaemia a majority of Beta cells have already been destroyed.

Major guidelines should direct future immunointervention strategies. The first involves the step from therapy to prevention, but diagnostic tests for the diabetic susceptibility background will be required. The second involves going from immunosuppression to tolerance induction. Inducing tolerance prior to the first activation of autoreactive T cells, as proposed in the case of insulin [147], will require defining human target autoantigens and the epitopes involved, which may prove tedious. However, intermediate approaches between conventional immunosuppression and antigen-mediated tolerance induction may be feasible (Table 1).

Past strategies may have been based on an oversimplified view of both immunosuppression and the diabetes process. The mechanisms of action of immunosuppressive agents can differ greatly. Some kill targeted cells, while others alter cell function, and ultimately opposite effects on immune responses may be seen. The disease process is complex, involving a sequence of activating or regulatory events; these include homing of T cells to the islets of Langerhans, followed by effector events at the onset of clinical diabetes when the immune system has already differentiated and Beta-cell destruction is occurring. From a therapeutic point of view, modification of regulatory T-cell function and elimination of effector T cells directly involved in Beta-cell destruction are both likely to involve more than one agent. Long-term protection may indeed require a combination of the two approaches. Finally, it is

likely that modulating autoreactive T-cell activation will require interference at the very first activation step of the autoimmune regulatory cascade, i.e. the interaction between autoreactive CD4+ T cells and antigen-presenting cells.

The requirements of an intermediate strategy

The first requirement is to dissociate induction and maintenance treatments. The goal of induction treatment is to kill activated T lymphocytes directly at the islet site, while maintenance treatment aims to restore or induce immune self-tolerance to islet antigens by modulating the activation of autoreactive T cells, thus bypassing the presented autoantigens. The next requirement is to target a restricted T-cell repertoire which includes islet-specific T cells. Finally, it will be necessary to intervene earlier in the natural history of Type 1 diabetes, before the onset of clinical hyperglycaemia, when autoreactive T cells are being activated against islet cell antigens.

Experimental bases

First, it is possible to eliminate insulinitis rapidly in the NOD mouse by using monoclonal antibodies such as anti-T cell or anti-CD45 RA in association with anti-CD8 monoclonal antibodies. Eight-week-old NOD mice treated with anti-T cell receptor antibody also show almost complete clearance of destructive insulinitis within 9 days. The same induction treatment reverses the diabetic state in animals with recent-onset hyperglycaemia [119]. A similar result may be obtained by targeting the T-cell receptor for interleukin 2 [148, 149].

The induction of tolerance by modulating T-cell activation while bypassing the autoantigen, is also possible in the NOD mouse. In the recent years several mechanisms have been proposed to account for tolerance to self antigens. The selection of the T-cell repertoire, including that of autoreactive T cells, is mainly a thymic process involving negative and positive selection of precursor cells. There is evidence for negative selection through the deletion of autoreactive clones as a mechanism of tolerance induction in the case of superantigens and class II MHC antigens expressed on bone-marrow-derived cells. Positive selection of autoreactive clones has been shown to involve class II antigens expressed by thymic cortical epithelial cells [150, 151]. The maintained tolerance of non-deleted self-reactive clones may involve various mechanisms: clonal anergy is one in which T cells are present but are incapable of secreting interleukin 2 when activated. Other mechanisms include peripheral T-cell suppression. The site of antigen expression largely influences self-tolerance-induction pathways. Antigens expressed or presented within the thymus may induce deletion, while those only expressed in the periphery are more likely to participate in tolerance through anergy, suppression or defective antigen presentation to specific T cells [152–154]. A wide antigen-specificity repertoire, but a small number of cells within each antigenic specificity or clone is an economic necessity of the immune system. The activa-

Fig. 4. New approaches to immunosuppression. The physiology of T-cell activation follows a two signal model in vitro. The absence of a "co-stimulatory" signal may induce active T-cell unresponsiveness and ultimate tolerance (e.g. anergy). IL, Interleukin; TCR, T-cell receptor

tion and proliferation of a specific clone in response to a given antigenic specificity is thus necessary for host defenses. The physiology of T-cell activation is similar to the two-signal model proposed in the early 1970s by Bretscher and Cohn for B-lymphocyte activation. One signal is mediated by the cognate interaction of T-cell receptor with specific peptides presented on MHC antigens. The occupancy or cross-linking of the T-cell receptor generates the activation of phospholipase C and tyrosine kinases. These pathways allow the activation of lymphokine genes such as interleukin 4, while others remain unactivated (e.g. interleukin 2). The second signal occurs via different interaction structures on cell membranes, possibly including CD28. Whatever the second co-stimulation signal, occupancy of the T-cell receptor, combined with the delivery of a co-stimulatory signal, leads to T-cell activation. By contrast, occupancy of the T-cell receptor in the absence of a co-stimulatory signal is not a neutral event: this form of signalling induces active T cell unresponsiveness [155].

The NOD mouse also provides clues on tolerance induction. Several approaches referring to tolerance induction at the thymic level using anti-CD3 antibodies [156, 157] or by generation of effector cells using streptococcal preparations [158] have been reported. Along the same line reversal of the diabetes process has been observed following adjuvant immunotherapy [159]. Long-term treatment of NOD mice with anti-class II antibodies prevents the development of Type 1 diabetes in female NOD mice. In addition, the same antibodies induce a state of unresponsiveness which is not a simple blockade effect on antigen presentation, but reflects an active induction of suppressor T cells [160]. By blocking antigen presentation and the normal delivery of activating signals by the T-cell receptor, anti-class II treatment may induce a protection state satisfying some of the criteria of tolerance. In protected animals, suppressor CD4⁺ T cells are generated which can transfer protection to untreated recipients. Animals that receive spleen cells from anti-class II-treated donors are protected against diabetes following injection of spleen cells from diabetic animals. Anti-

class II is not only effective in preventing diabetes when treatment is maintained up to the end of protection experiments, but also when injected within a very narrow time-frame. Anti-class II treatment was effective long-term when applied from 3 to 7 weeks of age in animals free of insulinitis at the start of treatment. By contrast, animals treated later (from 8 to 16 weeks of age) were only transiently protected. Spleen cells collected at 40 weeks from the animals treated at an early stage were unable to transfer diabetes, in contrast to spleen cells from non-diabetic animals treated with a control antibody, which rendered 88% of the recipients diabetic. The strong protection state induced by anti-class II treatment may be related to tolerance, since evidence has been obtained that protection is islet-specific in anti-class II-treated animals. Moreover, parenteral cyclophosphamide treatment of protected animals at 6 months of age failed to induce acute diabetes, but was successful in control animals. Similar evidence of tolerance induction in the mouse has been obtained in other models with anti-CD4. A major issue is whether the same protection that was induced with anti-class II can be achieved once the autoimmune process has started. Experiments are needed to induce tolerance in animals with an ongoing autoimmune process, but this may only be possible following induction treatment to eliminate insulinitis and target lymphocytes that are already committed. This is a prerequisite for new therapeutic strategies in man, and is half-way between conventional immunosuppression and antigen-mediated tolerance induction.

The tools for induction and maintenance treatment (Fig. 4) may already be available for human use (Table 2). Some are even aimed at a narrow T-cell repertoire which includes autoreactive islet-specific T-cell clones. A fusion toxin has recently been obtained as a recombination product associating the interleukin-2 gene and part of the diphtheria toxin gene. Interleukin 2 is the recognition unit and the toxin fragment A, the killing unit. Interleukin 2 delivers the toxin only to cells expressing high-affinity interleukin-2 receptors [148]. Less than 5% of circulating T cells usually express such high-affinity receptors, whereas most of those infiltrating islets are activated and

Table 2. New immunosuppression tools for use in man

Induction treatment:	Cytotoxic anti-CD4 Anti-CD3 Anti-T-cell receptor Fusion toxins
Maintenance treatment:	Low-dose cyclosporin A Anti-class II Non cytotoxic anti-CD4 Cytokines Anti-cytokine
Antigen-mediated tolerance:	Tolerance

thus express them. This approach exemplifies the strategies which will be developed both to affect a limited spectrum of T cells within the T-cell repertoire and as induction treatment of ongoing autoimmune processes. Maintenance treatments aimed at achieving long-term protection must also be developed. Candidate agents include low-dose cyclosporin and monoclonal antibodies against interaction structures expressed by antigen-presenting cells and CD4+ T cells; human monoclonal antibodies prepared by genetic engineering technology are already available for human therapeutics.

Conclusions

Defining adapted therapeutic strategies may require further insight into the pathophysiological mechanisms involved in anti-islet autoimmunity. The precise series of events which initiate the anti-islet T-cell autoimmune reaction is still hypothetical. The presence of autoreactive T cells with a genetic background defined by specific MHC genes and other undefined susceptibility genes is thought to be the prerequisite for the development of autoimmune diabetes. Triggering factors have been postulated which may initiate [154, 161] or prevent [162] the activation of autoreactive T-cell clones. The triggering role of environmental factors is supported by experimental evidence that autoimmune islet Beta-cell destruction can be induced by Beta-cell-toxic agents or β cytotropic viruses in animals, but proof has not been obtained in human diabetes. Recent studies suggest a role for interferon γ both on Beta-cell antigen expression and as inducer of an immune reaction at the islet site [163, 164].

Several non-exclusive hypotheses may explain the development of autoimmunity to Beta cells. The presence of autoreactive T cells may result from defective selection of the T-cell repertoire (e.g. absence of negative selection or abnormal positive selection). The role of T-cell selection in determining the peripheral T-cell repertoire and autoimmune susceptibility has been shown in the collagen arthritis model [165], but remains hypothetical in the case of diabetes. Extensive study of the T-cell repertoire in health and disease will thus be required before firm conclusions can be drawn.

In the presence of specific T cells, tolerance to self antigens has been shown to result from anergy (defined by the presence of autoreactive T cells, along with a T-cell incapacity to produce interleukin 2 in response to specific

antigens), T-cell mediated suppression, and unresponsiveness of effector (CD4 and CD8) T cells, each possibly overcome by the autoimmune process.

Our growing knowledge of the mechanisms of peripheral T-cell tolerance allows new immunointervention strategies. These may permit therapies aimed at inducing tolerance independently of defining autoantigens and peptides involved in the primary activation of autoreactive T cells and, possibly, the chain-reaction underlying anti-Beta cell autoimmunity.

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Prof. C. Boitard
 Immunologie Clinique
 Hôpital Necker
 161 rue de Sèvres
 F-75743 Paris Cedex 15
 France