Perspectives in Diabetes

T-Cell Responses to Autoantigens in IDDM
The Search for the Holy Grail

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IDDM (type I diabetes) is generally believed to result from T-cell-mediated autoimmune destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans. In the last few years, considerable progress has been made with regard to the identification and characterization of candidate autoantigens recognized by autoantibodies; several of these candidate autoantigens are recognized by T-cells, including insulin, GAD65 and GAD67, heat-shock protein 65 (hsp65), and islet-cell antigen 69 (ICA69). In addition to these, a number of unidentified β-cell antigens, including insulin-secretory granule membrane proteins and a 38-kDa protein, have been shown to stimulate T-cells of IDDM patients. However, T-cell autoreactivity to islet antigens is not specific for IDDM, and the T-cell target antigens are not specific for β-cells. Moreover, the autoreactive T-cells involved in the initiation of the insulitis must be defined, and the mechanism of the T-cell-dependent β-cell destruction remains to be unraveled. This review focuses on T-cell autoreactivity in IDDM in humans and the implications of the present knowledge for immunointervention and monitoring of immunotherapeutic trials. Diabetes 45:1147-1156, 1996

IDDM (type I diabetes) is thought to result from genetically controlled T-cell-dependent islet-cell destruction of an autoimmune nature. The disease is accompanied by the presence of a variety of circulating autoantibodies. Although a pathogenic role for autoantibodies has never been demonstrated (1,2), their (combined) predictive value makes them an invaluable marker for the identification of preclinical diabetes. Furthermore, targets of autoantibodies may help to identify molecules that are recognized by T-cells and therefore may have a role in the disease process leading to IDDM.

EVIDENCE FOR A ROLE OF T-CELLS IN IDDM

The role of T-cells in the pathogenesis of IDDM was initially based on the observation that they are detectable in the mononuclear cell infiltrates in the pancreatic islets at disease onset, the insulitis (3-5). Their role was subsequently illustrated by the effects of immunosuppressive drugs in delaying disease onset (6,7), the detection of autoreactive T-cells in IDDM patients, the recurrence of insulitis associated with destruction of pancreatic grafts in IDDM patients (8), and most recently the transfer of the disease by transplantation of bone marrow of a diabetes patient to an immunodepressed non-diabetic recipient (9). Animal studies have shown that T-cells are able to transfer diabetes and that the occurrence of the disease is thymus dependent and requires CD4 and CD8 T-cells (10,11).

T-cells are present in inflamed islets (insulitis) at disease onset (5,12-16), and several groups have demonstrated their presence in the peripheral blood of newly diagnosed IDDM patients that respond to human β-cells (17-21) or β-cell-associated proteins (22-29) in recent-onset or prediabetic IDDM patients. However, autoreactive T-cells are detectable not only in the circulation of newly diagnosed IDDM patients but also in healthy HLA- and age-matched nondiabetic control subjects, albeit they are less frequent and less reactive in the latter group. Nonetheless, this observation illustrates that the occurrence of circulating autoreactive T-cells does not mean autoimmune disease, and additional immune abnormalities must occur to induce the β-cell destructive process. The targets of the autoreactive T-cells vary. In fact, the list of candidate antigens is still increasing. Most of these candidate target antigens for potentially diabetogenic T-cells are not restricted to β-cells. For instance, the β-cell autoantigen glutamate decarboxylase (GAD65) is also expressed in other endocrine cells in the islets (α-cells) (30,31).

PERIPHERAL VERSUS INSULITIC T-CELLS

The vast majority of studies on T-cell reactivity to islet-cell antigen has been performed on peripheral, circulating T-cells, rather than T-cells from insulitis. Clearly, the insulitic lesions are most likely to contain the relevant pathogenic T-cells, but few studies have addressed these inflammatory cells. In IDDM patients with pancreas transplants, disease recurrence was associated with T-cell infiltration of the pancreas grafts, with a predominance of CD8+ over CD4+ T-cells (32,33). Antigen specificity of insulitic T-cells remains to be investigated. Phenotypic studies thus far have revealed that the T-cell repertoire is diverse rather than oligoclonal. Fluorescence-activated cell sorter (FACS) analysis of peripheral T-cells using monoclonal antibodies against the T-cell receptor (TCR)-Vβ5, TCR-Vβ8, and TCR-Vβ12 families...
showed heterogenic TCR-Vβ usage, even though oligoclonality in certain individuals was suggested by increased expression of TCR-Vβ8 (34). Studies on TCR-Vβ8 usage of inflammatory T-cells in IDDM are hampered by the poor accessibility of the target organ, the poor availability of postmortem pancreases, and the relative late state of the disease process (usually the clinical presentation of IDDM). The limited number of studies on TCR-Vβ8 usage of human islet-infiltrating T-cells has not elucidated whether or not preferential TCR-Vβ usage is associated with β-cell destruction. Hämminen et al. (14) observed heterogeneous usage of TCR-Vβ8 elements by infiltrating T-cells, with a slight overrepresentation of TCR-Vβ8. Somoza et al. (16) described restricted usage of TCR-Vβ of T-cells isolated from a postmortem pancreas of a newly diagnosed IDDM patient (16). Only 7 of the 20 Vβ families studied were detected. One study reported a predominant usage of TCR-Vβ7 by islet-infiltrating T-cells that was suggestive of exposure to superantigen (15). Membrane fractions of islets isolated from these diabetic pancreases were able to selectively stimulate TCR-Vβ7-expressing T-cell outgrowth of peripheral T-cell from healthy blood donors. However, β-cell reactivity or specificity of such TCR-Vβ7 T-cells was not tested. In fact, the TCR-V of T-cells reactive with islet antigens has been analyzed in only a few cases. In these studies, there was no evidence for oligoclonality, but note that the actual antigens and their epitopes differed for each T-cell line or clone investigated. We have sequenced the TCR of five β-cell antigen-reactive T-cell clones (A.A. Kallan, B.O.R., G. Duinkerken, P. van den Elsen, J.C. Hutton, R.R.P. de Vries, unpublished observations). Even though in two cases the TCR-Vβ was shared, the TCR-Vα and junctional diversity was different for each clone. However, we observed increases in TCR-Vβ8 expression in bulk T-cell cultures in response to islet antigens in 5 of 11 newly diagnosed IDDM patients, but additional increases were observed in 4 of these patients. In another study, T-cells that reacted with unknown islet antigens were cloned, and some expressed TCR-Vβ8 (14). This particular TCR-V family was also found to have increased expression on islet-infiltrating T-cells (14,35) and interleukin (IL)-2 receptor–expressing peripheral T-cells (34). Interestingly, part of the CDR3 region (which supposedly determines the specificity of the T-cell for antigen [36]) was shared with pathogenic T-cell clones isolated from NOD islets. The lack of further similarities in TCR-Vβ usage observed in T-cells in IDDM implies that immunotherapy selectively aiming at T-cells of par ticu-

T-CELL RESPONSES TO ISLET AUTOANTIGENS

TABLE 1
Comparison of T-cell responses to candidate autoantigens associated with IDDM in mice and men

<table>
<thead>
<tr>
<th>Autoantigen</th>
<th>Humans</th>
<th>Animals</th>
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| GAD65       | • Identified by autoantibodies  
• Recognized by T-cells  
• Limited tissue distribution  
• Immune response not disease specific | • Recognized by T-cells from unprimed prediabetic, diabetic, and nondiabetic NOD mice  
• Tolerization with GAD65 prevents diabetes |
| GAD67       | • Identified by autoantibodies  
• Recognized by T-cells  
• Not expressed in human β-cells  
• Immune response not disease specific | • Recognized by T-cells  
• Administration of GAD67 can prevent diabetes in NOD mice |
| Insulin     | • Identified by antibodies  
• Recognized by autoantibodies and T-cells  
• Immune response not disease specific  
• Protein synthesized and stored in β-cells, but widely distributed through circulation | • Recognized by T-cells; insulin-specific T-cell clones can transfer diabetes to nondiabetic recipients  
• Large fraction of T-cells in insulitis responsive to insulin  
• Tolerization with insulin prevents diabetes |
| 38-kDa      | • Identified by T-cells  
• Recognized by autoantibodies and T-cells  
• Several candidates (Yun-B; Inogen 38)  
• Widely distributed | • Homologous electroeluted fraction of 38-kDa insulin secretory granule membrane is recognized by diabetogenic T-cells of NOD mice  
• Prediabetic BB rats have autoantibodies to 38-kDa protein, which disappear after onset of diabetes  
• Mice infected with CMV develop autoantibodies to 38-kDa protein on islets |
| hsp65       | • Recognized by T-cells in several autoimmune diseases  
• Situation in humans unclear | • Spontaneously recognized by prediabetic NOD mice  
• T-cells reactive with hsp60 peptides p277 transfer diabetes  
• Immunization with p277 prevents diabetes |
| ICA69       | • Identified by antibodies  
• Recognized by autoantibodies and T-cells  
• Immune response not disease specific  
• Protein widely distributed | • Not yet known |
| Undefined candidate antigens | • Peripheral blood lymphocytes of newly diagnosed IDDM patients respond to β-cell homogenates and subcellular fractions  
• Immune response not disease specific | • Splenocytes and insulitic lymphocytes of (pre-) diabetic NOD mice response to β-cell homogenates and subcellular fractions  
• Responding T-cells are diabetogenic in adoptive transfer experiments |
lar TCR-Vβ families may be impossible. Studies on the CDR3 region of the TCR, however, may reveal more uniformity among β-cell antigen–reactive T-cells. Moreover, extensive analyses of the TCR-V of T-cells reactive with a given autoantigen have not yet been reported.

The stimulation of autoreactive T-cells may have arisen as a consequence of chronic insulitis. Indeed, inflammatory islet damage has been described in cases of pancreatitis patients (37). Interestingly, no signs of autoimmunity were noted in these patients, despite the presence of diabetes susceptibility alleles HLA-DR3 and/or DR4 in half of these patients. This argues that inflammation per se does not necessarily lead to autoimmunity, nor is it sufficient to explain selective β-cell death, since only a minority of patients with chronic pancreatitis became insulin dependent. This observation could be an argument in favor of a role of autoreactive T-cells in the β-cell destruction in IDDM, but in addition, it illustrates that inflammatory islet damage is not exclusively autoimmune T-cell dependent. Superantigen stimulation and polyclonal T-cell activation may suffice to initiate the destruction process (15). Insulitic T-cells need not be actively involved in β-cell destruction, as is illustrated by the presence of chronically activated T-cells around islets of Langerhans in nondiabetic patients (37) and, conversely, unactivated insulitic T-cells in recent-onset IDDM patients (12). In mouse studies, insulitis is often not accompanied by β-cell destruction—in particular in the case of “peri-insulitis”—despite the close proximity of activated T-cells and potential target β-cells. In humans, insulitis is not as dramatic as in diabetic mice or rats, and it is only observed in the presence of β-cells (16). This limits insulin in humans to the presence of β-cells.

**T-CELL SUBSETS**

What is the contribution of CD4- and CD8-expressing T-cells to the β-cell destructive process? In mice and men, the majority of lines and clones directed to β-cell determinants are CD4 positive. Blood lymphocytes are potentially cytolytic and have been shown to lyse islet cells (38). We have shown that autoreactive CD4+ T-cell clones isolated from the peripheral blood of newly diagnosed IDDM patients specifically lyse monocytes pulsed with β-cell antigen and that this lysis is HLA-DR restricted and CD4 mediated (39).

Recently, CD8+ T-cell clones have been generated from peripheral blood of IDDM patients against a specific HLA-A2 binding GAD65 peptide, which lyse autologous antigen–presenting cells pulsed with the peptide or infected with vaccinia virus expressing GAD65. However, these clones have not yet been shown to specifically (and preferentially) lyse β-cells (40). Furthermore, alterations in peripheral blood CD4 to CD8 ratios in relation to IDDM have been reported (41,42). One study reported a reduction of the CD4 to CD8 ratio before disease onset (43). In some studies, the increased ratio appeared to decline after disease onset (41,42). Similar increases associated with onset of IDDM were described for in vivo–activated T-cells (with double expression of CD45RA and CD45RO) (44,45; L. Douglas, G. Duinkerken, R. de Vries, B.R., unpublished observations). This might imply that the disease onset coincides with a rather general (aspecific?) activation of T-cells. In late stages of the disease process, the insulitic lesions in recent-onset IDDM patients, the majority of lymphocytes expressed CD8 cells (12,16). However, the role of CD4- or CD8-expressing T-cells in human IDDM is presently unresolved.

In animal models, it appears that CD4+ T-cells are required, but CD8+ T-cells clearly potentiate the diabeticogenic effects of CD4+ T-cells. Moreover, a NOD mouse–derived CD4+ T-cell clone successfully transferred diabetes to a nondoniabetic scid mouse (46). Arguments in favor of a role for CD8+ T-cells in the disease process in animals include the higher efficiency of disease transfer in case of combinations of CD4 and CD8 and the occurrence of diabetes in mice depleted of CD4+ T-cells (47,48). Even though transfer studies do not fully reflect the course of “spontaneous” diabetes and should be interpreted with caution, the aforementioned observations clearly illustrate the diabeticogenic potency of both CD4 and CD8 T-cells.

**T-helper (Th) cell subsets.** CD4+ Th cells can roughly be divided into two reciprocal subgroups with different biological activities on the basis of differential cytokine production after an immune response. T-helper 1 (Th1) cells are characterized by the production of IL-2 and γ-interferon (IFN-γ) and are mainly associated with cellular immune responses, whereas the Th2 subset is associated with humoral responses by the production of IL-4 and IL-10 (49–51).

It is unclear whether Th2 cells play a role in the pathogenesis of human IDDM. An inverse correlation between humoral and cellular responses to GAD65 was described, suggestive of selective activation of Th subsets in different individuals (52). The humoral responses against β-cell–associated proteins were associated with slow progression to the development of IDDM (52). This implies (but does not prove) that Th2 responses are activated. Inverse relationships between humoral and cellular responses to GAD65 and insulin in high-risk relatives of IDDM patients and in new-onset IDDM patients have also been found, but in this study, conversion to IDDM was not particularly associated with Th1-type responses (53). The vast majority of human islet cell reactive T-cells are Th1-like. However, this may represent a cultural artifact, since Th1 cells could be selectively isolated on basis of their preferential outgrowth with IL-2. In human insulitis, increased expression of cytokines was measured only for IFN-γ, whereas the expression of IFN-β, IFN-γ, tumor necrosis factor (TNF)-α, and IL-2 was similar to that in control pancreases (5).

Studies in NOD mice strongly support a role for Th1 cells in the pathogenesis of diabetes (54–57). β-cell destruction in the NOD mouse appears to be a staged process in which, before the destructive phase, an infiltration of the pancreas can be observed in which T-cells surround the islets but do not invade them. Characteristic of this “nondestructive” or “peri-insulitis” is the production of IL-4 and no IFN-γ by the surrounding T-cells (58,59). However, at the time of disease onset, the islets show massive infiltration with T-cells, the so-called destructive insulitis (58). Characteristic of this destructive insulitis is the high production of IFN-γ by the infiltrating T-cells, indicating the pathogenicity of Th1 subset. The disease can be prevented by monoclonal antibodies against IFN-γ (60,61). Upon cyclophosphamide injection, the subsequent islet-cell destruction was accompanied by increased frequencies of Th1 cells, even though the presence of Th2 cells was unaltered (59). A recent report dissected the contribution of Th1 and Th2 cells to the development of diabetes (56). Using Th1 and Th2 cells expressing the same diabeticogenic T-cell receptor, it was shown that diabetes in
the NOD mouse is clearly Th1 mediated. The Th2 cells could not induce diabetes, but moreover, failed to provide protection by downregulating Th1 cells.

**TARGETS OF AUTOACTIVE T-CELLS**

Several candidate β-cell antigens recognized by autoreactive T-cells that may be involved in the disease process have been described. The expression and distribution of these antigens is not exclusive to β-cells. Two approaches have been undertaken to define and characterize targets on β-cells that are recognized by circulating autoreactive T-cells of IDDM patients. The first and most successful approach focuses on T-cell responsiveness to defined targets of autoantibodies related to IDDM. Without exception, the antibody targets were shown to be recognized by varying frequencies in IDDM patients and, to a lesser extent, in nondiabetic control subjects. A second approach involves whole islets or subcellular fractions of islets or β-cell lines. These preparations were tested for (preferential) stimulation of T-cells derived from IDDM patients. T-cell lines and clones generated in such studies have never recognized any of the known candidate islet antigens defined by autoantibody reactivity. This closely resembles the situation in animal models, in which most islet-reactive T-cells responded to novel undefined antigens, with the exception of insulin (46, 62, 63).

**Insulin.** Insulin is a logical candidate antigen to be tested as a T-cell target antigen, since its production is exclusive to β-cells and autoantibodies to insulin are detectable in >50% of subjects with preclinical and recent-onset IDDM (64). Of course, secreted insulin is widely distributed throughout the bloodstream, but the concentration is maximal in and around the islets. Humoral recognition of insulin is most frequently found in younger children, in whom the β-cell destruction appears to be faster. Insulin is present throughout the body, including in the thymus. Nonetheless, insulin-specific autoimmune T-cell lines and clones have been generated from peripheral blood of newly diagnosed IDDM patients, as well as from healthy control subjects and patients suffering from insulin autoimmune syndrome (22, 23, 65). Insulin-reactive T-cells from diabetic patients treated with animal insulin have been cloned and have been shown to be HLA class II restricted (23, 66). Other reports on T-cells from IDDM patients and nondiabetic control subjects have described more frequent responses to insulin in IDDM patients than in control subjects (22, 67–69). It would be interesting to analyze T-cell reactivity to proinsulin, since this antigen is truly β-cell restricted and reactivity against proinsulin might relate to β-cell destruction and subsequent release of intracellular prohormone. In fact, autoantibodies to proinsulin have been described in prediabetic and diabetic humans (70), and diabetogenic T-cell clones have been generated from BB rats to a major histocompatibility complex (MHC)-binding peptide of proinsulin (71).

Although the trends in T-cell responses to insulin are similar, the assays to define this responsiveness vary greatly and result in qualitative and quantitative differences between the studies. **GAD65/67.** The 64-kDa autoantigen GAD, the biosynthesizing enzyme of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), is detected by autoantibodies as a hydrophilic soluble 65-kDa form and a 64-kDa hydrophobic form that can be both membrane-bound and -soluble (72–74). Brain and subpopulations of central nervous system neurons express high levels of this enzyme, whereas pancreatic islet cells express GAD at very low levels. GAD67 is probably less relevant to IDDM, since this form is not expressed in human islets and autoantibodies of IDDM patients specific to GAD67 are less frequent than those specific to GAD65, whereas nondiabetic subjects more frequently have antibodies to GAD67 (30, 31, 75). The majority of newly diagnosed patients and prediabetic subjects has been shown to display circulating autoantibodies reactive with various epitopes of GAD65.

After the identification of the 64-kDa humoral autoantigen as GAD65, the recombinant antigen was shown to be recognized by T-cells of approximately half of newly diagnosed IDDM patients and <10% of nondiabetic control subjects (26). Both CD4- and CD8-expressing T-cells have been selectively stimulated by GAD65-derived synthetic peptides (29, 40). A number of peptides were recognized by T-cells from both IDDM patients and healthy control subjects, even though some peptides may be more specific to IDDM (29, 76). Reactivity of T-cells of individuals at increased risk of developing IDDM to a GAD65-derived peptide with similarity to the P2-C protein of coxsackievirus B is of particular relevance, since this virus has been implicated in the etiology of IDDM (76). In fact, some individuals responding to the GAD65 peptide bearing homology with the viral P2-C antigen also responded to the equivalent viral peptide (76). This pattern of responsiveness at bulk T-cell levels does not necessarily implicate cross-reactivity, but the dual recognition of these two peptides clearly deserves future attention and asks for studies on the clonal T-cell epitope level.

The GAD67 form was shown to be recognized by 41% of recent-onset IDDM patients and a small fraction of nondiabetic individuals (27). Harrison et al. (52) found an inverse relationship between the titer of GAD-specific autoantibodies and peripheral T-cell responses to recombinant GAD67, which is suggestive of selective activation of different subsets of T-cells in different individuals.

Comparison of the different studies on T-cell reactivity to GAD65 suffers from variation in the T-cell assays and in modes of expression and purity of the recombinant antigen. **38-kDa autoantigen.** A T-cell clone generated against a crude insulinoma membrane preparation was shown to recognize a membrane protein of 38 kDa (24). Subsequent studies showed that the insulin-secretory granule membrane fraction of ~38 kDa was recognized by the majority of newly diagnosed IDDM patients responding to insulin-secretory granules, but not by age-matched nondiabetic control subjects (25). The expression of this antigen is not exclusive to β-cells. Interestingly, circulating autoantibodies against a 38-kDa islet-cell protein have been detected by immunoprecipitation of [35S]methionine-labeled human islet cells in newly diagnosed IDDM patients, whereas such antibodies were absent in healthy control subjects (72). The level of expression of this 38 kDa protein is extremely low in general, but it appears to be very immunogenic. Several candidate antigens of similar molecular weight (Yun-B [77], synaptophysin, and clathrin) were not recognized by the T-cell clone.

Studies in NOD mice showed that similar insulin-secretory granule fractions were recognized by diabetogenic T-cell clones. Again, these mouse T-cells were stimulated with heterogeneous preparations rather than with isolated candidate antigens. Furthermore, an antibody against a human pancreatic islet cell–specific 38-kDa protein was induced by
infecting mice with cytomegalovirus (78,79). Interestingly, ~15% of newly diagnosed IDDM patients had cytomegalovirus genome in their lymphocytes and islet-cell antibodies in their sera. In BB rats, autoantibody reactivity to a 38-kDa islet-cell protein precedes the onset of IDDM (80). The antibody disappeared within 3 weeks after diabetes onset.

Very recently, the peptide epitope recognized by the 38-kDa-reactive T-cell clone was mapped by a subtractive cDNA expression cloning procedure. The cDNA library was enriched for cDNA coding for β-cell proteins, rather than α-cell proteins (81). The parental gene was subsequently cloned and coded for a novel protein, which turned out to be targeted to mitochondria (82). The protein, designated Imogen-38 (islet mitochondrial antigen 38 kDa), was expressed to a greater extent in β-cells than in α-cells, but the tissue distribution was nonetheless widespread. The subcellular localization may imply that the protein serves as target for bystander autoimmune attack rather than as a primary autoantigen involved in the β-cell destruction process. Yet a putative role in the disease process is still feasible, since a number of mitochondrial antigens with a similar broad tissue distribution are implicated in other tissue-specific autoimmune diseases, including dilated cardiomyopathy (ADP/ATP transporter), biliary cirrhosis (dihydropipoyl acetyltransfearase), rheumatoid arthritis (heat-shock protein 60 [hsp60]), and IDDM (hsp60) (see below). Moreover, reactivity of autoantibodies was only evident in diseased tissue, suggesting that mitochondrial protein may be mistargeted to the secretory pathway in response to inflammation. A similar pattern of expression has also previously been shown for the eye lens protein αB-crystallin, a small heat-shock protein recognized by T-cells from multiple sclerosis (MS) patients (and healthy control subjects). The expression of this autoantigen was found to be elevated in active MS lesions, but also in glial cells of brains of patients suffering from Alzheimer's, Parkinson's, Huntington's, Pick's, and Lewy body diseases (83). Similar alterations in expression have been described for hsp60, which was found to be present in mitochondria as well as secretory granules (84,85). Insulitis-caused redistribution of hsp60 inside β-cells appeared to correlate with the induction of hsp60 autoantibodies (86).

**hsp60**. At this stage, evidence in support of hsp60 (60-kDa heat-shock protein, the mammalian homologue of the mycobacterial hsp65 protein) as a T-cell target in IDDM in humans is lacking. In rheumatoid arthritis and juvenile arthritis, hsp60 has been shown to be recognized by inflammatory T-cells (87,88). Cohen and Elias and colleagues (89,90) have provided evidence that an antigenic epitope of hsp60 is a critical β-cell target in the pathogenesis of diabetes in NOD mice. The peptide containing the epitope is termed p277. Their studies showed that the onset of β-cell destruction is associated with the spontaneous development of anti-hsp65 and anti-p277 T-cells and the appearance of hsp65 cross-reactive antigen and anti-hsp65 antibodies in the serum. These features decline with the development of overt IDDM. Furthermore, anti-hsp65 or anti-p277 T-cell clones injected into NOD mice could cause insulitis and hyperglycemia. The hsp65 antigen or p277 peptide could also be used either to induce diabetes in prediabetic NOD mice or to "vaccinate" them against diabetes, depending on the age of the animal and mode of administration (91).

**ICA69**. Recently, another humoral islet-cell antigen, ICA69, was identified by screening an islet-cell cDNA library with autoantibodies from prediabetic individuals (92). This 69-kDa protein was found to be expressed in human pancreas, heart, and brain and, to a lesser extent, in lung, liver, and kidney. Approximately 25% of recent-onset IDDM patients have detectable levels of antibodies to this protein (93). Surprisingly, high responses were also observed in rheumatoid arthritis patients but not in patients with other immune-mediated diseases, such as Crohn's disease, Hashimoto thyroiditis, Grave's disease, MS, and colitis ulcerosa, or in healthy individuals (95).

We recently observed that T-cell responsiveness to ICA69 was significantly higher in recent-onset IDDM patients compared with IDDM patients after disease onset, non-diabetic first-degree relatives, and rheumatoid arthritis patients (94). Surprisingly, an HLA-DR3-associated inverse correlation between T-cell and autoantibody responsiveness to ICA69 was observed. These results indicate that HLA-associated T-cell reactivity to ICA69 is increased at onset of IDDM, which is suggestive of a genetically controlled selective activation of Th subsets to a specific autoantigen in humans.

A previous study indicated that primary T-cell recognition of ICA69 was undetectable in peripheral blood unless IL-2 was added to the culture simultaneously with the antigen. It was proposed that ICA69 may stimulate early stages of T-cell activation (IL-2 receptor transcription) but that it results in insufficient IL-2 production and thus anergy (95). Again, the differences in the assays to determine T-cell responses to ICA69 may explain part of the inconsistencies described.

**Other T-cell targets**. Previously, we have shown that the majority of newly diagnosed IDDM patients respond to proteins that are present in insulin secretory vesicle membrane preparations (20). Peripheral blood T-cells reacting with β-cell membrane preparations enriched for insulin-secretory granule antigen were detectable in the majority of newly diagnosed IDDM patients. Such reactivity was reduced in IDDM patients tested at least 2 years after disease onset proportionally to the duration of the disease. Nondiabetic age-matched control subjects showed no or moderate responses to the granule preparation. These results imply that T-cell recognition of undefined insulin-secretory granule antigens is associated with IDDM and, in particular, with the immune-mediated process of β-cell destruction.

T-cell reactivity, often at the clonal level, has further been observed against a number of unidentified proteins expressed on β-cells (19,21,28,35,96,97). The tissue distribution of such T-cell targets is often more widespread than that of β-cells. These T-cell lines and clones were raised in the presence of heterogeneous islet- or β-cell line membrane preparations. Interestingly, this approach never resulted in the generation of T-cells reactive to a known candidate β-cell autoantigen, i.e., a humoral target. In addition to the undefined proteins recognized by T-cells of IDDM patients, β-cell proteins that are upregulated by IL-1β, viral infection, or inflammation may prove to be stimulators of autoreactive T-cells (98). Finally, any protein preferentially recognized by IDDM sera, such as the protein tyrosine phosphatase IA-2 (ICA512) (99,100) or carboxypeptidase H (101), or implicated in the particular functional activity of β-cells (102) deserves to be evaluated in T-cell assays.
IMPLICATIONS: A HOLY GRAIL NO MORE?
The results obtained thus far on autoimmune T-cells reactive with β-cell antigen in humans imply that multiple antigens may conceivably be involved in the disease process. Thus far, T-cell responsiveness to islet autoantigens is not IDDM specific, and the expression or distribution of target antigens of such autoreactive T-cells is never limited to β-cells. Moreover, the mechanism by which β-cell-reactive T-cells are involved in the β-cell destruction process is an enigma. Are all the proposed antigens involved, or does their involvement in autoimmune responses represent a general imbalance of the immune system? Is IDDM a consequence of failure in suppressive or regulatory T-cells? Should we expect the definition of a holy grail? Does an outgrowth of autoreactive T-cells with various reactivities result from defective apoptosis? These issues are still open and deserve critical evaluation.

At this moment, there are several lessons to be learned from animals. The similarities with the results of studies on T-cells in NOD mice and humans are striking. Islet cell antigen autoreactive T-cell clones have been isolated from both spleen and inflamed islets of NOD mice. Again, such T-cells were stimulated either with whole islets or islet-cell preparations or with isolated purified candidate autoantigens. Stimulation with crude islet preparations generated T-cell clones that were capable of adoptive transfer of diabetes to nondiabetic recipient mice. In some cases, such diabeticogenic T-cell clones were specific for NOD islets (103,104) and not cross-reactive with islets of other mouse strains. In most cases, undefined β-cell–associated antigens rather than any of the previously defined candidate islet autoantigens were recognized (57,103,105–108), with the exception of insulin (46,62,63,109) and a 38-kDa fraction of insulin-secretory granule membrane proteins in which particular antigens may be repeatedly recognized (25,57). However, autoreactive T-cells of NOD mice could be selectively stimulated with several candidate antigens of autoreactive T-cells that may be involved in the disease process described, including insulin, insulin-secretory granule protein 38-kDa (57), GAD65 (55,110), or hsp60 (89,90). Even though the expression of most of these antigens is not exclusive to β-cells, most of the aforementioned T-cell clones reactive to insulin, secretory granules (105), 38-kDa (57), or hsp60 could transfer diabetes to nondiabetic recipients, but apparently no other tissues were affected. In other words, such T-cells reacting with an autoantigen not specific for β-cells still can cause a disease that is tissue specific.

Interestingly, similar to the apparently nondestructive T-cell reactivity to GAD65 and insulin measured in nondiabetic humans, NOD mice spontaneously responded to GAD65, regardless of their progression to overt diabetes (55,110). Immunization with candidate target antigens in nondiabetic mice thus far has not led to accelerated development of diabetes. Conversely, prophylactic insulin injection (111–113) or oral insulin administration (114), immunization with GAD65 intravenously or intrathymically (110,115,116), or subcutaneous injection with hsp60 peptide (91) delayed or even prevented the onset of IDDM. In other words, even though several different antigens may be involved, antigen-based immunotherapy is still a potential approach to intervene in the T-cell–mediated autoimmune destruction. However, aspecific immunomodulation by, for instance, bacillus Calmette-Guérin (BCG) (117) or injection of insulitic T-cells reactive with autologous splenocytes (118) led to similar results, which illustrates that the effects obtained with β-cell–related proteins may not necessarily be antigen or T-cell specific.

Standardization. The results of the various assays described above may seem different, but they are not truly comparable because of considerable experimental differences. In our efforts to detect and characterize relevant autoreactive T-cells involved in β-cell destruction and their targets on the β-cells, it is essential to standardize the assays used to define T-cell responsiveness. For future clinical trials for immunointervention in the pathogenesis of IDDM, a consensus assay is required. During the last few years, T-cell reactivity to the various autoantigens that are candidates in the pathogenesis of diabetes has gained considerable interest. Assays to detect circulating autoreactive T-cells are still quite difficult, and the results obtained are not always consistent with those published earlier because of a number of variabilities in the assays and antigens used. The number of individuals tested was often small; and the concentrations, expression vectors, sources, and species of the antigens varied greatly, which conceivably explains a considerable part of the differences in results. Comparison of T-cell proliferation assays, candidate (islet) autoantigens, and patient and control populations is therefore essential. The recently established Immunology of Diabetes Society supports the initiative to start a workshop that aims to compare the various T-cell proliferation assays currently used.

The notion that T-cells reactive to β-cell autoantigens are not unique to IDDM patients implies that for diagnostic purposes, positive T-cell responses per se are of low diagnostic value. However, qualitative (Th1, Th2, cytotoxic T-cells) and quantitative differences in (types of) T-cell responses measured in longitudinal studies may provide useful information during monitoring of immunointervention trials or islet transplantation, even if they merely reflect secondary responses as a consequence of β-cell disruption.

PERSPECTIVES FOR IMMUNOTHERAPY
Several immunointervention strategies have been applied to prevent or intervene in IDDM in humans. General immunosuppression suffers from the lack of specificity (6,7,119) and the risk associated with the immunosuppressive drugs. Induction of oral tolerance appears to be an attractive alternative, even though the mechanism by which oral administration of candidate autoantigens affects the autoimmune response has not yet been fully resolved. The same applies for prophylactic insulin therapy—which is specifically directed to an autoantigen—nicotinamide consumption, and BCG vaccination. The specificity of the latter two approaches needs to be defined. Nevertheless, the start has been made, the side effects seem acceptable, and we are now in a situation where we can try to resolve the mechanism and monitoring of these immunotherapies.

To apply specific immunointervention, we need to define the relevant autoantigen, the responsible pathogenic T-cells, and the mechanism by which these T-cells play a part in the disease process. Furthermore, since intervention will be most effective in the earliest stages of the disease process, we need to identify at-risk individuals with high specificity and sensitivity, preferably in the general population rather than in family members of IDDM patients. Already, individuals with highly increased risk of developing IDDM can be
identified by the presence of multiple autoantibodies, predisposing HLA alleles and impaired glucose tolerance, but not yet on the basis of cellular immune abnormalities. Finally, we need to monitor the efficacy of the immunointervention and establish the surrogate endpoints.

At this stage, immunotherapy for IDDM in humans on the basis of autoantigens recognized by autoreactive T-cells is hampered by various factors. First, the expression or distribution of the presently known target autoantigens is not tissue specific, an antigenic holy grail is (still) lacking, and the frequency of patients with T-cells responsive to a given candidate β-cell autoantigen rarely exceeds the 50% level. With a minority of patients responding to a given islet autoantigen, antigen-based immunotherapy may require the use of several candidate autoantigens. Second, in view of the spectrum of HLA associations, consequent diversity of HLA-binding peptide epitopes, and the HLA-shaped T-cell repertoire differences, the autoantigens stimulating autoreactive T-cells will differ in different individuals. Recognition of multiple β-cell targets by T-cells and high frequencies of such T-cells may increase the risk of developing IDDM, as has been shown in the case of autoantibodies (120,121). At this stage, it is not known which candidate target antigens are involved in the initial autoimmune β-cell destruction process and which may be secondary to β-cell destruction. Yet T-cells responsive to antigen that became accessible to the immune system upon β-cell deterioration may be involved in the destruction of the remaining β-cells. Third, there is insufficient knowledge about the actual mechanism and characteristics of T-cells involved in the T-cell-dependent β-cell destruction. The hypothesis that deviation of Th1 responses to Th2 may be sufficient to prevent the onset of IDDM is premature. Indeed, even though the majority of β-cell antigen reactive T-cells appear to be Th1 cells, Th2 T-cells have not been shown to prevent Th1-mediated immunopathology. Furthermore, it may be harmful to intervene in the delicate balance between autoimmunity and autoprotection in individuals at risk of developing IDDM. The observed inverse correlation between T- and B-cell responses to a given antigen illustrates that the imbalance may result in predominant Th1 responses or preferential Th2 cell activity. There are considerable risks associated with manipulations of immune balances, in particular when they are undertaken without understanding of the mechanism of T-cell dependent β-cell destruction. In fact, conversion of Th1 responses to GAD65 to Th2 theoretically could lead to stiff-man’s syndrome-like immunopathology rather than to IDDM (122).

CONCLUSIONS

The identification of T-cell targets in IDDM remains one of the major research goals for the near future. Even though several defined proteins have been shown to be important cellular targets in NOD mice, as-yet-undefined β-cell determinants also play an important role in the pathogenesis of IDDM in these animals (103,107,108). Therefore, the identification of novel antigens involved in the β-cell destruction process may provide additional (perhaps primary) antigens involved in the initiation of the disease process. T-cell responses to candidate target antigens related to the pathogenesis of IDDM should be distinguished from those secondary to the disease process. Even though the cellular and autoantigenic holy grails have not been defined, we should not concentrate on only this. The time has come to focus attention on the cause of autoimmunity. This implies that research on T-cell subsets, cytokines, and the mechanism of T-cells in the pathogenesis of IDDM awaits. Intervention in an ongoing disease process may not be feasible. Definition of the initial hit and characterization of the initial insults may in fact show that T-cells are not the initiating factors in β-cell destruction (123). Still, if mice teach men the right lessons, specific (T-cell or autoantigen-based) immunomodulation may be effective early in the insulitides.

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