Novartis Foundation Symposium 292

DEFINING OPTIMAL IMMUNOTHERAPIES FOR TYPE 1 DIABETES

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Contents

Novartis Foundation Symposium on Defining optimal immunotherapies for type 1 diabetes, held at The Novartis Foundation, London, 11–12 July 2007

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Chair's introduction

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Diabetes is a disease where we still have many gaps in our knowledge. It is a special disease because we can't access the organ very well, especially during the prediabetic phase in humans. Perhaps by linking animal studies with *in vitro* studies of human cells and then actual human studies we can close some of these gaps during this meeting. This pertains to both the basic pathogenesis of the disease as well as clinical translations.

There are many areas that are important to me in this field. I want to learn more about how the human disease actually comes together. I want to understand the kinetics. There are certain things that continue to puzzle me: I don't understand how an immune-mediated disease is sustained for such a long time (in some cases the prediabetic phase can last more than seven years). How can it be that cells are continuously regenerated to attack islets in this chronic fashion? That a comparatively low-grade inflammatory immunological process can continue like this for several years puzzles me.

Understanding these types of kinetics will not only translate into understanding the pathogenesis, but also devising an optimal therapy: for example, we do not know for how long we would have to stop aggressive cells for in order to circumvent recurrence of disease. Does immunosuppressive or immune modulatory therapy have to be administered continuously, even if bystander regulation and other immunological control mechanisms that can be self-sustained by autoantigens are being invoked? Here we should discuss these issues, and others, for example with the question of the number of important autoantigens in type 1 diabetes: is there just one antigenic 'driver'pathway? I would also like to see parallels made with other diseases, where applicable, and we have therefore invited speakers whose main expertise is in multiple scerosis and other autoimmune disorders.

Retrospectively, this conference turned out to be a treat in many respects even for those who would consider themselves to be seasoned investigators in the pathogenesis of type 1 diabetes. We uncovered crucial 'forgotten' human data sets that should be revisited and expanded, we learned much more about the human aspects of type 1 diabetes pathogenesis which will be important to properly adjust current animal models, and we better comprehended crucial therapeutic and kinetic issues of the disease.

Pancreatic pathology in type 1 diabetes in human

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Abstract. In type 1 autoimmune diabetes there is a selective destruction of insulin-secreting β cells. Around the time of clinical presentation, insulitis, a chronic inflammatory infiltrate of the islets affecting primarily insulin containing islets, is present in the majority of cases. The inflammatory infiltrate consists primarily of T lymphocytes; CD8 cells outnumber CD4 cells, there are fewer B lymphocytes and macrophages are relatively scarce. β cell death may involve the Fas apoptotic pathway since they have been shown to express Fas, infiltrating T lymphocytes express Fas-L and apoptotic β cells have been described. Hyperexpression of class I MHC by all the endocrine cells in many insulin-containing islets is a well recognized phenomenon, characteristic of the disease. It has been argued that this is an earlier event than insulitis within a given islet and appears to be due to secretion of interferon α by β cells within that islet. A recent study has found evidence of Coxsackie virus infection in β cells in three out of six pancreases of patients with recent-onset type 1 diabetes. Coxsackie viruses are known to induce interferon α secretion by β cells and this could initiate the sequence of events that culminates in their autoimmune destruction.

2008 Defining optimal immunotherapies for type 1 diabetes. Wiley, Chichester (Novartis Foundation Symposium 292) p 2–18

There are a number of different ways of obtaining pancreas specimens from patients with type 1 diabetes. Historically, the most common source was retrospective collections of autopsy pancreases from children who had died around the time of clinical diagnosis (Foulis et al 1986, Gepts 1965). The disadvantage of this approach was that there was usually a degree of autolysis in the tissues and the pancreas would almost certainly have been fixed in formalin and paraffin embedded. These factors and the lack of access to peripheral blood of the patient limited the range of possible studies on these pancreases.

A radical departure from this historical practice has been the approach of the group from Osaka. They performed laparoscopic pancreatic biopsies on patients who had been diagnosed with type 1 diabetes in the previous three months. A great range of tests has been done on this tissue and the results have been correlated with clinical findings. The disadvantage is that the biopsies were small

(20–30 mg) leading to a possible sampling problem. Thus the biopsies of three out of the first seven patients had no insulin-containing islets (Hanafusa et al 1990). While pancreatic biopsy has proven to be safe, no other research group has adopted this practice.

Finally, in the last 15 years a number of patients with recent-onset disease have died in intensive care units and permission has been given to remove organs for transplantation. The pancreas has thus been removed immediately after death, there has been no shortage of tissue and a full range of tests could be done (Dotta et al 2007).

Insulits

If the pancreas of a patient who has had type 1 diabetes for more than five years is studied, the great majority of islets will be seen to be insulin deficient. They consist of a normal number of the other hormone-secreting cells found in the islets of the pancreas (pancreatic polypeptide-secreting PP cells, glucagon-secreting A cells and somatostatin secreting D cells) (Foulis & Stewart 1984). There has thus been selective loss of the β cells. If the pancreas is studied at or within a year or two of clinical diagnosis, three types of islet are found (Gepts 1965, Foulis & Stewart 1984). Firstly, approximately 70% of the islets are insulin deficient (identical to those found in patients with prolonged disease). Secondly, there are islets containing β cells that are affected by insulitis (a chronic inflammatory infiltrate within the islet, Fig. 1) and, thirdly, there are insulin-containing islets which appear essentially normal. The finding that 18%

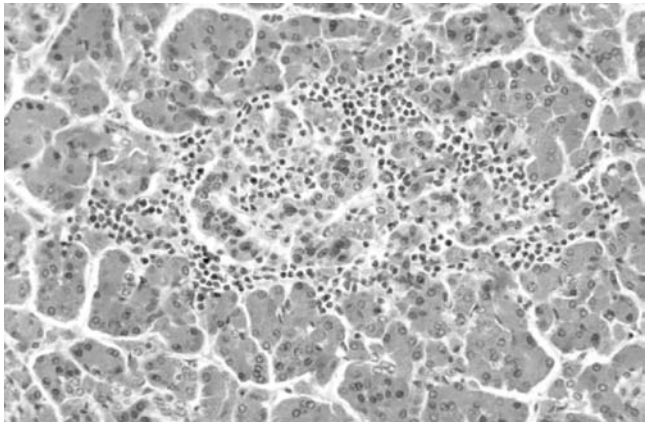


FIG. 1. Insulitis. There is a predominantly lymphocytic infiltrate in this islet.

of insulin containing islets but only 1% of insulin-deficient islets were affected by insulitis helped support the concept of there being an immunologically mediated destruction of β cells in the pathogenesis of type 1 diabetes (Foulis & Stewart 1984).

It can be seen therefore that within a given pancreas at clinical presentation there are islets where the β cells have been destroyed (insulin deficient), islets where the β cells are being destroyed (insulitis) and islets where the β cells are yet to be destroyed (normal). It has been argued that the pancreas in type 1 diabetes at clinical presentation is very similar qualitatively to the pancreas a few years after clinical presentation and also to the pancreas before clinical presentation. All three types of islet described above are present but the proportion of the islet types varies greatly with duration of the disease (Foulis 1989). Insulits affecting insulin containing islets has been observed six years after clinical presentation and in this pancreas 95% of islets were insulin deficient (Foulis et al 1986) By contrast in a pre-diabetic pancreas only 4% of islets were insulin deficient but insulitis was also observed (Foulis et al 1986). Thus it seems that the disease process in the pancreas is remarkably similar over a long period of time, with clinical presentation occurring when two thirds of the islets are insulin deficient (Foulis et al 1986). It follows that study of disease phenomena in the pancreas at clinical presentation should help to elucidate the pathogenesis of type 1 diabetes both at clinical presentation and in the pre-diabetic period.

Inflammatory cells in insulitis

Bottazzo et al (1985), in their case report, were the first to study the nature of the inflammatory infiltrate in insulitis. It consisted essentially of lymphocytes, with macrophages being inconspicuous. The majority of the lymphocytes were cytotoxic T cells. All studies on autopsy pancreases have repeated the observation that macrophages represent a minor population of the infiltrate. In a study of 87 affected islets from 12 autopsy pancreases the ratio of lymphocytes to macrophages was 10:1 and the average number of lymphocytes per inflamed islet was 85 (Foulis et al 1991). The first study of pancreatic biopsies reported no evidence of insulitis even in the four pancreases with residual β cells (Hanafusa et al 1990). Subsequent studies from the Osaka group however did report insulitis. Interestingly, their definition of insulits in the later studies was an islet infiltrated by two or more inflammatory cells (Itoh et al 1993) Even in this minimal (significant?) form of inflammation the predominant inflammatory cell was the $CD8^+$ T cell. These findings are consistent with destruction of β cells by cell-mediated cytotoxic T cell attack and do not support a major role for bystander damage by cytokines released by macrophages.

Fas and Fas ligand expression

Two groups have looked at Fas and Fas ligand (Fas-L) expression in insulitis. Fas-positive endocrine cells were detected in islets affected by insulitis but not in non-inflamed islets in diabetics or in normal control pancreatic islets (Stassi et al 1997, Moriwaki et al 1999). Interestingly, Moriwaki et al (1999) showed that while most B cells were Fas positive a significant minority of A cells also expressed this receptor. Infiltrating lymphocytes were Fas-L positive while islet endocrine cells were Fas-L negative. These observations have led to the hypothesis that cytokines such as interferon (IFN)γ, tumour necrosis factor (TNF)α or interleukin (IL)1, which induce Fas expression by islet endocrine cells *in vitro*, could be released in the insulitis process and cause the same effect *in vivo*. In this manner Fas-L-positive infiltrating cells in the inflamed islets could destroy Fas-positive β cells.

β cell apoptosis

A number of groups have looked for evidence of β cell apoptosis. No affected β cells were seen in pancreatic biopsies by the Osaka group (Moriwaki et al 1999), while others found evidence for plentiful apoptosis in β cells in autopsy pancreases using the TUNEL method (Meier et al 2005, Stassi et al 1997). In view of the lack of evidence of β cell regeneration one has to view an apoptosis prevalence of 6% of β cells (Meier et al 2005) as being extremely unlikely given the fleeting nature of apoptotic bodies and the very long time over which β cell destruction appears to take place clinically.

Aberrant expression of class II MHC by β cells

It was hypothesized that aberrant expression of class II MHC by insulin-secreting β cells (Fig. 2) could lead to their presenting self antigens, with resulting autoimmunity (Bottazzo et al 1983). β cells do not normally express class II MHC but they did show this phenomenon in pancreases of 21 out of 23 cases of recent-onset diabetes (Foulis et al 1987a). In these cases aberrant expression of class II MHC was found in 12% of insulin-containing islets, and double stains showed that it was confined to β cells being not present in A, D or PP cells. The phenomenon has also been described in pancreatic biopsies of two Osaka patients (Imagawa et al 1996). Half the islets in which β cells expressed class II MHC had no evidence of inflammation, raising the possibility that in a given islet this abnormality preceded insulitis (Foulis et al 1987a). β cells expressing class II MHC were not seen in 95 control pancreases from patients with a variety of diseases including type 2 diabetes, graft versus host disease, chronic pancreatitis, cystic fibrosis and enteroviral pancreatitis (Foulis et al 1987a).

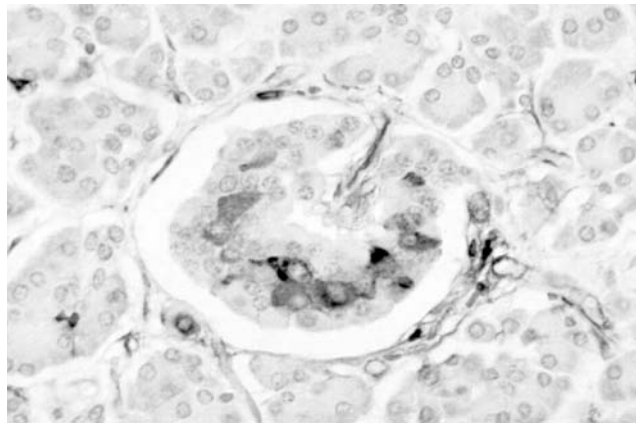


FIG. 2. Aberrant expression of class II MHC on endocrine cells. Double stains showed that these were β cells.

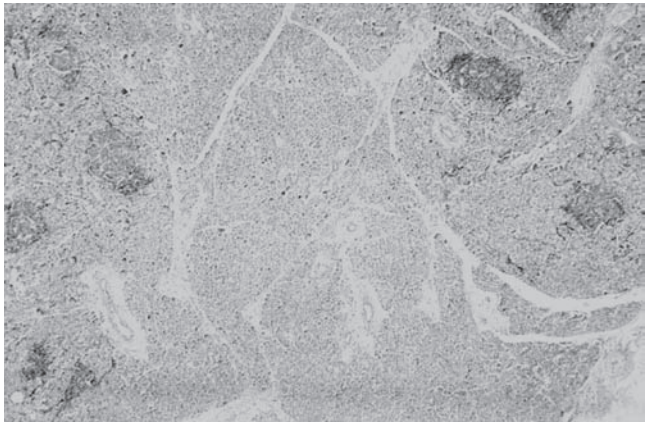
An antigen-presenting cell must express co-stimulatory molecules such as CD80 and CD86 as well as class II MHC to successfully present antigen to $CD4^+$ Th cells. Evidence against a pathogenetic role for aberrant expression of class II MHC on β cells has been the failure to demonstrate expression of either of these co-stimulatory molecules by β cells in pancreatic biopsies of patients with recent-onset type 1 diabetes (Imagawa et al 1996).

Hyperexpression of class I MHC by insulin-containing islets

Cytotoxic ($CD8^+$) T cells, which are the dominant cell type in insulitis, recognize antigen when it is presented in association with class I MHC by a target cell. Hyperexpression of class I MHC by the target cell is likely to enhance this engagement. A phenomenon unique to type 1 diabetes is hyperexpression of class I MHC by all the endocrine cells in insulin-containing islets (Foulis et al 1987a). 92% of insulin-containing islets hyperexpressed class I MHC in contrast to only 1% of insulin-deficient islets (Fig. 3). The phenomenon was not seen in islets in any of the 95 control pancreases in that study. Class I MHC hyperexpression of islet endocrine cells was induced *in vitro* by IFNα, IFNβ or IFNγ (Pujol-Borrell et al 1986). Forty per cent of the lymphocytes in the insulitis infiltrate expressed IFNγ

FIG. 3. (a) Islets in two lobules hyperexpress class I MHC. (b) This is a serial section to Fig 3a stained for insulin. Insulin-containing islets hyperexpress class I MHC in type 1 diabetes. (c) This serial section has been stained for glucagon. Numerous shrunken insulin deficient islets are present in the centre of the photograph which do not hyperexpress class I MHC.

(a)



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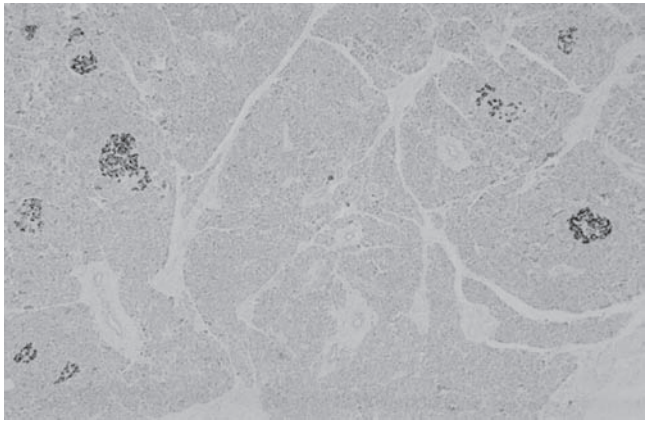

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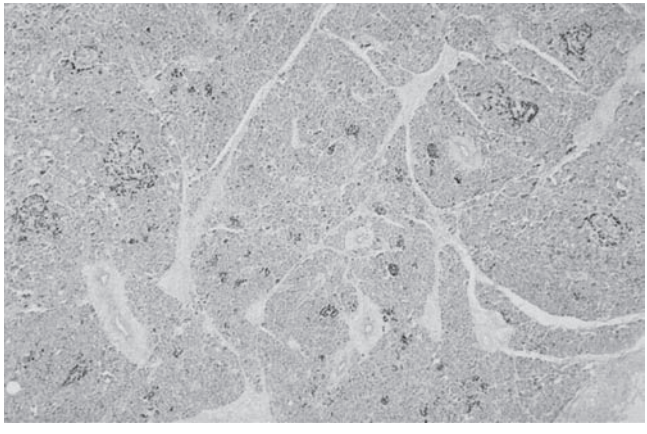

(Foulis et al 1991) so it might be supposed that this hyperexpression of class I MHC would be a secondary event following insulits. However, even when whole islets in multiple serial sections were studied it was clear that over half the insulin-containing islets which hyperexpressed class I MHC had no evidence of insulitis whatsoever. Thus it was argued that hyperexpression of class I MHC by insulin-containing islets was an earlier event in the disease process than insulitis. Comparison of class I hyperexpression and aberrant class II expression by β cells showed that all islets where the latter phenomenon was seen hyperexpressed class I MHC. By contrast 73% of islets which hyperexpressed class I MHC showed no evidence of aberrant expression of class II MHC on β cells. Thus hyperexpression of class I MHC also appeared to be an earlier event in the disease process within an islet than class II MHC expression by β cells. Finally it was noted that A and D cells hyperexpressed class I MHC when they lay adjacent to β cells in insulin-containing islets of type 1 diabetes patients, but not when they were physically divorced from β cells in insulin-deficient islets. This raised the possibility that the β cells were releasing a type 1 interferon that was causing the hyperexpression through a paracrine effect (Foulis et al 1987a).

β cells express IFNα in type 1 diabetes

An immunohistochemical analysis of IFNα expression in type 1 diabetes was therefore undertaken. β cells, but not A, D or PP cells expressed IFNα in all 28 pancreases from patients with recent onset type 1 diabetes. This expression was closely related to class I MHC hyperexpression. β cells expressing IFNα were found in 94% of islets which hyperexpressed class I MHC but only in 0.2% of islets which did not hyperexpress this complex. Among 80 control pancreases, β cells expressed significant IFNα in four cases of Coxsackie B viral pancreatitis but not in other pancreatic diseases (Foulis et al 1987b).

Possible sequence of immunological events in islets (Fig. 4)

The conclusion of the studies outlined above is that the first abnormality in an islet in type 1 diabetes is expression of IFNα by β cells. Secretion of this cytokine is likely to cause hyperexpression of class I MHC by all the endocrine cells within that islet. Aberrant expression of class II MHC is a later event, which probably occurs in a minority of islets, but it too appears to precede insulits. The finding that β cells secreted IFNα in enteroviral pancreatitis as well as type 1 diabetes raises the possibility that a non cytopathic viral infection of β cells is the initiating event in the disease process leading to autoimmune destruction of β cells and type 1 diabetes (Foulis 1989).

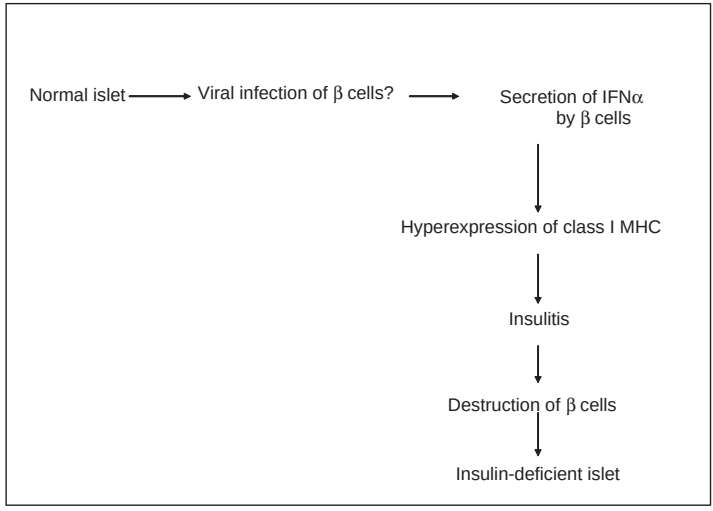

FIG. 4. Possible sequence of events in islets in type 1 diabetes.

Enteroviral infection and type 1 diabetes

There has long been speculation that enteroviruses, particularly Coxsackie B viruses, are involved in the pathogenesis of type 1 diabetes. Famously, a Coxsackie B4 virus was cultured from the pancreas of a child who died of recent-onset diabetes and this virus was capable of inducing diabetes in mice (Yoon et al 1979). In spite of many attempts no other reports of such a virus being cultured under these circumstances was published in the following 25 years (but *vide infra*).

In a survey conducted in three different countries 30% of patients at the time of clinical onset of the disease had increased levels of IgM antibodies to Coxsackie B viruses, suggesting recent or continuing infection (Banatvala et al 1985). Enteroviral mRNA with sequence homology to Coxsackie B3 and B4 was found by RT-PCR in serum of nine of 14 children with recent onset diabetes, all of whom were 6 years old or less (Clements et al 1995).

It is recognized that there may be a pre-clinical period lasting years during which there is evidence of islet cell autoimmunity but no evidence of clinical diabetes. Several groups have looked for evidence of viral infection in the period immediately before autoantibody seroconversion by surveying young siblings of diabetic patients (Hiltunen et al 1997) or studying genetically high-risk infants from birth (Salminen et al 2003). Both approaches showed significantly more enteroviral infections in the months preceding seroconversion in patients who became

autoantibody positive than among controls, although this observation was not repeated in a North American study (Graves et al 2003).

The search for enteroviruses in pancreases of type 1 diabetic patients

Initially an immunohistochemical study looking for enteroviral capsid protein Vp1 was performed on autopsy pancreases. Vp1-positive cells were found in pancreatic tissue of seven of 12 infants who had died of neonatal enteroviral myocarditis. The virus showed marked tropism for the islets rather than exocrine tissue and, while A cells were sometimes affected, β cells particularly frequently showed a cytopathic effect. Study of pancreases of 88 young patients who had died at clinical presentation of type 1 diabetes showed no evidence of infection using this technique (Foulis et al 1990). In retrospect it could be argued that the immunohistochemical technique used may have had poor sensitivity as it was done prior to the development of the technique of antigen retrieval from formalin fixed tissue. A similar study in which *in situ* hybridization for enteroviral RNA was performed also found no evidence of viral infection in the diabetic pancreases (Foulis et al 1997). This contrasts with a more recent *in situ* hybridization study of autopsy pancreases of 65 diabetic patients aged 18 to 52 years in whom evidence of enterovirus infection was found in islets in four pancreases (Ylipaasto et al 2004). In this study it is not clear whether the virus detected was in insulin-containing islets or not and the duration of diabetes in those affected was not known.

A recent study offers further evidence of enteroviral infection in the pancreas in type 1 diabetes (Dotta et al 2007). These authors studied six pancreases removed from patients with type 1 diabetes immediately after death, at the time of organ harvest for transplantation. Two patients had died as a result of accidents and they had had diabetes for 8 and 9 months, respectively. Three patients had died of complications of ketoacidosis at their first clinical presentation of diabetes. The pancreas of the remaining patient was an allograft that was removed from a patient with type 1 diabetes because of septic complications.

Evidence of enteroviral infection was sought using a number of techniques. Firstly, immunohistochemistry, using antigen retrieval on formalin-fixed tissue, demonstrated enteroviral Vp1 capsid protein in β cells but not A cells in the majority of islets in three of the six patients. Secondly, electron microscopy showed viral inclusions in over 75% of β cells in Vp1-positive islets, but not in A or D cells. Thirdly, a virus was extracted from one pancreas and sequence analysis showed that it was a Coxsackie B4 virus.

There are a number of caveats to this study. Firstly, one of the pancreases in which Vp1 positivity was found was a transplanted organ from a patient who was being immunosuppressed up until organ extraction. Secondly, there was no

reported loss of β cells in the islets of the three patients in whom enterovirus was detected. The patient in whom enterovirus was isolated had had type 1 diabetes for 9 months and it is distinctly unusual to find normal numbers of β cells in type 1 diabetes of this duration. The three pancreases in which no virus was seen had more typical findings of type 1 diabetes, with reduced numbers of β cells.

Interestingly, the isolated virus was able to infect β cells in islets cultured from non-diabetic donors. Infected β cells showed little evidence of cell death but did show reduced insulin secretion on stimulation with a variety of secretagogues.

Conclusion

Dotta et al (2007) concluded that a non-cytopathic enteroviral infection of β cells could occur in type 1 diabetes causing functional impairment of glucose metabolism. Such an infection has been shown to cause secretion of IFNα by β cells *in vitro* (Chehadeh et al 2000). Secretion of IFNα by β cells *in vivo* probably causes hyperexpression of class I MHC and signifies activation of the innate immune system. Loss of tolerance to β cell antigens in genetically susceptible individuals may be provoked by a degree of damage to β cells as a result of the viral infection, with subsequent presentation of β cell antigens. Professional antigen presenting cells within islets may perform this role although conceivably β cells themselves, by virtue of class II MHC expression, may be involved (Fig. 5).

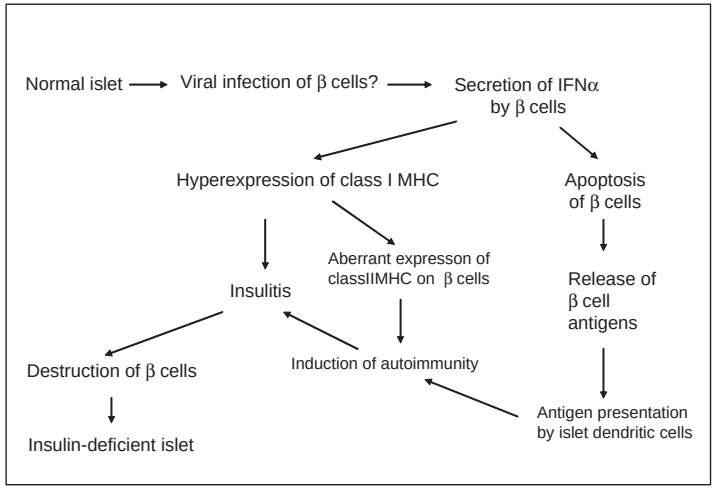

FIG. 5. Possible sequence of events in islets in type 1 diabetes.

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DISCUSSION

Lew: Have you looked at IFNα expression in those cells that were staining for enterovirus protein?

Foulis: Not yet.

Lew: Your viral infections seem so patchy, yet your class I expression is widespread throughout the islet. I know IFNα is an 'altruistic' cytokine, so it can go to lots of other cells, but *a priori* it seems that there is a link between it and the expression of the viral protein.

von Herrath: There might be a detection issue here. In the earlier studies you didn't find virus. It is probably not so easy to find these viral patches. What is known about these Coxsackie B4 strains that selectively infect the islets? Do you know what receptor they use? Is it the Coxsackie virus CAR receptor?

Foulis: Yes, it has been shown that is the case *in vitro*. They use PVR and integrin αVβ3 enterovirus receptors in cell lines (Ylipaasto et al 2004).

von Herrath: Presumably the Coxsackie virus B3 strains don't do this.

Roep: And not all the B4s do. There is a remarkable similarity between the isolate of Francesco Dotta which was β cell tropic, and another isolate which is also β cell tropic. They have sequence similarities that are different from some other virus strains that do not infect β cells.

von Herrath: Have they mapped this to receptor binding?

Roep: No.

Eisenbarth: We have to be very careful that the monoclonal antibody is only seeing a virus. Roberto Gianani is seeing Vp1 staining in 'normal' cadaveric donors. I'd bet that monoclonal cross-reacts with other molecules, not only the virus. I love the idea that pre-onset, onset and post are all the same: these data are clear. There is only one prediabetic in your data, and this individual had very few cells with IFNα or MHC up-regulation. An alternative hypothesis is that the up-regulation of class I MHC occurs because of insulitis. What is the difference between the prediabetic having so few cells, and all the others having class I MHC in almost all the islets? My bias is that it might well be that viruses aren't involved at the islet level. Class I MHC might still be due to insulitis at some point in time. I don't understand why the prediabetic had so few cells with class I MHC increased, or IFNα.

Peakman: We don't know where it is on the progression.

Eisenbarth: We need more prediabetics, but it is striking that it is so different.

Foulis: It may be that the two pancreases that I studied were from patients who would have been years from developing diabetes, or may never have developed diabetes. But there is evidence of autoimmunity in one of them (autoantibodies). The islet phenomena are the same as seen in diabetes but apparently at an earlier stage.

D Hafler: Have other antibodies against other Coxsackie viruses been used in this system? Immunoprecipitation and mass spectrometry on the tissue, to confirm the finding, would be of interest. George's point is critical: the degree of cross-reactivity among monoclonal antibodies is tremendous, but there is something there the antibody is cross-reacting with. This is of interest. You don't need much tissue to do mass spectrometry any more.

Foulis: All the samples I have are from autopsies and are fixed in formalin which limits the range of tests that can be done.

Insel: 20 years ago you performed *in situ* hybridization and immunocytochemistry. What do you do now that is different to what was done then?

Foulis: We now use a different antibody and also antigen retrieval. The latter is a kind of dark art. We heat the section up in a variety of solutions, such as citrate and EDTA. This enables us to stain things in formalin fixed tissue that we can't stain otherwise. You then have to prove what you are staining is the right thing, but you have to do that in any event if you do not use antigen retrieval.

D Hafler: So this process denatures proteins.

Foulis: The formalin fixation appears to hide certain epitopes and antigens. Heat-induced antigen retrieval exposes these hidden epitopes again, but you need good controls to prove that you are staining the correct antigen.

Butler: In the context of a virus infection, is it known that it this Coxsackie virus is a β cell tropic virus? Are the receptors you are referring to on β cells and not α cells? It is intriguing that some β cells are positive and others aren't. This implies some heterogeneity of vulnerability among β cells. This might give some insight into the slowness of what you are referring to, if a lot of the cells are somehow resistant to the infection even if the cell next door is infecting. As someone who has looked at some recent-onset type 1s, the frequency of β cell apoptosis strikes me as being incredibly low compared with what I would have expected. I expected type 1 diabetes to show high rates, but they are no higher than in type 2 diabetes, which itself is subtly higher than non-diabetics. It doesn't make the slightest bit of difference whether you look at an islet that is decorated with lymphocytes or one that has no lymphocytes. The frequency of apoptosis bears no relationship with that lymphocytic infiltrate. The other thing that was intriguing to us is that we see islets that have no β cells. We stain them up with a mixed cocktail that you mentioned. Some of these have a lymphocytic infiltrate, which puzzled me because I had though that β cells were needed to drive that process. In general, looking at these recent cases we have rediscovered everything you discovered 20 years ago: there is a lobular pattern, some lobules with no β cells and others with normal islets. Again, looking at apoptosis rates, there is not a lot of difference between those that are inflamed versus those that aren't. I am intrigued by the patchy infection by the virus within the islet, with one cell affected and the next cell not. Since we have so much immunology brain power in here I am intrigued to know if anyone has insight into this.

von Herrath: It is frequently seen following viral infections when we look for viral antigen that it is not homogenous within an organ, even if the virus has tropism for many different cells. I would consider this to be a normal situation. What is curious here is that mostly the Coxsackie B4 strains seem to be getting into β cells.

Roep: That is not true. What Roivainen shows is that of any enterovirus strain, some are doing it but the majority are not doing it. I am a co-author on Francesco's paper (Dotta et al 2007). He isolated the virus and then he infected other normal islets, and all of the β cells became infected. Also, the islets that were affected had an impaired first-phase insulin release, but when the virus is removed it is possible to restore normal β cell function. There is an apparent reversibility in the process.

Butler: Your electron micrographs made it look like pretty much all the cells that were positive.

Roep: That is *in situ* staining of the diabetic patients, not after infection *ex vivo.*

Foulis: I'd like to back up the comments on viral infection. Hepatitis B infection in the liver is patchy. It appears random. Some patients have many infected hepatocytes while others have few. I think many viral infections are like that.

Peakman: I wanted to comment on Roivainen's paper (Ylipaasto et al 2004), which supports what you see. This is a different technique—*in situ* hybridization—tested on a bunch of negative normal pancreas and then tested on pancreas from type 1 diabetes patients. I also have a question about the class I reagent. Does this distinguish classical from non-classical MHC?

Foulis: I don't know.

Peakman: The reagent you are using is a polyclonal. Does it stain a framework that may be present on things like MICA?

Foulis: I have no idea. I showed that an antibody to β2 microglobulin stains islets in type 1 diabetes in exactly the same way as the antibody to class I MHC.

Herold: I have a few questions. First, the only way you knew the islets from patients with diabetes had β cells or not was the presence or absence of insulin. Is it possible that there were β cells present there that didn't express insulin? Second, the next most common cell in the islets apart from the CD8s is the B cell. Is it uncommon for viral infections to see a predominance of B cells? What are they doing there? Are there mouse data about the susceptibility of damaged islets to viral infection? Could you clarify your model: this initiating event must have occurred years before, yet you show us data from new-onset patients. Aren't we looking at epiphenomena here?

Foulis: I try to make the argument that the islet phenomena seen in pancreases of prediabetics, recent-onset diabetics and patients with diabetes for several years are the same. In other words when 4% of the β cells have been knocked out the phenomena are the same as when 80% of the β cells have been knocked out, and this is the same as when 99% of the β cells have been knocked out. Thus one can extrapolate back to talk about early events in the disease process.

Kay: Is it expected that an enterovirus infection seven to 10 years previously would still be present at diagnosis? Say we accept the data that there is Vp1 staining reflecting enteroviral infection at the time of diagnosis: is this the continuation of an initiating event?

Foulis: That's an important question. Previously, people thought that enteroviruses came and went quickly. They probably thought this about a lot of viruses. It now seems that many viruses seem to persist in the body for long periods. Clearly more work needs to be done in examining this phenomenon with respect to enteroviruses.

Roep: There could be a problem in the design of these experiments. Type 1 diabetes patients normally don't die at diagnosis. This need not mean that these patients would have 10 years of prediabetic stage. There is some ascertainment bias in the Dotta study. You cited one case that was an allograft and another that

had an infection at onset of diabetes. There could be an ascertainment selection bias that would affect the prevalence of this enterovirus infection.

Foulis: That is true in respect of Dotta's cases (Dotta et al 2007). Among the patients I have studied the case with the most Vp1 expression is a 42 year old diabetic of 9 months duration woman who died of a brain tumour. She did not die of a complication of type 1 diabetes.

Herold: Still, there is this question about the susceptibility of injured β cells to viral infection.

Roep: The work of Nora Sarvetnick (Horwitz et al 1998) is interesting here. She did some studies on Coxsackie virus, and showed that enteroviral infection doesn't cause β cell destruction, but if there is pre-existing autoimmune insulitis and then have an infection of the Coxsackie you actually precipitate the disease. This way you could have a convergence of different disease mechanisms ending up in the β cell destruction.

von Herrath: The finding that there is all this class I MHC and IFNα without profound cellular islet infiltration is striking. Tom Kay and I published a paper (Seewaldt et al 2000) about class I up-regulation on β cells following viral infection. This 'unmasking event' enabling recognition by cytotoxic $CD8^+$ T cells is definitively experimentally provable. In addition, these are highly dynamic systems and MHC or IFNα once induced do not stay up-regulated for life: this would be terrible after a virus infection. If the virus is present the interferon goes up, and then when the virus is gone it goes down along with class I. You can infect the whole pancreas with the virus, unmask the islets, the virus gets cleared and class I goes back down. This argues strongly that you have a chronic viral infection that pushes class I up. I would argue that if the virus is gone, class I and IFN should all go back to baseline, especially if there are no T cells in there. These considerations make the search for such agents quite important.

Foulis: The key for this is the IFNα. It seems to be expressed by every β cell in the islets where it is found. It is intimately associated with islet hyperexpression of class I MHC in type 1 diabetes.

Roep: The different morphology of islets with or without insulitis, or with or without β cells implies that T cells must circulate during the disease process. It is therefore not inconceivable that circulating T cells are reflecting insulitis. It is a pity that Jeff Bluestone isn't here, because he laughed at me when I commented that human insulitis isn't the same thing as mouse insulitis. You have shown that they are different. Can you comment on these differences?

Butler: We see periinsulitis around the islets in humans, but this is much less common than in mice when we compare them side by side. Immunologists didn't rise to my bait when I said that there is no correlation between β cell apoptosis and any of these appearances in terms of lymphocytic infiltrates. I'll throw out here the point that this might not be an immunology disease. Perhaps it is all virus.

Roep: That is material from obduction. You could be seeing non-specific infiltrates in the pancreas of people who have been in intensive care for a long time.

Foulis: Yes, some of the pancreas samples we have show signs of acute pancreatitis, as well.

Flavell: What is known about the polymorphism of the IFN response in diabetes? We have talked a lot about virus and interferon responses, and Matthias made the point that the IFN response should wane rapidly, but everything is polymorphic. Has anyone screened diabetics versus MHC matched controls?

Foulis: A paper from the Lille group looked at circulating levels of IFNα in patients with diabetes. 70% of them had raised IFNα. They then also looked for enterovirus RNA in the blood and found it in half of the diabetic patients who had a raised IFNα level, but not in any of the patients who had normal IFNα levels (Chehadeh et al 2000).

Flavell: Someone should take fibroblasts, stimulate them and compare them with controls. It may be that there is greater sensitivity or greater prolongation of response.

Butler: Didn't the Dotta group find that healthy ordinary islets were attacked by the same isolates?

D Hafler: Yes, but that would be expected.

Roep: But he didn't have IFNα expression as a readout.

Staeva: It is important in the context of this discussion to mention that JDRF has just launched a large initiative, termed nPOD, to procure and characterize pancreas, pancreatic lymph nodes and spleen from three types of organ donors: prediabetics, people with recent onset diabetes, and people with long-standing diabetes. This initiative should be able to address many of these questions.

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The β cell population in type 1 diabetes

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Abstract. Type 1 diabetes is often considered as a disease where more than 90% of the β cells have been destroyed at clinical onset and where β cell antigen-driven autoimmune reactivities progressively destroy remaining β cells as well as newly formed or implanted β cells. This view will be evaluated in light of histological observations in the pancreas of type 1 diabetic patients and of antibody-positive non-diabetic organ donors.

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Variable degree of β cell loss at clinical onset and of β cell survival in long-term type 1 diabetes

Type 1 diabetes is caused by a massive reduction in the insulin-producing β cell mass. Few quantitative data are available on the loss in human organs that were analysed immediately before or after clinical onset of the disease. Gepts was the first to compare β cell numbers per pancreatic surface area in normal controls with those in patients who died shortly after diagnosis of 'juvenile diabetes' (Gepts 1965). When selecting the data from the 12 cases studied within 15 days after clinically detected onset, a reduction of 88% becomes apparent versus the numbers counted in sections from non-diabetic controls in the same age category (0 to 30 years of age) (Gepts 1965, reviewed in Pipeleers & Ling 1992). Analyses at later time points indicated more islets devoid of β cells and predominantly composed of glucagon-containing α cells or pancreatic polypeptide-positive PP cells (Gepts 1965, reviewed in Gepts 1981). These observations have maintained for many years the view that at least 90% of β cells are destroyed in type 1 diabetes and that the disease process goes on until all β cells are destroyed. In reviewing the cases reported by Gepts and by other pathologists, we noticed that shortly after clinical onset, virtually all cases presented β cells in the

pancreas but that the β cell number per surface area was higher in older patients (Pipeleers & Ling 1992). An age difference was also observed in the proportion of cases with complete loss of β cells following diagnosis: for a clinical onset under age 7, all β cells seem to disappear within the first year while long-term survival of β cells became more and more frequent when overt diabetes appeared at an older age (Pipeleers & Ling 1992). This histopathological finding correlates with measurements of β cell function: a residual C-peptide secretion is present in most patients at diagnosis, rapidly disappears in young patients and remains detectable in increasing proportions with older age at diagnosis (Madsbad 1983, Wallensteen et al 1988). Use of autoantibody assays has shown that the onset of type 1 diabetes is not restricted to the age category under age 15 years. Although Gepts did use the term 'juvenile diabetes' for patients up to 30 years (Gepts 1965), type 1 diabetes was long considered as a disease that most often affected young children and late infants (Marker & Maclaren 2001). Influenced by the microscopic observations in the sections collected by Gepts (Pipeleers & Ling 1992) we decided to extend the antibody measurements to older age categories, up to 40 years. The Belgian Diabetes Registry thus became one of the first to describe that after the well-known incidence peak of type 1 diabetes around puberty the incidence remains relatively high between age 15 and 40, in particular in males (Vandewalle et al 1997). This led to the conclusion that in the Belgian population more cases are diagnosed after age 20 than before that age (Weets et al 2001). Overall, the acuteness and severity of clinical presentation significantly decreased with age at presentation. With older age at diagnosis a decrease was seen in prevalence of insulin- (IAA), islet cell- (ICA) and insulinoma-associated antigen 2 antibodies (IA2-Ab), in multiple antibody positivity and in presence of the high risk genotype *HLA DQ2/DQ8*, whereas an increase was measured in the prevalence of glutamate decarboxylase anti-bodies (GAD-Ab) and in residual C-peptide levels (Gorus et al 2001). These observations cannot be taken as evidence for age-dependent differences in underlying causes and mechanisms, although they do not exclude this possibility. They do however demonstrate that the degree of β cell loss varies among patients, with a marked age-dependency. The residual β cell mass at clinical onset is often not negligible: in a series of 80 C-peptide positive patients investigated within four weeks after diagnosis, the insulin secretory capacity averaged 25% of that in normal controls (Keymeulen et al 2005). Furthermore, surviving and functioning β cells remain present in a significant fraction of patients who developed the disease after the age of 15 years, where also the majority of cases are diagnosed. It is conceivable that the lifespan of these β cells is lower than that in normal controls (Meier et al 2005), and that their function is abnormal or dysregulated. Their mere existence nevertheless demonstrates that the disease process in humans can leave a window for neoformation and/or survival of β cells. This may in itself not be

sufficient for recovery of physiological functions but could serve as target for therapeutic aid.

β cells with lymphocytic infiltrate, an uncommon but pathognomonic islet lesion

The presence of an inflammatory infiltrate in islet tissue has long been recognized as characteristic for a pancreas of a juvenile diabetic patient but its rare occurrence left doubts on its pathogenic significance (Stansfield & Warren 1928). In 1965, Gepts reported that the lesion was more frequent than originally thought, being present in 68% of the organs he examined soon after diagnosis (Gepts 1965). Since he did not detect it in cases with a longer clinical duration, he interpreted it as a glimpse of a final stage of a process that had been going on for an indefinite time, perhaps from birth on (Gepts 1965). He considered the process as a chronic inflammatory reaction, possibly involving an immunological derangement (Gepts 1965). Numerous subsequent studies on the immune system in type 1 diabetic patients and in animal models with insulitis have demonstrated the presence and pathogenic significance of autoimmune reactivities against β cell antigens (Liu & Eisenbarth 2002, Yang & Santamaria 2006). They led to the concept that the β cell destruction in type 1 patients is caused by a β cell antigen-driven lymphocytic infiltration and cell death that goes on until all islet β cells have disappeared and that also interferes with the survival of newly formed β cells later in life (Meier et al 2005). The insulitis lesion described by Gepts became regarded to be a hallmark of the autoimmune pathogenesis of type 1 diabetes. It remains however unknown whether it occurs in all patients and when and why it appears in the disease process. The problem is that so little information is available on the islet pathology in human type 1 diabetes, and that reported data are not always easy to interpret or compare. Since the work of Gepts only a few histopathological studies have been performed on pancreatic tissue from type 1 diabetic patients, mostly in the late phase of the disease i.e. after clinical onset. The representativity of the data is often uncertain as the examined surface area was either small or not mentioned and the criteria for a leucocytic infiltration not defined or questionable. Within the limitations of these restrictions, it can be concluded that islets with lymphocytic infiltrations have so far not been commonly encountered in the pancreas of type 1 diabetic patients while their pathogenic significance appears related to disease phases in children and young adolescents.

In an analysis of 178 autopsy observations, we found that insulitis was reported in all patients younger than 15 years and examined within one month after clinical onset, but only in 38% of patients between 15 and 30 years of age at that stage (Pipeleers & Ling 1992). In both age categories, the lesion was mostly confined to

a minority of islets; it was much less frequently observed during the year following diagnosis, and was not found at all at later time points, irrespective of the presence or absence of β cells (Pipeleers & Ling 1992). Since no information was available on circulating autoantibodies in these cases, it cannot be ruled out that some correspond to a non-autoimmune form. On the other hand, data from the Belgian Diabetes Registry have indicated that this form represents less than 5% of cases diagnosed under age 30. Instead of inferring different forms of disease, we attribute the observed age-dependent variability in insulitis and in β cell losses as signs of a disease process that can start at all ages with generally a faster progression in children or start at young age and progress differently depending on the genetic or environmental variations; this process does not necessarily affect all islets, and is not synchronized in all islets that will be affected. It is thus conceivable that more islets have presented an inflammatory infiltrate in the prediabetic phase, perhaps many years before clinical onset in adolescents and young adults, leading to losses of β cells in infiltrated islets and to rapid or slow progression to diabetes depending on the percent of affected islets. The presence of islets with insulitis in the diabetic pancreas is considered as a remnant of a process that has affected many more islets in the prediabetic phase and then disappeared. Their absence is a sign that not all islets attract inflammatory infiltrates, or at least not within the same time frame. We believe that the heterogeneity in the pancreatic β cell population can explain, at least in part, this heterogeneity in the disease process (Pipeleers & Ling 1992). Another potential variable is of course the type and severity of the inflammatory and immune reactivities that can vary with age, as can also the β cell properties.

It has been proposed that the lymphocytic infiltration of the islets is preceded by an MHC-class I hyperexpression on the β cells as may be induced by a virus (Foulis et al 1987, Harrison et al 1989). Foulis found this hyperexpression in both islets with and without insulitis (Foulis 1987) and associated it to a higher local expression of interferon α (Foulis et al 1987). These observations were confirmed by others (Itoh et al 1993, Huang et al 1995) and supported the view that MHC-class I hyperexpression by β cells drives interactions of their autoantigen(s) with $CD8^+$ T lymphocytes. Since these studies were conducted on organs from diabetic patients the possibility should also be considered that elevated glucose levels may have contributed to the MHC up-regulation, as was observed *in vitro* (Pavlovic et al 1997). The observation that not all β cell-containing islets presented a MHC class I hyperexpression is another sign of heterogeneity in the β cell population in the diabetic pancreas and supports its participation in the disease process (Pipeleers & Ling 1992).

Since the time of clinical onset corresponds to a late phase of the disease where most of the insulitis lesions may have disappeared, in particular in adults,

we reasoned that the lymphocytic infiltrations might be more frequently encountered in the prediabetic phase. Use of autoantibody assays allows us to identify individuals at risk for type 1 diabetes, and these assays were therefore used to select donor organs which may exhibit signs of this phase (In't Veld et al 2007). 62 non-diabetic pancreas donors between 25 and 60 years were positive for at least one auto-antibody. They were examined for the presence of insulitis, with 62 age-matched antibody-negative donors as control. Insulitis was only detected in two antibody-positive donors. Interestingly, these two cases belonged to the group of three that was triple-antibody positive, i.e. positive for ICA, IA2-Ab and GAD-Ab; moreover these three donors also presented a susceptible HLA-genotype (In't Veld et al 2007). Only a minority of islets presented a leucocytic infiltrate (9 and 3%); this was composed of CD8-positive lymphocytes and CD68-positive macrophages (Plate 1). It can be concluded from this study that the detection of one or two diabetes-related autoantibodies in adults is not correlated with the presence of insulitis. Positivity for three antibodies was associated with the occurrence of insulitis; the percentage of affected islets is low whereas no decrease in β cell mass was noted. These observations relate for the first time a set of circulating prediction markers in a non-diabetic individual with an immune process in the endocrine pancreas. They also indicate that this combination can occur without apparent reduction in β cell mass. It is so far unknown whether this reflects the remnants of a distant disease process in which the β cell population has not been seriously affected and may have regenerated, or whether it represents a stage in which an activation of the immune system or a shift of the β cell phenotype can result in higher infiltration rates and subsequent losses in β cells.

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DISCUSSION

Herold: We have interpreted the β cell mass of children differently. The critical issue for the interpretation is 'what is normal?' Our data are based on DPT1

follow-up data which have the limitation that the subjects are not true normals—they do not yet have clinical diabetes and are relatives of people with type 1 diabetes. When we followed both children who did not progress to diabetes as well as those who did, there was an increase in β cell function over time. This is mimicked in rodent data which show an increase in β cell mass with growth. Related to your studies, therefore, the question that I have is what is normal β cell mass for a six-year-old? Could the reason why you have found such low numbers be because you have compared with an adult normal rather than an age-matched normal individual?

Pipeleers: The only data I have seen where β cells have been counted in children with recent-onset diabetes come from the study of Gepts (1965). They list the number of β cells per unit surface area for a group of patients and normal controls, both ranging from age 0 to 30 years. As a group, patients presented a 90% reduction in β cell count. When stratified according to age, this reduction was much more pronounced in recent-onsets under age 7 years than in older patients. There are not enough cases to conduct a comparison with normal children under age 7 years. It is however unlikely that this difference would then disappear given the present expression per surface area and the knowledge that the relative β cell area is higher in young children.

Butler: I assume this question is about normal growth. We have looked at that. There is a huge growth in β cell mass, and by the time a child is 3 years old it is 80% of what it will be by adulthood. You want your babies to be fat and insulin resistant rather than your adolescents, because you grow almost all of your adult β cell mass by the time you are five. We included more than 70 cases. We were looking at sources of β cells, and reproduced what has been published previously. I am sure β cell function gets better with age so more insulin is made per cell, but almost all the β cells are present early on in childhood.

Flavell: What is the rate of turnover?

Butler: From birth to age three or four, healthy islets have a high frequency of β cell replication. It is an inverted hyperbole, dropping precipitously from birth to age three or four. By the time the child is five it is low, but still there. Through teenage life there is little further replication. If you look at 100 islets in section you'll find on average one $KI67^{+}$ insulin cell. It works out as β cell survival of about five years. There is a growth phase, then in adults it is maintained. In animals there is fierce debate as to whether there are other sources of β cells. Since this requires lineage studies, which I can't do in humans, I can duck out of this issue.

Kay: What is the interpretation of Danny's data with the KI67-positive insulin cells in the islets with insulitis? Clearly there are technical issues there to do with whether the apparent co-localization of the staining is true or not. Have you tried to address this with confocal and other technologies?

Pipeleers: Correct. Double-positive cells in classical immunocytochemistry can be misinterpreted when the staining corresponds to overlaying cells. Confocal or electron microscopy can exclude this possibility. In the presented case, islets still contained many aggregated β cells while not being diffusely infiltrated. In addition, the 5% KI67^{+} cells in these aggregates is high which makes it unlikely that this would correspond to non-β cells.

Butler: That is incredibly high. But there are other circumstances where it is possible to drive adult β cells into cell cycle. This has been shown in relation to gastrinoma. The problem is, if you drive cells into cycle under a pro-apoptotic environment, this is the basis of cancer. Attempted therapy for cancer relies on the predisposition of replicating cells to enter apoptosis.

von Herrath: One consideration about the findings on replicating β cells is that they only replicate in islets that are under attack. This is unfortunate, in a sense, because as a consequence, the regenerating islets also contain the attacking cells. It seems natural, because the body always regenerates if there is inflammation.

Butler: My analogy to that is that the worst possible thing to do is to try to drive β cell replication in type 1 diabetes. This would drop the β cell mass. If I had a therapy that I knew would make β cells replicate and I infused that into a patient with early onset type 1 diabetes, my prediction is that β cell mass would drop unless we could protect the newly forming β cells.

Bonifacio: If you take away the inflammation, you may no longer get the stimulus.

Butler: Precisely. A lot of effort is focused on finding cures for type 1 diabetes that drive cells out of G0 into cell cycle. All those other cells that are currently sitting at G0 and are relatively protected would suddenly be vulnerable. This is a concern.

Insel: I wouldn't assume that a regenerated β cell in a metabolically stable environment would necessarily be recognized similar to a 'stressed' β cell by the immune system in type 1 diabetes.

Roep: I'd like to discuss the discordance between seropositivity and insulitis, with regards to the kinetics or chronicity of the disease process, and also the consequences for immune intervention. We know that antibodies can predict disease, and in the DPT1 and ENDIT studies the predictions were right in terms of progression. But it is striking that less than 10% of islets express insulitis in the best case, and this is in very few patients. If we would like then to do prevention therapy with anti-CD3, for example, this would be bound to fail. How do we deal with this?

Eisenbarth: The findings are consistent with Bayes theorem. You have selected individuals over age 25 from the general population, not relatives of patients with

type 1 diabetes. I think from these data, if you use just the biochemical autoantibodies and get rid of ICA, which is worthless, and get greater than or equal to two of the antibodies, you have identified insulitis with a pretty high hit rate in that group. But this group will be one in 300 to one in 500 of individuals you screen from the general population. I don't think that is surprising that those with multiple biochemical autoantibodies are those with insulitis, and not others with low risk autoantibody profiles.

Roep: It is clear now that there is a big discordance between seropositivity and insulitis.

Eisenbarth: Let's go back again. You need to get greater than or equal to two of the antibodies to get enough specificity in a general population over age 25 in order to figure out insulitis. This would be my suggestion.

von Herrath: This makes sense. If there is high specificity of the islet antibodies, this could correlate with insulitis. But what happens if you tinker with your cut-off for insulitis? If you count every lymphocyte, in the normal individuals and in the ones with one islet antibody and so on, is there a type of physiological insulitis? If so, how much is there? Is there any difference between the normals and the ones that have one islet antibody?

Pipeleers: As with any test, thresholds need to be first set for the normal population before interpreting data in the test population. The presence of an inflammatory infiltrate can then be based on the appearance of higher numbers of particular cells in the experimental group. When using Leucocyte Common Antigen (LCA) as marker for infiltrating cells, we found in the normal pancreas up to five of these cells per islet; the presence of one or a few of these cells should thus not be considered as a sign of insulitis.

von Herrath: If we look at it not in terms of distinguishing between normal individuals and type 1 diabetes, but just look at what is there, is the situation different?

Pipeleers: There are residual lymphoid cells in normal islets. They can occur in the capillaries, but also in connective tissue. Their presence in islet transplants is considered to contribute to rejection (Faustman et al 1984).

Kay: Did you try class I MHC as a sort of sensitive read-out of insulitis somewhere, or disease somewhere?

Pipeleers: No, we have not and that would certainly be interesting. I would like to pick up on the statement that there is a correlation between antibody positivity and insulitis. What is the basis for this conclusion?

Eisenbarth: I'd say we don't have the data yet to conclude this. But your data suggest that there is a correlation. When you get greater than or equal to two antibodies forgetting ICA, you are down to five or six people, two of which have insulitis.

Roep: In less than 10% of the islets and with normal β cell mass.

Santamaria: I think we are getting ahead of ourselves here. We can't make conclusions on a sample of two patients with insulitis.

Eisenbarth: It's two out of six versus none out of 56. We also know that the antibodies predict disease.

Santamaria: There is definitely some suggestive value here, but we can't get carried away yet. Someone has made a statement that there is a correlation between autoantibody positivity and insulitis, and we have to be careful about this.

Foulis: Equally, I have an *n* of 2 when it comes to prediabetic patients. In the prediabetic patient with Addison's disease whose pancreas I studied there were 62 islets that hyperexpressed class I MHC but only two that had insulitis (Foulis et al 1988). It might be that hyperexpression of class I MHC is a more sensitive marker of pre-diabetes in a pancreas than looking for insulitis.

Eisenbarth: And IFNα.

Foulis: Yes, they correlate well.

Flavell: Peter Butler, what is it that makes you believe that replicating cells are going to be more likely to die?

Butler: It's based on the cancer biology. Any cells going through cell cycle are targeted for apoptotic treatment because they are more vulnerable.

Flavell: This is after initiating DNA damage. It's a DNA damage response.

Butler: Not necessarily. There is a big chunk of cancer biology work that looks at pro-replication and pro-apoptosis. They screen for compounds by driving the cells into cell cycle and putting small molecules on them. They look for cells that are killed. In the context of the β cell, we have shown that the IAPP oligomers which cause β cell apoptosis in the context of type 2 diabetes are more likely to kill cells in cell cycle. If you put cytomix on β cells in culture, the cells replicating are more likely to die. Quite a lot of papers have shown that in human pancreas as well as rodent models that apoptotic cells are frequently found in post-mitotic pairs, which is consistent with this concept of replicating cells undergoing apoptosis.

Flavell: We have to be cautious. p53-driven mechanisms are going to play a role here. This is where the checkpoints are.

Butler: The G2/M checkpoint is the point where a cell makes a decision: does it continue to divide or does it die? In a pro-apoptotic environment it is likely to make the decision to die. This is the key discrimination between these two, because if you drive any cell into cell cycle and damage DNA by any means, the cell pair will go into apoptosis at that time.

Flavell: We have to be analytical about it, and consider specific apoptotic stimuli and activate specific apoptotic pathways. Do you know what is doing this? It isn't sufficient to say we treat cancer cells with chemotherapy and the cells die. Of course they do.

Butler: We treated the human islets with cytomix and showed that cells that were replicating died under those conditions, and the ones that weren't didn't.

Flavell: What cytokines were involved?

Butler: IL1β 50 U/ml; TNFα 1000 U/ml; IFNγ 1000 U/ml (Meier et al 2006).

D Hafler: I am still intrigued by the presence of insulin in the presence of the class I MHC and interferon-expressing islets. Could the class I be protecting the islets from damage by inducing KIR receptors on $CD8^+$ cells? There could be a loss of class I as diabetes occurs that allows the $CD8^+$ cells to do their damage.

Roep: This was the interpretation that Sarvetnick made in the *Nature Immunology* paper (Flodström et al 2002). The cytokine profile that Francesco found in some of those cases was NK dominated. The cytokines were IL10 and TNFα which by themselves are not destructive. It could be that the first hit is a protective one. The innate immune system is also there for surveillance and protection.

von Herrath: I have a hard time believing that all this IFN and class I has no consequences for the islets.

D Hafler: You'd think not, but there is a lot of insulin there.

Roep: And there is a different effect on the adaptive versus the innate immune system of course.

von Herrath: The insulin might be there because just the up-regulation of IFN and class I, as long as no T cells go in there, is probably OK for a while.

D Hafler: What experiments can one do to address this hypothesis, looking at the nature of the $CD8^+$ T cells?

Kay: Most things that inhibit class I expression on β cells are generally protective. This is of course in mouse models. In general, I'd support what Matthias is saying: most things that dampen class I expression inhibit progression of diabetes.

von Herrath: The reverse doesn't seem to be the true, that β cells with little class I are all of a sudden susceptible to the innate immune system. This would make no sense at all, because we would have a lot of constitutive insulitis.

Peakman: Your cases aren't typical of what we have seen, where the majority of cells are CD8 and CD3 positive.

Flavell: There are probably additional mechanisms at play here. Almost all tissue is low in MHC class I. Presumably there is a dominant mechanism behind this NK protection.

Pipeleers: We shouldn't exclude the fact that chronic exposure to high glucose increases class I expression in β cells.

Peakman: The lobularization of some of the features, such as killing or hyper-expression, you commented on simply make this seem a focal, segmented disease. Is this because of the blood supply?

Pipeleers: This could indeed be the case but the occurrence of insulin-positive islets in particular lobes of the diabetic pancreas may also correlate with the presence or survival of precursor cells in these lobes.

Eisenbarth: One additional hypothesis. If you look at vitiligo, there are patches of destroyed melanocytes. We don't ascribe this to differences in the skin. It is likely to be a plaquing assay, so there is a certain amount of randomness to it.

D Hafler: Thinking about plaques, what one sees is MS is not dissimilar. It is chronic, long-term and it may well be that there are waves of lymphocytic infiltration going on in diabetes early on similar to what is seen in MS.

Tree: Coming back to your definition of insulitis across the whole pancreas, have you looked in individuals with a high level of autoantibodies to see whether or not there is an unequal distribution in the normal number of T cells in different lobes of the pancreas? You might be losing true insulitis by describing the whole pancreas as a single distribution.

Pipeleers: We had only access to tissue from one part of the pancreas. It was, however, always the same part.

Tree: With type I, at the end of disease we can see one lobe that seems to be untouched. Perhaps disease begins with just one lobe being touched.

Pipeleers: This is possible but it cannot be documented with currently available data. We have analysed many more islets than in most of the studies on human pancreas but can certainly not address this question.

von Herrath: The lobular pattern of disease really argues for a viral involvement.

Roep: It could also be the circulation.

Tree: What is known about the lymph nodes that drain individual lobes? This is surely extremely important for the collection of pancreas and draining lymph nodes that you are doing.

Insel: With respect to the observation of MHC class I-positive cells in the absence of insulitis, it is my understanding that some viruses have the ability to up-regulate MHC class I and dampen inflammatory responses by stimulating the release of anti-inflammatory cytokines. It is possible that some viruses may also have an anti-apoptotic effect. Conceivably you could have virally infected cells with up-regulation of class I and increased IFNα, and yet the virus itself could be preventing an inflammatory response and keeping the β cell alive.

Roep: That is quite possible.

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Bone marrow expressing a diabetes resistance MHC class II allele: diabetes deviation by chronic immune stimulation

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Abstract. The major histocompatibility complex (MHC) of the type 1 diabetes-prone NOD mouse lacks a functional class II *H2-Ea* gene such that antigen presenting cells (APCs) are I-E null. Transgenic expression of *Ea* in NOD mice both restores I-E expression and confers complete protection from diabetes progression. Non-myeloablative neonatal transplantation of bone marrow cells from such I-E$^+$ transgenic donors into NOD recipients resulted in low-level but long-term haematopoietic stem cell (HSC) engraftment. Despite low levels of I-E antigen expression in blood (averaging 0.4–3.8% of total MHC class II-positive population), chimeric recipients were protected from overt diabetes, although not insulitis development. Adoptive transfer of diabetes into immunodeficient NOD-*Rag* recipients that received chimeric splenocytes from primary recipients confirmed the presence of an autoreactive T cell repertoire. The demonstration that purified T cells from these weak chimeras were not tolerant to irradiated transgenic I-E$^+$ splenocytes indicated that I-E$^+$ donor cells provide a constant, low-level immune stimulation capable of up-regulating nominally deficient immunoregulatory networks. This study raises the possibility that cord blood HSCs from infants with high risk HLA haplotypes and a family history of type 1 diabetes might be re-introduced without myoablative treatments following transfection with a single HLA class II allele associated with diabetes resistance.

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In both the BB rat and NOD mouse models of spontaneous, T cell-mediated type 1 diabetes (T1D), the primary aetiopathological factor is the abnormal development of an immune system from haematopoietic stem cell (HSC) precursors. Certain clinical cases in humans indicate that inadvertent transfer of T1D may have been produced by HSCs from donors that subsequently developed T1D. In NOD mice, both MHC and non-MHC genes contribute to a failure of HSC-

derived antigen presenting cells (APCs) to fully mature and acquire tolerogenic signalling capacity. Multiple MHC loci within the $H2^{g7}$ haplotype of NOD, collectively termed *Idd1*, and including two class II alleles, are major determinants of HSC diabetogenicity. One is the *H2-Ab*^{g7} allele, the orthologue of the human risk conferring *HLA-DQB1-0302* allele. Diabetogenicity in the NOD allele is the product of a nested set of five nucleotide substitutions between position 248–252 converting a conserved proline at amino acid position 56 to histidine and converting a charged aspartic acid at position 57 to an uncharged serine. Reconstitution of lethally irradiated NOD mice with autologous HSCs retrovirally transduced with either an aspartic acid^{57}-containing *Ab*^{k} or *Ab*^{d} cDNA prevented development of insulitis and both spontaneous and cyclophosphamide-induced diabetes (Tian et al 2004). This protection was accompanied by stable chimerization in peripheral blood of progeny of the transduced HSCs at 5–8% as detected by a green fluorescent-protein (GFP) reporter (Tian et al 2004). In addition to *H2-A*^{g7}, a second diabetogenic class II contributor is represented by a null mutation in the *H2-Ea* locus such that no I-E molecules are expressed on APCs. Direct injection of a functional *H2-Ea*^{d} gene into NOD/Lt zygotes suppressed insulitis and prevented both spontaneous and cyclophosphamide-induced diabetes (Hanson et al 1996). This protection did not entail central deletion of autoimmune T-effectors; diabetes was adoptively transferred into standard (I-Enull) NOD irradiated recipients by T cells from these *H2-Ea*^{d} transgenic mice following depletion of I-E expressing APCs (Hanson et al 1996).

The strong resistance to diabetes development in NOD mice conferred by insertion of a single, protective MHC class II allele either by retroviral transduction of HSCs or by transgenesis has implications for human medicine. The increased diabetes risk associated with the human *HLA-DQA1-0301/DQB1-0302* (DQ8) alleles can be mitigated by linked H2-E orthologous DR alleles. For example, DQ8 in combination with *DRB1-0401* or *DRB1*-0405 is associated with increased diabetes risk, but DQ8 in combination with DRB1-*0403* or *DRB1-0406* is associated with protection (Undlien et al 2001). Human cord blood cells obtained at birth contain HSCs that could serve as vehicles for genetic manipulation of newborn infants deemed to be at high TID risk because of family history and inheritance of a high-risk set of T1D susceptibility genes. However, any such contemplated manipulation would require non-myeloablative procedures. Use of bone marrow transplantation to treat non-malignant disorders is limited by morbidity and mortality associated with host myeloablative preparatory regimens. This is of particular concern during neonatal development, when rapidly dividing cells are more susceptible to ablative agents, highlighting the need for safer treatment protocols in infants. In the present study, we investigated whether a single injection of HSCs from NOD-I-E^{+} transgenic donors into NOD/Lt neonates could provide sufficient engraftment under non-myeloablative conditions to circumvent diabetes development.

Methods

Mice

Bone marrow donor strains were either standard NOD/Lt (Thy1.2$^+$, I-E^{null}) (JR#1976) or NOD/Lt-Tg(*H2-Ea^d*)5Lt (Thy1.2$^+$, I-E$^+$, JR#2034, Type1 Diabetes Repository, The Jackson Laboratory). Henceforth, the latter transgenic strain will be abbreviated as NOD-I-E$^+$. Where noted, a fully diabetes-susceptible stock expressing the allotypic Thy1.1 allele (NOD.NON-*Thy1^a*/1LtJ N21, JR#4483, Type1 Diabetes Repository, The Jackson Laboratory) was used as transplant recipients. T and B cell-deficient NOD.129S7(B6)-*Rag1^{tm1Mom}*/J mice (JR#3090), henceforth referred to as NOD.*Rag*, were used in diabetes adoptive transfer studies as described. CBA/J mice (H2^k, I-E$^+$, JR#656) were used as positive control immune stimulators in mixed lymphocyte reactions.

All mice used were bred and maintained in our specific pathogen-free research animal facility at The Jackson Laboratory. Mice were allowed free access to NIH-31 diet (Purina Mills, Richmond, IN, 6% fat) and acidified drinking water. All procedures involving the use of animals were approved by the Animal Care and Use Committee of The Jackson Laboratory.

Bone marrow transplantation

Whole bone marrow was collected from the femurs and tibia of donor female mice. In one experiment, marrow was divided into two equal portions with one sample receiving 2000 rads from a ^{137}Cs irradiator to serve as a negative control for allotypic marrow engraftment. Either untreated or irradiated cells were injected into the superficial temporal vein (Sands & Barker 1999) of two-day-old recipients (both sexes) that had not been exposed to any prior myeloablation (Soper et al 2001). Mice received 1.2–2.4 $\times$ 10^7 cells in a 100 µl volume of phosphate buffered saline.

Flow cytometry

All monoclonal antibodies were purchased from BD Biosciences (San Jose, CA) except where noted. Cells were analysed on a multicolor flow cytometer (FACS-Calibur; Becton Dickinson, San Jose CA) using CellQuest 3.0 analysis software. All analysis for chimerism was redone under consistent gating using FlowJo (Tree Star, Ashland, OR) software. NOD-I-E+/Thy1.2$^+$ donor chimerism in recipients was determined initially at 4, 5 or 6 weeks of age and again at 8, 12, 15, 30 or 40 weeks depending upon the experiment. In all experiments, degree of chimerism was assessed by flow typing I-A^{g7}-positive APC in peripheral blood (identified by PE-conjugated mAb AMS-32.1) for transgenic I-E expression (FITC-conjugated

mAb 14-4-4S). Thus, all chimerism data are expressed as the percentage of I-E^+ cells in the I-A^{g7}-positive population. In an initial experiment where NOD.NON-Thy1.1 females were used as recipients of either standard or I-E^+ (transgenic) marrow from Thy1.2^+ donors, peripheral blood leukocytes (PBL) were typed for recipient-type Thy1.1 (anti-CD90.1 PE-conjugated Mab OX-7) vs. donor-type Thy1.2 (anti-CD90.2 APC-conjugated Mab 53-2.1) as an additional marker to assess multi-lineage HSC engraftment. At necropsy, mice were also typed for I-E chimerism in spleen, and a subset of mice for transgene expression in thymus and bone marrow as well. One group aged to 12 weeks was analysed for percentage of pancreatic β cell autoreactive $CD8^+$ NY8.3 clonotypic T cells detected by specific tetramer staining as previously reported (Chen et al 2005). Tetramers were kindly provided by Dr Pere Santamaria (University of Calgary, Alberta, Canada). At 8 weeks of age, a group of 13 mice were necropsied to assess percentages of regulatory T cells in spleen by staining for co-expression of CD4 (APC-conjugated Rm4-5) and CD62L (FITC-conjugated MEL-14), CD25 (PE-conjugated PC-61) and GITR (PE-conjugated DTA-1) (3 of the 13 were not tested for GITR expression). Splenic $CD4^+$ T cells with putative immunoregulatory phenotypes are expressed as percent of total $CD4^+$ cells.

Incidence of diabetes

Transplant recipients and controls were tested for changes in urinary glucose (Diastix, Bayer, Elkhart, IN) at weekly intervals beginning at 10 weeks of age. Those mice transiting to diabetes were necropsied and chimerism determined at that end-point. Mice remaining non-diabetic were sacrificed at 30 or 40 weeks and pancreata were fixed in Bouins solution. Histological sections were stained for granulated β cells by aldehyde fuchsin and counterstained with haematoxylin and eosin. Pancreata were examined and scored for insulitis, where 0 = no insulitis and 4 = severe insulitis, as described previously (Chen et al 2005).

T cell tolerance assay

T cells were isolated from splenocytes of eight-week old unmanipulated NOD females and NOD chimeric recipients of I-E^+ transgenic bone marrow cells. $CD3^+$ T cells were purified by negative selection from three spleens pooled per group using a streptavidin-conjugated magnetic bead system according to manufacture's protocol (Miltenyi Biotec, Auburn CA). Purity of $CD3^+$ T cells was 83–94%. Mixed lymphocyte reaction (MLR) was performed as described previously (Serreze & Leiter 1988). T cells were resuspended at 5×10^6 cells/ml and 100 µl of cells were incubated 3 days in a 96-well flat-bottom plate with an equal volume and concentration of irradiated (2000 rads) splenocytes or T cells alone. Irradiated

stimulators from transgenic NOD-I-E^+ mice, and CBA/J were also included as positive controls. All assays were in triplicates with 1 μCi ^{3}H-thymidine (Perkin Elmer, Boston, MA) added to each well and cells incubated for the last 24 hours. After freezing the cells overnight, DNA was transferred to a filter and [^{3}H]thymidine incorporation determined by a Wallac plate counter (Turku, Finland).

Adoptive transfer of diabetes by splenocytes

Pooled splenocytes from three eight-week-old unmanipulated NOD-I-E^{null} females and three eight-week-old NOD-I-E^{null} females engrafted with NOD-I-E^+ bone marrow were transferred into at least 10 female NOD.*Rag* recipients (6–7 weeks old). Pancreata were taken from the donors to determine degree of insulitis as described above. Diabetes onset in recipients was measured by testing for urinary glucose every week from 6–20 weeks post-transfer.

Statistical analysis

Significance of differences in diabetes-free survival was analysed by Kaplan-Meier survival analysis (JMP® software, SAS Institute, Inc. Cary, NC). Statistically significant differences in a given phenotype among the genotypes tested were assessed by one-way ANOVA with significance accepted at P of ≤ 0.05.

Results

The level of micro-chimerization over time by donor I-E^+ leukocytes (as a percentage of the I-A^{g7}-positive subpopulation) in lymphoid organs is shown in Table 1. Consistent with the absence of any myeloablative treatments, the data show a very low, but relatively stable level of micro-chimerism over time in all lymphoid sites. The decreased mean percentage of PBL micro-chimerism recorded at 5 weeks is likely to represent an actual transient drop as this value represents data from three separate transfers. Chimerism in the various lymphoid compartments increased modestly with age; in the group aged to 40 weeks, spleen chimerism averaged 5.75%, with a range between a high of 31.8% and a low of 0.04 (standard NOD negative control background level = 0.06 ± 0.04). The lowest percentage detected in mice with documented stable chimerism was 0.3% at 40 weeks. The detection of I-E^+/I-A^{g7} double positive cells in bone marrow at both 8 and 12 week of age indicated low-level seeding by donor HSCs, a finding reinforced by low but detectable chimerism in the thymus at 8 and 12 weeks of age. The use of the Thy1.2 allotypic marker for the donor (I-E^+ transgenic) marrow also permitted assessment of population by donor-origin T cells; in general, these values correlated well with the degree of I-E chimerization.

TABLE 1 I-E$^+$ microchimerism in different lymphoid compartments over time indicates low, but stable chimerization in many individuals

Age (weeks)	*3*	*4*	*5*	*6*	*8*	*12*	*15*	*30*	*40*
PBL	1.91 ± 0.42 (20)	1.93 ± 0.30 (28)	0.43 ± 0.05 (27)	2.16 ± 0.36 (45)	3.77 ± 0.93 (13)	0.48 ± 0.11 (7)	3.11 ± 1.06 (7)		1.76 ± 0.93 (11)
Spleen			2.29 ± 0.23 (3)		1.66 ± 0.27 (16)	2.24 ± 0.39 (7)		3.60 ± 1.03 (11)	5.75 ± 1.28 (29)
Bone Marrow					0.23 ± 0.07 (13)	0.52 ± 0.04 (7)			2.09 ± 0.47 (12)
Thymus					0.81 ± 0.26 (3)	0.65 ± 0.13 (7)			

Data are mean percentage ± SEM (*n*). Chimerism is expressed as the percentage of I-A$^+$ cells that are also I-E$^+$.

Previous studies wherein lethally irradiated NOD post-weaning recipients were reconstituted with different combinations of standard NOD marrow and marrow from the NOD-I-E$^+$ transgenic stock showed that a minimum of 40% I-E chimerism was required to achieve protection from diabetes development (Johnson et al 2001). Given the considerably lower level of I-E$^+$ chimerization achieved by neonatal transfer in the absence of myeloablative treatments, the strong diabetes-retarding effect achieved in treated female mice was unexpected (Fig. 1). Injection of standard NOD marrow into neonates failed to retard diabetogenesis . Similarly, transfer of 2000 Rad-irradiated NOD-I-E$^+$ marrow into NOD-Thy1.1

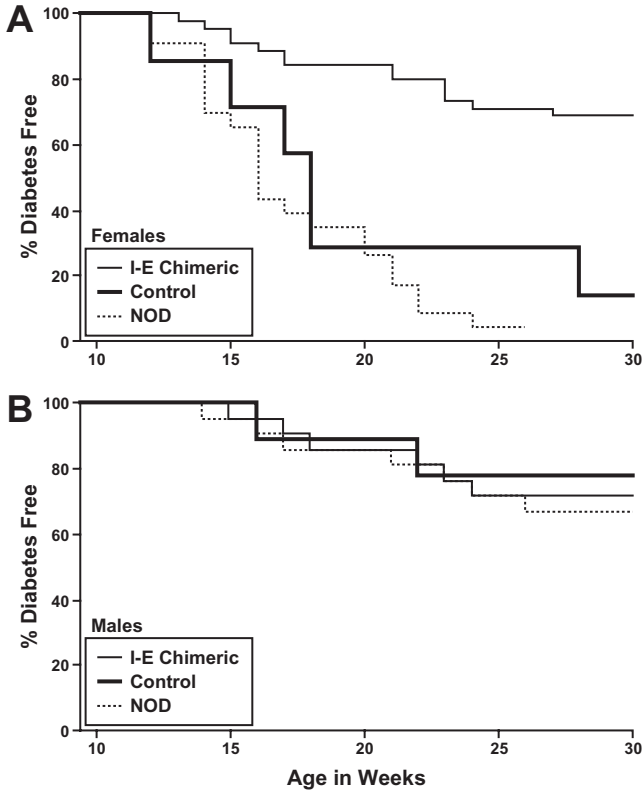

FIG. 1. Neonatal bone marrow transfer from NOD-I-E$^+$ donors significantly extends diabetes-free survival in NOD female recipients (A), but does not further enhance the native resistance of male recipients (B). Controls (females, $n = 7$, males, $n = 9$) are a mix of two separate control experiments that yielded similar results (see Methods). I-E chimeras are a meld of several groups (females, $n = 45$, males, $n = 21$). Control and I-E$^+$ chimeric diabetes-free survival curves are shown in comparison to that for standard, unmanipulated NOD mice in our colony over the same period (females, $n = 23$, males, $n = 21$).

neonates also produced no chimerism (data not shown) and had no effect on diabetes-free survival when compared to unmanipulated NOD (these controls combined with standard NOD marrow recipient controls, Fig. 1A). In contrast, female recipients of the unirradiated I-E+ marrow showed a highly significant increase in diabetes-free survival ($P = 0.0009$ vs. controls; $P < 0.0001$ vs. unmanipulated NOD females). This diabetes suppression was female-specific; standard NOD males are naturally more resistant to type 1 diabetes development than are females, and the low level of I-E chimerism achieved did not enhance this differential resistance (Fig. 1B). The control groups confirmed that protection was not a function of the transplantation procedure nor a result of a one-time early immune stimulation by irradiated I-E$^+$ cells. Among the recipients of I-E$^+$ bone marrow, 31% of the females ($n = 45$) and 28.5% of males ($n = 21$) became diabetic by 30 weeks. Among those recipients of I-E$^+$ marrow that developed diabetes between 15–30 weeks of age, 15 were assessed for splenic chimerism at the time of necropsy. Of these, 7/15 showed extremely low I-E$^+$ chimerism (<1%) that approached background staining. At the 40 week termination point for non-diabetic survivors, splenic I-E$^+$ chimerism less than 1% was recorded in only 8/29 individuals.

One of the strain-specific characteristics of diabetes-free NOD mice aged to 30 weeks and beyond is the development of thymic lymphomas (Leiter 1990, Prochazka et al 1992). In the 40 week necropsies of I-E$^+$ marrow recipients, metastatic thymic lymphomas, characterized by large numbers of splenic CD4$^+$/CD8$^+$ double-positive lymphocytes, was independent of the level of chimerization.

The diabetes-retarding effect of neonatal I-E$^+$ marrow administration was evidenced by the significantly lower mean insulitis scores over time (Table 2). However, the fact that insulitis was present at all ages in these mice, and became progressively more severe with age, showed that autoreactive T cell effectors were not eliminated

TABLE 2 Insulitis development over time (weeks)

	Sex	*8*	*12*	*40*
Chimeras	Total	0.85 ± 0.15 (16)[a]	1.23 ± 0.21 (6)[b]	2.80 ± 0.39 (14)
	Females	0.77 ± 0.18 (13)[a]	1.20 ± 0.41 (3)[a]	3.06 ± 0.41 (12)
	Males	1.18 ± 0.20 (3)	1.26 ± 0.24 (3)	1.27 ± 0.74 (2)[b]
NOD	Total	1.29 ± 0.14 (37)	2.27 ± 0.18 (20)	3.54 ± 0.23 (12)
	Females	1.38 ± 0.17 (26)	2.55 ± 0.25 (12)	3.76 ± 0.95 (2)
	Males	1.08 ± 0.23 (11)	1.89 ± 0.23 (8)	3.50 ± 0.14 (10)

Data are mean percentage ± SEM (*n*). Insulitis score for chimeras are compared to unmanipulated NOD controls over time.
[a]$P \leq 0.05$ and [b]$P \leq 0.01$ for chimeras vs. age and sex matched NOD. Insulitis is suppressed in the chimeras, correlating with the decreased incidence of overt diabetes. The 40 week time point includes only diabetes-free survivors.

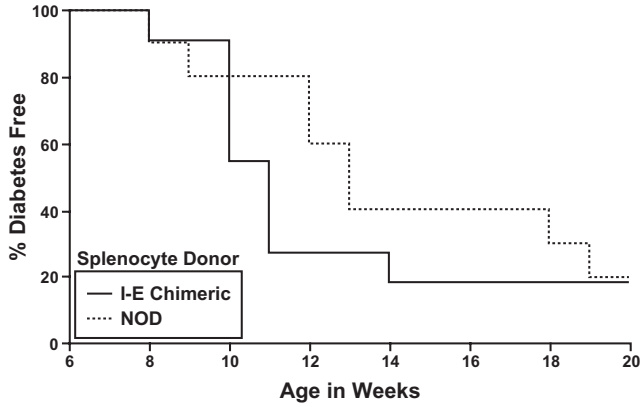

FIG. 2. Splenocyte transfer from eight-week-old unmanipulated NOD or I-E$^+$ chimeric donors ($n = 3$) into NOD.*Rag* recipients ($n = 10$, $n = 11$, respectively) exhibit comparable development of diabetes. This indicates that the T cells in the spleen of the I-E chimeric donors are fully auto-immunogenic, but are functionally suppressed.

or completely suppressed. Indeed, splenocytes from pooled pre-diabetic 8-week-old I-E$^+$ chimerics injected into NOD.*Rag* females transferred diabetes as efficiently as did splenocytes from age-matched unmanipulated NOD donors (Fig. 2, survival curves not significantly different). Thus, in contrast to the retrovirally mediated I-A gene therapy described above where central tolerance was reported in the absence of lymphomagenesis (Tian et al 2004), our results recapitulated the findings with the NOD-I-E$^+$ transgenic stock where APC-dependent peripheral T cell suppression was induced (Hanson et al 1996, Johnson et al 2001). Frequency of splenic CD4$^+$ cells with a T regulatory phenotype (CD4+, CD25+, CD62L+) did not differ between eight-week-old unmanipulated NOD (2.4 ± 0.2%) and recipients of neonatal injections of I-E$^+$ marrow (2.2 ± 0.3%), although both were modestly, but significantly lower than the 3.5 ± 0.3% frequency observed in age-matched NOD-I-E$^+$ transgenic females. Although FoxP3 was not used to phenotype putative Tregs in the present study, no increase in percentage of CD4$^+$CD25$^+$FoxP3$^+$ cells distinguished the fully diabetes-resistant *Ea*^{d} transgenic donor strain from standard diabetes-susceptible NOD mice (Dr D.V. Serreze, The Jackson Laboratory, personal communication 2007).

Similarly, enumeration of the diabetogenic IGRP β cell autoantigen-specific NRP-V7 clonotype (Lieberman et al 2003) by tetramer staining showed no significant difference in spleen of NOD mice versus I-E$^+$ chimeric mice at 8 weeks of age, reinforcing results from adoptive transfer showing that autoreactive clonotypes were not centrally deleted. Tolerance to I-E in the NOD recipients engrafted with NOD-I-E$^+$ marrow was tested by a mixed lymphocyte reaction (MLR).

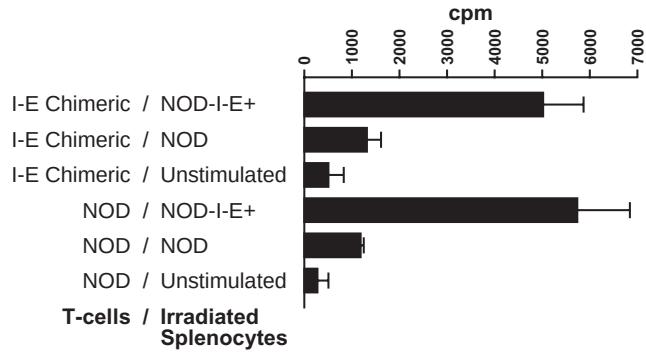

FIG. 3. Mixed lymphocyte reaction (MLR) demonstrates that purified T cells from eight-week old NOD-I-E chimeric mice, like those of standard ($I\text{-}E^{null}$) NOD mice, are not tolerant to irradiated splenocytes from transgenic NOD-$I\text{-}E^{+}$ mice. The source of the responders ('T cells') is shown in the left column and the source of the stimulators ('irradiated splenocytes'), if any, in the right-hand column. 'Unstimulated' denotes T cells only. These data suggest that the diabetes retardation mediated by the micro-chimerism is a consequence of chronic allo-reactivity to the introduced MHC class II antigen.

Purified T cells from NOD or chimeric donors were stimulated with irradiated NOD splenocytes as a negative control, irradiated CBA/J splenocytes as a positive control (the latter producing a strong MLR, data not shown), or left unstimulated (second negative control). Purified T cells from both naive NOD and $I\text{-}E^{+}$ chimeras were challenged with irradiated splenocytes from NOD-$I\text{-}E^{+}$ transgenic marrow donors. Strong, comparable MLR responses were mounted by both responding populations (Fig. 3) showing that I-E expression in the chimeras failed to elicit tolerance. Because low, but relatively stable chimerization was found over an extended period in most chimeras, these data suggest that the mechanism of protection was a chronic low-level immunostimulation to the I-E alloantigen expressed on HSC class II-positive progeny.

Discussion

Mouse disease models permit investigation of prophylactic interventions that cannot now be considered for use in human medicine for ethical reasons. An excellent example is the complete circumvention of diabetes development in both NOD mice and BB rats by destruction of the host immune system and its replacement by bone marrow bearing diabetes-protective alleles (Serreze & Leiter 1995). Although full allogenic chimerization of post-weaning, lethally irradiated NOD mice represents an effective means of diabetes prevention, the procedure results in an immunosuppressed state because donor-derived T cells cannot effectively be

selected on thymic epithelium expressing host-derived MHC molecules (Serreze et al 2006). Obvious ethical considerations preclude transgenic manipulation of the human embryo, as was done in the NOD embryo to restore I-E expression in nominally I-E^{null} NOD mice (Hanson et al 1996). Although retroviral-mediated HSC transduction to introduce diabetes resistance-conferring MHC class II offers interesting possibilities, the efficacy study in NOD mice entailed lethal irradiation of recipients. By transplanting I-E^{+} bone marrow from transgenic, diabetes-resistant NOD donors into diabetes-susceptible newborn NOD pups without irradiation or other myeloablative preconditioning, our hope was that a sufficiently high level of engraftment by I-E^{+} APCs would alleviate diabetes progression without producing the generalized immunosuppression produced by full allogenic chimerism. This approach also bypassed the ethical constraints on complete ablation of the recipient's immune system. The current literature describing HLA contributions to genetic risk for type 1 diabetes in humans (Reijonen & Nepom 1997, Undlien et al 2001) provided the rationale for expecting that a mouse orthologue for a protective human HLA-DR allele (such as DRB1–0602) might modulate the diabetogenic contributions of a 'high-risk' DQ allele as modelled by the orthologous Ab^{g7} in NOD mice.

Engraftment of donor marrow without any myeloablation was first reported in adult mice by Micklem et al (1968). Recipients were injected with 2×10^7 whole marrow cells (~8×10^8 cells/kg) and the donor cells were tracked in blood by a chromosomal marker. Recipients maintained 3–8% donor 'micro-chimerism' in blood. When cell dose was both increased and repeated (4×10^7 cells per day for 5 days), donor engraftment was increased to 16–25% (Brecher et al 1982). Subsequent reports using this approach support the hypothesis that multiple high-dose donor cell injections competitively displace host cells and maintain long-term, durable engraftment (Blomberg et al 1998, Rao et al 1997, Saxe et al 1984, Stewart et al 1993). Using neonatal injection of HSCs from normal donors, we successfully treated mice with a recessive lysosomal storage disease mutation (Lessard et al 2006, Soper et al 2001). In the present study, only a single high dose of marrow cells were inoculated into NOD neonates, and accordingly, a very low 'micro-chimerism' was achieved in most individuals over time. Previous studies in our laboratory did not suggest that such a low level would provide the surprising degree of protection that, in fact, we observed. Competitive repopulation using variable mixtures of Ea^d transgenic and standard NOD marrow to reconstitute lethally irradiated four-week-old NOD recipients showed that I-E expression on over 40% of the macrophages/dendritic cell APC population was required to functionally suppress activation of diabetogenic T cells, and under experimental conditions where I-E expression was limited to B lymphocytes, 100% chimerism of this subset was required to suppress its diabetogenic contribution (Johnson et al 2001). A similar result was obtained in competitive

repopulation studies using marrow from NOD mice congenic for an MHC haplotype ($H2^{nb1}$) that not only expressed a functional *Ea* allele, but also a diabetes-protective *H2-Ab* allele and a disparate *H2-K*^{b} class I allele (Chen et al 2007). However, in this latter study employing an entire protective MHC haplotype as compared to a single protective MHC allele (*Ea*^{d}), it was found that 50% chimerization of the macrophage/dendritic APC population produced a complete deletion rather than a peripheral suppression of a highly diabetogenic T cell clonotype (Chen et al 2007).

The diabetes protection elicited by sustained micro-chimerism in multiple lymphoid compartments in the present study clearly did not entail central deletion of diabetogenic effectors, as evidenced both by the continued presence of diabetogenic $CD8^+$ clonotypes, and the ability of splenocytes from these mice to adoptively transfer diabetes. Nor could changes in immunoregulatory subsets (Tregs or iNKT cells) be demonstrated. Rather, the MLR results determined that the recipient T cells were not tolerant to I-E^+ targets. This indicated that protection from overt diabetes was not due to a re-programming of the NOD immune system, but to a constant low-level immunostimulation elicited by I-E^+ cells recognized as foreign by the host immune system. The diabetogenic processes in the NOD mouse model are notoriously sensitive to deviation by treatments providing generalized immunostimulation, such as treatment with complete Freund's adjuvant or exposure to microbial agents (Atkinson & Leiter 1999, Bowman et al 1994). Our detection of low but demonstrable micro-chimerism in the bone marrow of neonatal recipients of I-E^+ marrow suggests that allostimulation in the most diabetes-resistant mice was chronic in nature. The absence of diabetes retardation in our control groups show that neither *ex vivo* manipulation of the marrow cells or a single pulse immunostimulation by irradiated, mitotically inert I-E^+ marrow cells could account for the protection afforded by sustained micro-chimerism in recipients of unirradiated I-E^+ marrow cells.

In summary, the diabetes protection afforded by the micro-chimerism elicited by neonatal injection of *Ea*^{d} transgenic bone marrow is not due to the reprogramming of the immune system, but rather due to chronic immune stimulation that diverts T cells away from autoimmune attack of the islets. This diversion is by no means complete, as insulitis (and diabetes) develop, albeit at a protracted rate. Nevertheless, because infant cord blood HSCs are readily available, and genetic prediction of Caucasian children at highest risk for type 1 diabetes is becoming increasingly more accurate (Aly et al 2006), some prophylaxis might be achieved by early introduction of an infant's cord blood-derived HSCs stably transduced *ex vivo* to express a resistance allele such as *HLA-DR-0602* associated with dominant protection. Rather surprising recent reports indicate that autologous HSCs from cord blood re-introduced into children with recent-onset T1D and without

any myeloablative preconditioning lessened syndrome severity in association with an increased number of T cells with T regulatory phenotypes (Haller et al 2007, Viener et al 2007). The even more surprising report entailed HSCs isolated from the blood of recent onset type 1 diabetic adults, following mobilization by cyclophosphamide and cytokine treatment. Following their collection by leucophoresis and cryogenic storage, these autologous HSCs, were thawed and re-introduced into the donors following only a brief immunosuppressive preconditioning. These manipulations provided remissions from symptoms in some of the recipients (Voltarelli et al 2007). Although it remains to be established how lasting these remissions will be, these early studies provide encouragement for HSC-based therapies that do not require destruction of the recipient's immune system (Skyler 2007).

Acknowledgements

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DISCUSSION

Bonifacio: Are you suggesting that autologous is not going to work?

Leiter: None of the control experiments we have done in the NOD mouse, in which we put NOD marrow back into lethally irradiated NOD recipients have ever protected from development of type 1 diabetes.

Bonifacio: In your model, have you looked at sialitis, or any of the other conditions of potential lymphocytic infiltration? Does this also get reduced in the chimeric mouse?

Leiter: We haven't looked. With regard to the issue of why the NOD mouse is different from the Brazilian patients we have looked at or these type 1 diabetic children that Mark Atkinson and Desmond Schatz have recruited to come to Gainesville to have their cord blood stem cells put back in, all I can suggest is that some manipulation of either the immobilized stem cells from the Brazilian patients or something about plastic adherence or the serum that is being used is having some effect. Matthias von Herrath has results on what happens to mouse dendritic cells (DCs) after they have seen fetal calf serum (they become activated).

von Herrath: Yes, this was when we did tolerogenic interleukin (IL)10-treated DCs. If we grow them in fetal calf serum (FCS) we get all kinds of non-specific effects and strong immune responses to FCS antigens and, as a result, non-specific immunosuppressive phenomena.

Leiter: So I would suggest that it may be an immunostimulation that they got in Brazil or Gainesville. The cord blood cells had been cryopreserved in DMSO (dimethylsulfoxide). The antigen-presenting cells must be responding to being frozen down and warmed up with up-regulation of certain heat shock proteins and other defence mechanisms, not to mention the potential effects of the isolation procedures themselves. All I can suggest is that the cord blood stem cells recovered from cryopreservation are not the same as when they were circulating in blood.

Butler: Does Freund's adjuvant prevent diabetes in the BB rat?

Leiter: Yes. This is published.

Roep: Can I bring the maternal chimerism into the picture. Van Rood has shown that maternal HLA antigens can induce some store of tolerance in the child. The mechanism for this is not known. He has just finished a meta-analysis in rheumatoid arthritis where the protective non-inherited genes of the mother affect the predisposition of the child to the disease in the sense that they prevent. There is some sort of haplotype-matched microchimerism. It is difficult to detect but this would fit with the observations in your presentation.

von Herrath: I am curious about this theme of chronic immune stimulation, which in many cases seems to prevent type 1 diabetes. It might be associated with the ability of viruses to prevent diabetes. Does anyone have any mechanistic ideas about how this works? The action of complete Freund's adjuvant (CFA) in preventing type 1 diabetes is probably different: it could be involving tumour necrosis factor (TNF) and apoptosis. But how do these chronic immune stimulatory phenomena work in preventing type 1 diabetes?

Leiter: In the NOD mouse and your example with the FCS and the DC activations, we know that the innate immune system of NOD mice maintained in high level specific pathogen-free (SPF) environments is simply not as fully mature as it should be for normal immunoregulatory communication with the T cells. Anything you do to activate the innate immune system so that it is sending out stronger co-stimulatory signals seems to be showing better peripheral regulation and central deletion if that is required. The first time we got our NOD mice at The Jackson lab, we thought we could inject some IL2 into young prediabetics to accelerate diabetes onset rather than having to wait in excess of 14 weeks to get 90% of the animals diabetic. We thought we should be able to get them all diabetic more rapidly because macrophages, $CD8^+$ T cells, and B lymphocytes are already present in intra-islet infiltrates beginning at 4–5 weeks of age. To the contrary, weekly injections of recombinant IL2 protected NOD mice from clinical diabetes development. When we simulated viral infection with injections of poly-IC at 50 μg twice a week to young prediabetic NOD mice, they were similarly protected from developing clinical diabetes.

Herold: There is good evidence in humans that chronic stimulation does generate Tregs. They can be found in viral hepatitis or TB.

von Herrath: This is true. But in Ed Leiter's scenario there aren't any Tregs.

Leiter: Unfortunately we didn't phenotype for FoxP3 expression, but there was no difference in percentages and numbers of splenic $CD4^+CD25^+$L-selectin$^+$ cells in our diabetes-resistant chimeric mice when compared to diabetes-developing controls.

Kay: Was that in numbers or in function?

Leiter: This was in numbers; we didn't do function.

Insel: If this is just low-level immune stimulation, technically you wouldn't need the H2-Ea protective genes. You could try to mimic this with other particular single gene itself. Have you tried non H2-Ea genes to see whether you can create microchimerism and low level immune stimulation?

Leiter: I am not sure if the stimulation would be as strong as in the MHC class II gene. We have GPI allotypes and CD45 allotypes—there are all kinds of allotypes on the NOD background that we could try. I haven't tried an allo-class 1. Dr Dave Serreze at The Jackson Laboratory has studied a NOD stock transgenically expressing the HLA A2.1 on its normal promoters. This seems to enhance

the panoply of β cell autoantigenic peptides that are presented in an A2.1-restricted fashion such that these mice develop accelerated diabetes onset. So you can't just put in any MHC class I or class II gene and down-regulate the severity of the diabetes as we were able to do with bone marrow expressing the H2-Ea transgene.

Flavell: Does this work if you use purified $CD34^+$ cells? You are transferring quite a big mixture of some mature cells, with mature lymphocytes of various kinds.

Leiter: No, we did not use purified stem cell populations from marrow. We wonder whether our data showing a decrease in Ea-positive PBL at the 5 week age point may reflect loss of pluripotency of more mature progenitors present in the unfractionated inoculum. Interestingly, Mark Atkinson's data, in which they put unfractionated autologous cord blood cells into the new-onset diabetic children, also showed a dip in $CD4^+CD25^+FoxP3^+$ T cells at about the same time that we see a dip. We think this is a real dip because numbers of Ea-positive PBL came back up to earlier levels at the later time points. Thus, I wonder whether the transient decline reflects a loss of cell progeny from the more differentiated class II Ea^+ progenitors that were in the admixture.

Bonifacio: We have some data on this. We took female NODs of 11 weeks, paired them with semi-MHC matched and fully MHC mismatched males. We get some protection in the females that are paired with the semi-mismatched males. We though this might be something to do with chimerism.

Leiter: Perhaps there is some microchimerism in the fetus.

Roep: There was also a Japanese study (Kagohashi et al 2005) in which they transplanted fetuses into discordant mice, which protected the offspring.

Herold: One of the points made from the Brazilian study was that in humans there is an increase in C peptide over time. The largest increase was even after six months. This raises a question about some effect on β cell function. The data that Danny Pipeleers presented suggest that if anything they have a supportive role, not a direct one.

Flavell: I'd like to return to the transfer study in which you transfer T cells from the protected mouse and cause diabetes in the Rag recipient. On face value, this suggests that it is not the T cells that are protective. But there is a big caveat: you have a massive homeostatic expansion in a Rag mouse, and it doesn't have to be proportionate expansion of effector cells versus anything that is protecting. Have you any thoughts on this?

Leiter: We have no way of resolving that issue at present. We know that peripheral suppression (or anergy) of T-effectors is the reason why the NOD-H2-Ea transgenic mouse itself remains diabetes-free. We assume that in our neonatal transfer chimera model, it could easily be an altered cytokine profile emanating from the transgenic marrow-derived macrophages and DCs. In the NOD mouse with a mutant leptin receptor gene ($NOD\text{-}db^{5J}$) where the insulitis is kept at bay

and Type 1 diabetes development is suppressed, we can't show that this has anything to do with Tregs. If we isolate splenic macrophages, they suppress mitogen activation of T cells. Out best data associate this suppression with higher macrophage IL10 production. It is this altered metabolic milieu changed by the leptin receptor mutation in combination with APC functional alterations that underlie reduced activation of T-effectors.

Flavell: Can you protect the prediabetic NOD from developing diabetes by transferring cells from these mice? Then you would have an assay.

Leiter: We haven't tried this. I would doubt it. I qualified my presentation to state that the treatment only retarded development of diabetes, based upon the severity of the insulitis in these clinically normoglycaemic chimeras at 40 weeks of age. They may well have lived diabetes-free up to a year at which point they probably would have died of lymphoma or some other tumour. The fact that we are losing β cells in this model argues against some complete protection of the sort that would lead me to believe that we could protect a prediabetic mouse with splenocytes from these mice. We might delay onset a little, but we wouldn't get the long-term protection we got with I-E constantly coming out at microchimeric levels from bone marrow.

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Resuscitating adaptive Tregs with combination therapies?

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Abstract. Induction of 'adaptive' regulatory T cells (Tregs) using islet-specific antigen vaccinations has been shown to prevent disease in various animal models for type 1 diabetes (T1D). Even though translation from bench to bedside has been unsuccessful so far, this non-invasive approach is the Holy Grail to safely achieve immune tolerance in humans. We will discuss here the fact that every immune response appears to contain a balance of adaptive effector and Treg cells. The evolution of these population and their antigen specificities over time during diabetes development will determine at which time and route a given islet antigen can be chosen to augment such adaptive Tregs most efficiently. Their 'resuscitation' will be crucial for long-term tolerance and homeostasis in the islet micro-environment, which is ultimately needed for a cure from T1D. Recent insight from our studies shows that short-term creation of a systemic milieu that favours Treg propagation, as it occurs after systemic administration of non Fc-binding anti-CD3, can strongly enhance this process. We propose that combination therapies with anti-CD3 or similar systemic immune modulators that lower effector cells and enhance Tregs with vaccines that induce adaptive Tregs will be a crucial step in developing successful immune-based intervention in T1D.

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Immunization with islet antigens has been shown to prevent disease in multiple animal models of type 1 diabetes (T1D). One key mechanism underlying these therapies is the induction of adaptive, antigen-specific regulatory T cells (aTregs). Such Tregs frequently exhibit functional features also seen with natural Tregs (nTregs) such as transforming growth factor (TGF)β dependence, fork-head transcription factor FoxP3 expression and secretion of immune modulatory cytokines (IL10 or IL4). As far as we currently understand, mucosal or peripheral vaccination with islet autoantigens (aAgs) can activate pre-existing precursors of adaptive Tregs, which then recognize the same antigen *in vivo* in the islets or pancreatic lymph node (PLN) if inflammation is present. As a consequence they act locally restricted as bystander suppressors, thus circumventing systemic immune suppression and the need to precisely know the scope and nature of islet-destructive

effector responses, which might vary from mouse to mouse and person to person. One major question has emerged from the growing literature on antigen-induced Tregs in T1D: which islet antigen(s) is (are) best suited to induce protection in humans?

Insulin, glutamic acid decarboxylase 65 (GAD65) or islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) are among the candidates. For several years, many scientists have vigorously defended their 'favourite' islet antigen for protecting from autoimmune diabetes. In this review, we will attempt to provide some insights into the design of potent antigen-specific immune interventions and perhaps reconcile some of the different currents of thought. We would argue that the context (route, dose, timing, microenvironment or MHC background) in which the antigen is delivered matters as much as the islet antigen itself.

A different and perhaps broader view on Tregs and immune regulation

Currently, the Treg field is dominated by investigations centred around 'professional' or 'intrinsic' Tregs. These cells were initially characterized and discovered by Sakaguchi and colleagues, who had observed that depletion of $CD4^+CD25^+$ T cells can elicit gastritis in mice (Sakaguchi et al 1985). Intrinsic $CD25^+$ Tregs emerge from the thymus and their main task is probably to work as a rheostat that keeps overall immune reactivity in check. They function, in mice, through expression of the FoxP3 and their impairment or depletion affects the course of many autoimmune disorders in a negative way. Conversely, too many intrinsic regulators can interfere with antiviral responses and have been associated with viral persistence. It has become clear, however, that $CD25^+$, which constitutes the β chain of the interleukin (IL)2 receptor, is not an optimal marker for Tregs, since it is also increased on many cells upon activation. Therefore, additional markers have been sued to characterize 'intrinsic' nTregs, most recently CD127, which is low on Tregs and highly expressed on memory lymphocytes (Liu et al 2006, Seddiki et al 2006). While, undoubtedly, such professional Tregs are crucial for balancing the immune system, we would like to contest that therapeutic application of Tregs in particular should maybe adopt a more expanded view on immune regulation.

This appears particularly important, because antigen-induced or adaptive Tregs have been much more heterogeneous in function and phenotype compared to $CD25^+$ nTregs. Some appeared similar to TH2 cells, aTregs frequently express IL10 ('TR1' cells) and other cell types such as NK-DCs, CD8γ/δ and NKT cells have also show clearly attributable Treg function in *in vivo* systems (Godfrey & Kronenberg 2004, Harrison et al 2003, Homann et al 2002). Thus, taking a step back, one has to consider how the phenomenon of immune regulation is best defined. In our opinion, it is best to adopt an operational concept, wherewith

immune regulation is the active dampening of a given *in vivo* immune response, which should be attributable as an effect of a specific cell, for example following transfer or introduction of a cell population. This broader view allows us to circumvent some of the Treg lineage dogmas or the temptation to label only cells that constitutively express CD25 as Tregs. It follows that Tregs can have a variety of effector functions and phenotypes and their ability to regulate will depend on the precise location and quality of the effector response under observation. In other words, a good Treg that is operational in preventing asthma attacks might not be suitable to prevent arthritis or T1D. It follows that there might be fewer genes and proteins that are ALWAYS attributable to Tregs and their function, many proteins that have regulatory function only for one or a few given diseases and some essential but more housekeeping type of proteins and genes, which will be important for both Tregs and effector T cells. Figure 1 illustrates this operational concept.

The practical value of looking at immune regulation like this is that regulatory cytokines, genes and cell types can be defined operationally for a specific disease and their modulation is therefore likely associated with a lesser risk for side-effects. For example, if IL4, IL5 and IL10 are known to dampen islet destruction in several T1D models, their production by islet-specific T cells will likely result in an adaptive immune regulation process and thus one could define an islet reactive CD4 cell that dampens T1D upon transfer into prediabetic recipients and does so by

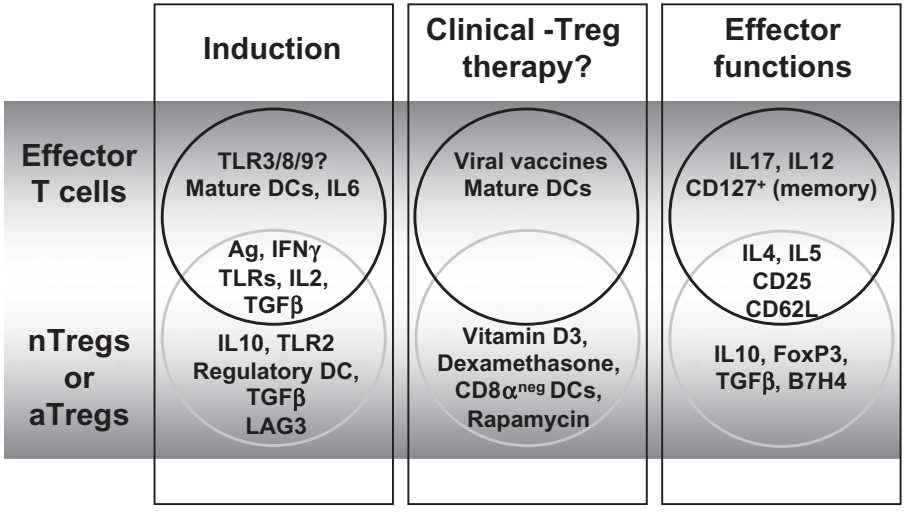

FIG. 1. Common pathways and divergences between the molecules utilized by effector and naïve or adaptive regulatory T cells (nTregs or aTregs). TLR, Toll-like receptor; DCs, dendritic cells; IL, interleukin; Ag, Antigen.

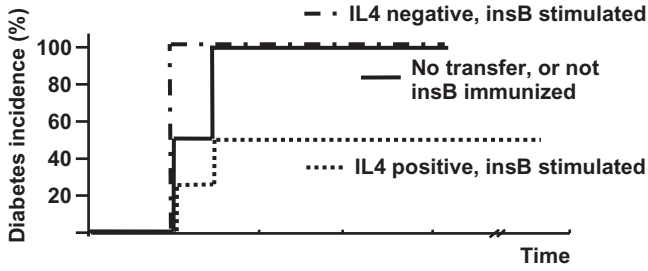

FIG. 2. Adoptive transfer of insulin B-chain (insB)-specific T cell producing IL4 protects from diabetes. Pre-diabetic recipient RIP-LCMV mice received 2×10^5 $CD4^+IL4^+$ T cells with or without *in vitro* stimulation with insulin B-chain (insB) or were left untreated. Diabetes incidence is followed over time.

producing IL4 and/or IL10 as an aTreg. This functional definition of Tregs has the additional advantage that the markers used to define and isolate the Tregs are directly associated with their effector function. This is not necessarily the case for the IL2 receptor, except for the rather general concept that presence of the IL2 receptor is important for T cell growth and survival in general, and therefore also for Tregs (Fehervari et al 2006). Figure 2 illustrates how this concept could be applied experimentally by isolating Tregs from suitable donors by their effector cytokine production rather than surface markers. In the shown example IL4-producing cells were sued to regulate autoimmune diabetes in pre-diabetic recipients.

Common features and differences between natural and adaptive Tregs

Autoimmune disorders are characterized by a breakdown in the mechanisms of tolerance to self-antigens. The negative selection of autoreactive thymocytes is the first self-tolerance mechanism occurring in the thymus but, even though this process is extremely efficient, a not insignificant number of self-reactive cells overcome this selection barrier, possibly many with intermediate or lower affinities for self antigens. Thus, autoreactive T cells can be activated in the periphery by tissue- or self-mimicking exogenous antigens leading to subsequent autoaggression. Although, autoreactive T cells are found in virtually all individuals, autoimmune diseases only affect a minor proportion of the population. This suggests that mechanisms of peripheral tolerance, involving Tregs, operate to silence potentially pathogenic T cells.

Tregs with diverse functions and phenotypes have been described and shown to act *in vivo* and *in vitro* through a variety of mechanisms. Natural Tregs (nTregs) are generated in the thymus and escape the organ possibly after being positively

selected on antigens. These nTregs are characterized by their expression of CD25 which is also up-regulated on activated $CD4^+$ and $CD8^+$ T cells. Accordingly, one has to consider that during autoimmune diseases many subsets of T cells may express CD25, therefore, making difficult the purification of nTregs. In 2001, the *Foxp3* gene defective in mice with *scurfy* mutation and involve in the regulation of T cell activation and differentiation is cloned (Schubert et al 2001). In 2003, Hori and colleagues report that Foxp3 is a key regulatory gene for the development of Tregs in mice (Hori et al 2003). The murine nTregs are classified as $CD4^+CD25^+Foxp3^+$ lymphocytes and were shown to suppress immune responses by direct cell-cell contact and production of suppressor cytokines such as TGFβ and IL10 (Roncarolo et al 2003). In the mean time, there has been some controversy regarding the phenotype of nTregs in humans. Indeed, human $CD4^+CD25^-$ T cells were shown to acquire Foxp3 expression upon activation (Allan et al 2007, Walker et al 2005), raising questions about the specificity of Foxp3. A recent report shows that expression of Foxp3 in activated T cell leads to hyporesponsiveness, but not necessarily to acquisition of suppressor function (Wang et al 2007).

In contrast, aTregs are generated in the periphery and upon antigen encounter. They are not by default $CD25^+$ and can be generated from the pool of peripheral $CD4^+CD25^-$ T cells. Once activated, aTregs as well as nTregs express Foxp3 and are capable to suppress undesired heterologous immune responses in a bystander fashion. Recent studies pointed out the importance of TGFβ in the conversion of peripheral $CD4^+CD25^-$ T cells into aTregs (You et al 2007, Zheng et al 2007), IL2 is required for TGFβ to generate and expand naïve $CD4^+CD25^-$ T cells to aTregs (Zheng et al 2007). Two main differences were noted in the generation of nTregs as compared to aTregs. First, TGFβ is not required for the generation of the nTregs (Piccirillo et al 2002) and second, whereas positive CD28 co-stimulation is required for the generation of nTregs, TGFβ-induced aTregs require negative CTLA4 co-stimulation.

There is still much debate on whether nTregs and aTregs arise from the same or different cell lineage(s). The finding that human $CD4^+CD25^+Foxp3^+$ Tregs are generated from rapidly dividing and highly differentiated memory $CD4^+$ T cells and have limited capacity for extensive self-renewal, would argue that a large proportion of aTregs and thymic-derived nTregs are generated as a separate functional lineage (Akbar et al 2007).

Assay development to choose the correct antigen to induce aTregs on an individual basis

The key issue here is to gather sufficient knowledge to establish improved safeguards (e.g. improved predictive assays) that will help in directing the immune

responses toward tolerogenic and away from pathogenic pathways (Fousteri et al 2007). This information should reduce the risk for possible adverse events in humans. The main reasons why this has proven to be difficult is because immunity comprises not one single but a multitude of potential classes of responses and the outcome of immunization depends on pre-existing immune status as well as aAg delivery (amino acid sequence, dose, timing and route of administration). For instance, it was reported in a phase II clinical trial in patients with multiple sclerosis that immunization with an altered-peptide ligand (APL) derived from the myelin basic protein (amino acids 83–99) aggravated the disease (Bielekova et al 2000). Although, such an outcome was never reported upon antigen-therapy in T1D patients, we observed that the route of immunization (subcutaneous versus nasal, the timing of administration (early or late in the autoimmune process) as well as the peptide sequence itself (insB9-23, insB9-23 APL or proinsulin B24-C36) may influence diabetes incidence in NOD mice (G. Fousteri, D. Bresson and M. von Herrath, unpublished data). These observations underscore the necessity to administer the right antigen at the right time and the right place. Insulin is the final product of a maturation process transforming the proinsulin, containing a leader sequence and three domains (A and B chains linked by a C-peptide), into insulin only composed of linked A and B chains. A recent review highlighted the broad diversity of epitopes identified in mice and humans with T1D (Di Lorenzo et al 2007). It is striking to realize that a minority of $CD4^+$ T cells epitopes is reported inside the A and B chains of insulin, which are the domains used to test the therapeutic efficacy of insulin (full-protein) in mice and humans. The vast majority of MHC class II epitopes encompasses amino acids within the leaders and C-peptide domains (which is the opposite for MHC class I epitopes). One could argue that using proinsulin instead of insulin might tip the balance of MHC class I/class II epitopes presented *in vivo* towards the $CD4^+$ T cell compartment and ameliorate the therapeutic outcomes.

The aAg GAD65 is another player in the pathogenesis of T1D. A phase II randomized, double blind placebo-controlled clinical trial was conducted with 35 patients recently diagnosed with T1D to test the efficacy of GAD65 (two subcutaneous injections with 20 μg of either GAD65 or placebo). Diamyd Medical described that the GAD65 treatment group showed higher C-peptide levels at 15 months than the placebo group (unpublished data). Even thought GAD65 is not considered as the primary Ag in murine T1D (Kent et al 2005, Nakayama et al 2005), one has to agree based on this report that it might be a potent antigen to induce tolerance. This fits interestingly with the observation that in the virally-induced rat insulin promoter-lymphocytic choriomeningitis virus (RIP-LCMV) model for T1D, antigen-specific tolerance is never obtained with the driver viral antigen (i.e. nucleoprotein or glycoprotein of LCMV) but rather with insulin B-chain- or GAD65-specifc aTregs upon bystander suppression (Homann et al 1999,

Wolfe et al 2002). Lastly, although these data obtained with GAD65 in humans are certainly very encouraging, one has to keep in mind that one of the inclusion criteria for this trial was a positive GAD65 antibody (Ab) response at screening. Since approximately 60 to 70% of newly-diagnosed patients are GAD65Ab positive, we would like to raise the question of whether GAD65Ab-negative patients would profit from this antigen-specific therapy. If not, one has to assume that another antigen such as insulin should be tested.

It is well documented that genetic susceptibility is one major component in the pathogenesis of T1D. The major histocompatibility complex (MHC) class II genes, known as human leukocyte antigen (HLA) in humans, are the most prominent susceptibility genes (Maier & Wicker 2005). HLA class II molecules are expressed on epithelial cells in the thymus and on antigen presenting cells (APC). Unfortunately, the precise mechanism by which certain HLA alleles predispose to T1D is still unknown. Consequently, the majority of diabetic patients share a common genetic background and approximately 95% of people with T1D carried either HLA-DR3 or -DR4 susceptibility alleles. However, it is surprising to see that with a relatively restricted repertoire their autoreactive T cell repertoires display different patterns of reactivity (Arif et al 2004). For instance, a study by Oling and colleagues showed that diabetic patients and at-risk subjects have a significantly higher prevalence of GAD65- and proinsulin-specific $CD4^+CD25^+$ T cells than the control subjects, but the frequency for one or the other aAg markedly fluctuated in the blood of each individual (Oling et al 2005). Such observations indicate that it might be difficult to discover a single antigen-specific therapy that will be equally efficient for all patients with T1D. To overcome this problem in developing potent antigen-based immune-interventions for T1D, we propose that an individualized antigen therapy for each patient will be more appropriate (Fig. 3).

Ideally, the first diagnosis should include a systematic detection of the autoreactive T cell repertoire (looking at $CD4^+CD25^+$ T cells as in Oling et al 2005) in the blood by using *in vitro* T cell assays (such as ELISPOT, proliferation assay and/or tetramer technology). Based on these data, a customized per patient antigenic treatment could be realized and should have a positive impact to expand more forcefully islet-specific aTregs *in vivo*.

Designer Tregs

Once the factors for optimal function of nTregs and aTregs to operate in T1D have been sufficiently defined, one could envision engineering islet-specific Tregs that can act locally in the pancreas or PLN as 'bystander suppressors'. To achieve this, one would insert a suitable T cell receptor into T cells and ensure that important homing receptors as well as effector cytokines are being expressed. If such

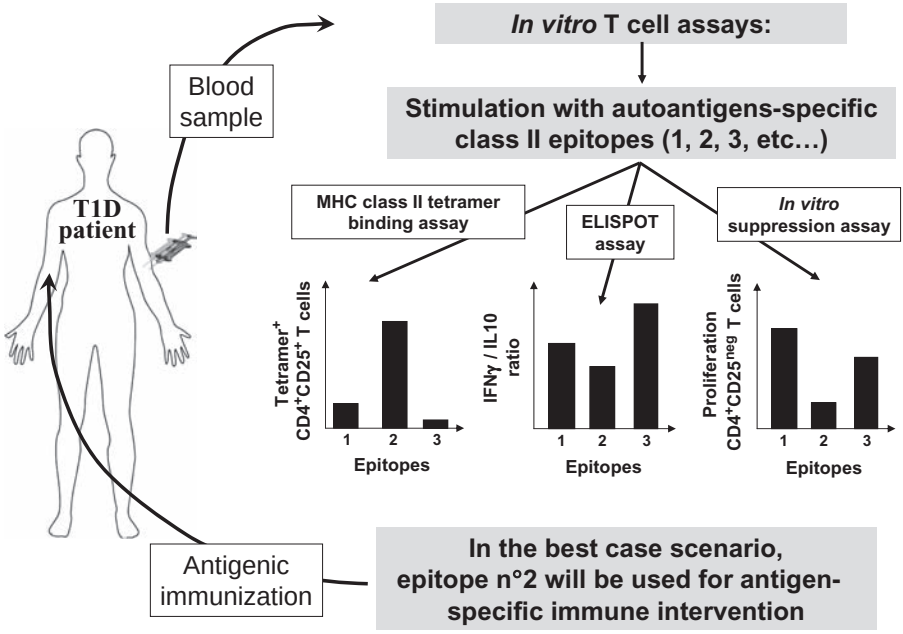

FIG. 3. Customized antigen-specific treatment in patients with type 1 diabetes (T1D). Blood samples are obtained from newly-diagnosed T1D patients and processed to stimulate *in vitro* autoantigen-specific T cells by using human MHC class II-specific epitopes. Subsequently, expanded T cells will be tested by *in vitro* assays (ELISPOT, MHC class II tetramer staining and/or proliferation assay). The epitope expanding T cells showing the 'best' regulatory phenotype and function ($CD4^+CD25^+$, low IFNγ/IL10 ratio and suppression of $CD4^+CD25^-$ proliferation) should be used for antigen-specific therapy *in vivo*.

cells that could be used for autologous cell therapy exhibit a stable phenotype, they could be transferred to protect from diabetes development in one individual multiple times.

Combination therapies will accelerate clinical translation

While many therapies have been successful in animal models, none of the clinical trials conducted in humans have yet reached the ultimate goal of achieving euglycaemia without conventional insulin administration. After several attempts to cure T1D in humans, we would argue that a monotherapy will not reach the necessary efficacy needed to maintain permanent tolerance. The genetic and pathological heterogeneities observed from patient to patient at trial entry complicate the discovery of a single therapy that will be similarly efficacious and safe when administered into all patients. To progress on this issue, we propose to combine

compounds that will expand islet-specific aTregs and regenerate the β cell mass to halt C-peptide decline (Bresson & von Herrath 2007).

As previously described, anti-CD3 constitutes an extraordinary systemic immune modulator that has showed repetitive efficacies even when injected at relatively low doses after new onset. Anti-CD3 therapy exerts its effects by promoting a milieu for generation of $CD4^+CD25^+$ aTregs. However it is conceivable that only a small fraction of these Tregs possess a specificity for islet antigens. These islet-specific aTregs are the most prone to impact the effectiveness of the treatment. Therefore, expansion of islet-specific aTregs will augment the odds for a permanent remission from diabetes by redirecting these cells to the target organ under attack. Based on this rationale, we hypothesized that combining systemic anti-CD3 with islet-aAgs immunizations might synergize and specifically expand islet-specific aTregs that will be recruited to the site of inflammation in the pancreas or the PLN, where they will attenuate the autoimmune aggression by effector T cells. We have recently shown that among several aAgs tested, mucosal vaccination with proinsulin in conjunction with low dose of anti-CD3 exerted the best synergy to cure T1D after new-onset in two animal models (Bresson et al 2006). The concomitant therapy enlarged the number of proinsulin-specific aTregs that produce regulatory cytokines (IL10 and TGFβ) and migrate to the PLN where they block autoreactive $CD8^+$ T cells by bystander suppression. Recent experiments also pointed out a good synergy between anti-CD3 and the full-human insulin protein administered orally or nasally (D. Bresson and M. von Herrath, unpublished observations). This approach constitutes a great hope towards the development of safer and more effective immuno-interventions. Furthermore, this concomitant therapy could also be part of protocol aiming at increasing islet graft survival after transplantation.

Lastly, clinical signs of diabetes often appear in T1D patients when more than 80% of the β cell mass is destroyed. This observation denotes that (i) at diabetes onset a significant percentage of β cells still remain in the pancreas and (ii) euglycaemia can be restored by regenerating only a small proportion of those β cells. The question of whether or not β cells have the capacity to regenerate/proliferate *in vivo* is still open for debate (Desai et al 2007, Phillips et al 2007, Trucco 2005). However, nowadays it seems commonly accepted that additional therapies are urgently needed to promote regeneration and reach a sufficient threshold of β cell mass (Bresson & von Herrath 2007, Herold et al 2007). So far, glucagon-like protein 1 (GLP1) and its long-acting analogue (exendin 4 also known as exenatide) have been the only drugs able to stimulate β cell expansion *in vivo*. This should prompt us to put more effort into the development of other compounds to enable potent and safe *in vivo* proliferation of β cells.

Therefore, in newly diagnosed patients forthcoming therapies would profit from the combination of a safe blockade of the pathogenic immune responses (by using

anti-CD3 and aAg immunizations, for instance) with an expansion of residual β cells using GLP1, exendin 4 or future drugs.

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DISCUSSION

Peakman: I am a little worried about human viruses being used in these models. We know from the HIV field that a family of viruses does completely different things in different hosts. Your interpretation of the Coxsackie virus 3 may need to be revised.

von Herrath: It depends what you look at. If you look at basic things, such as whether PD1 is up-regulated, this can then be transferred to the human situation. However, I agree that viruses do different things in different hosts. For example, the difference whether you die from a viral infection or the virus does nothing can depend on the host, and in many other scenarios it is just the immune kinetics. An example would be SIVmac239 which results in a rather acute infection in rhesus macaques compared to a much more chronic decline of immune function in many human HIV cases. There, immune kinetic factors strongly differ. However, I think we still can draw decent analogies: for Coxsackie B viruses, you can analyse whether these viruses are tropic for β cells in the murine model, and what happens if they only infect the exocrine pancreas, and then these things can be tested in human cell systems.

Leiter: In the BB-DR (diabetes resistant) rat model, where there is no lymhopaenia and generally no diabetes, years ago a sporadic outbreak of diabetes in that colony at the University of Massachusetts, Worcester, was traced to a parvovirus exposure. Following this up, the investigators found that it wasn't the virus attacking the β cells, but really the APCs. I think more attention should be given to the rat models where experimentally introduced rat viruses are clearly potential diabetogenic triggers. Perhaps they impair innate immune responses required to keep autoreactive T cells in balance.

Lew: The DNAX scientists seemed to think that Th17 cells have no role to play in NOD diabetes. Have you looked at Th17 in your model?

von Herrath: We haven't looked at this much. We know that there are probably some Th17 cells generated. We studied IL21 receptor knockouts in the NOD, which are completely free of diabetes. This could still be due to genetic factors, but also the RIP-LCMV model is completely free of diabetes if you cross the IL21 receptor knockout with this model. Obviously, IL21 does many other things in addition to affecting IL17 production, so we don't know yet.

D Hafler: Getting back to the Tregs and their influence over what the virus is doing in the model, have you looked at what anti-TGFβ does in this system? How much is mediated by TGFβ and how much is mediated by cell–cell contact? Is there any LAP expression?

von Herrath: We haven't looked at LAP. We have looked at TGFβ *in vitro* and it can account for about 50% of the immune suppression. We are testing the role for TGFβ *in vivo* at the moment.

Atkinson: Which one of your models do you think would explain the fivefold increase in the incidence of type 1 diabetes over the last 50 years? And which of these models would explain the profound geographic differences in incidence, where 50 miles of distance can lead to a sixfold difference in disease incidence, such as is found between Finland and Russia (Kondrashova et al 2005)?

von Herrath: I don't fully understand the vaccine situation in Finland and Estonia. Genetically, the populations are still closely related. Of course, they have had very

different types of industrialization over many years. The population in Estonia is 1.3 million, so it is relatively small. It could go both ways: if you undergo a full Coxsackie B virus infection without prior vaccine, this might be good for training your immune system. Conversely, it could be that when there is a good vaccine, a full blown infection with an islet-tropic strain is much less likely. Someone would have to isolate the virus strains from these individuals and clearly look at their tropism.

Atkinson: It could also be a bit like the situation with HIV, where there is an emerging virus that could explain the increase. There are other major questions with type 1 diabetes models involving viruses. Why do we have more diabetes than ever occurring in children under the age of five? Furthermore, if we are as mobile a society as ever, one would presume a situation where the incidence of disease would work towards homogeneity. However, this does not occur and we are left with even more questions, such as why does this disease increase every decade in every country?

Roep: The studies in the Belgian cohort and other countries with national registries show that there is an earlier presentation. If this is the case, it raises the issue of the hygiene hypothesis. The baseline immune response is important, so we should never keep children cleaner than is strictly necessary. If children become cleaner and there is more antibiotic use each decade, the viral triggering would be the same all through, but it is the other pathogens and the intestinal flora that are changing. The presentation of the disease is earlier and the disease accelerates more because there isn't a high enough baseline immune response to distract the autoreactive T cells.

D Hafler: A lot of the discussion in Estonia/Finland is to do with the vaccination differences between the populations, and the public health differences. In general the Estonians haven't been vaccinated but the Fins have.

Insel: This is not specific for type 1 diabetes per se. All autoimmune diseases have been rising in frequency, so we need to consider this as a more general phenomenon.

D Hafler: The immune system evolved in conjunction with microbes. The price we pay for defeating parasites and viruses could be autoimmunity. It might be a price worth paying, because these are usually horrible diseases with a high fatality rate. It is likely that an immune system that has developed to fight these common infections and which then finds it has nothing to fight might result in problems. But how do we get hard evidence for this?

von Herrath: The Finland/Estonia scenario is one, where we could look at the viral vaccine responses.

Peakman: There are data on enterovirus antibodies and ICAs in Estonia. There is quite a lot of evidence of infection there. The antibodies are generally lower than would be seen in Finland (Juhela et al 1999, Viskari et al 2004, 2005).

Bonifacio: Heiki Hyoty has various hypotheses on this, which include protection against virus. One of the hypotheses is maternal protection, where mothers aren't exposed as much during pregnancy to Coxsackie virus in Finland. Therefore the children aren't protected during the first years of life. When he originally looked for frequency of Coxsackie B infection in various countries, he was hoping to see more in Finland, but in fact he saw less which would fit with the concept of weakened maternal protection leading to increased risk.

Eisenbarth: I have a bias about viruses. Whatever is in the environment triggering has to be ubiquitous. We are all exposed to it; it is nothing rare. I like the models where things are induced, such as the Kilhamrat virus, where the immune system is activated and then you trigger. But it doesn't explain why things are increasing.

Roep: What is the NOD mouse story? The cleaner the colonies the worse the disease. This fits with your earlier arguments about baseline immune triggering being protective.

von Herrath: There might be two effects. There might be baseline activation of the immune system by pathogens that is immunoprotective—in addition, there could be an ubiquitous triggering virus that is islet-tropic and induces class I. This would drive the process and make it possible to develop type 1 diabetes on a susceptible genetic background.

Eisenbarth: It doesn't have to be islet-tropic. We can model this where we put B7.1 on an islet or give poly IC. If you give enough of it the rat develops diabetes.

Peakman: You have to have B7 in the islets.

Eisenbarth: Not in the rat. All you need is enough poly IC given to the DR without lymphopaenia, and you can induce diabetes.

von Herrath: The B7.1 studies in the mouse argue for islet tropism because it is a local effect. The β cells are, in a sense, rendered to be professional APCs.

Eisenbarth: In B7.1, when you give poly IC, if you block the induction of IFNα you block the disease. You can give IFNα systemically and create disease. The question is what the B7.1 is doing. I think it is creating a hair trigger in the islet, but systemic activation can do this. But there is something wrong with the islet in B7.1—it is an artificial system. Our epidemiology doesn't tell us well enough what is going on. There are pilot studies to have families monitored for fever because I think we will have to look close to the time in these young children at which they actually get infection to when they develop autoantibodies. Just looking every three months as children develop autoantibodies hasn't told us well enough whether Coxsackie viruses really are associated with the development of diabetes.

Roep: One thing I'd like to recall from the work of Francisco Dotta is that the virus itself doesn't cause diabetes. It does cause impaired β cell function,

and it can even cause hyperglycaemia, but without autoimmunity this can be reversible.

von Herrath: That is why I propose that it has to be the local effect that somehow affects the islet. It doesn't have to be in the islet, perhaps, but the virus has to do something very close to the islet so that it up-regulates class I or something else, and then autoagressive T cells are also needed.

Roep: The infection need not be local, either. If it is the autoimmunity that determines the local consequences, this would also fit nicely with the selectivity of the destruction.

von Herrath: The data we saw earlier showed clearly that MHC class I up-regulation was present first.

Roep: It is a minority of cases where we can see evidence of a viral infection. People think that perhaps the VP1 study is an overestimate, or it could be other viruses. So far, we have a minority of cases where there is virus. Hyperexpression of class I is ubiquitous, so there is a discrepancy.

Flavell: What percentage of virus infections are detected?

D Hafler: What percentage of viruses do we know of?

Roep: Most information is on surrogates of infection. I'm sure the first thing you will do when you get home is look at IFNα in your series of prediabetic samples, if this cytokine mirrors viral infection.

Pipeleers: I would be careful in calling these individuals pre-diabetics.

Insel: Ezio, is it not correct that you and Olli Simell's group in Finland have data suggesting that diabetes manifesting in the first decade of life represents the clinical outcome of an immune-mediated insulitis that occurred in the first 2–3 years of life?

Bonifacio: If we look at when these children develop their autoantibodies, there is a peak incidence after the first year, which is contributed to by high affinity insulin autoantibodies. 2% per year will develop this. Either the children are programmed and there is no trigger for this autoimmunity, or there is something happening in this first year of life that is causing this peak.

Butler: This peak coincides with the wave of β cell replication in normal development.

Bonifacio: We have always wondered about this. There is something happening at the level of the β cell that doesn't require viral infection or the other factors. I also think that there is a type of autoimmunity that we see that represents a subtype. There will be potentially others that happen later, including Coxsackie virus type events.

D Hafler: Can one think of a child that hasn't had some sort of viral infection in their first year of life, with fever?

Insel: It is a limited number of fevers, however, that a child has in that first year of life.

D Hafler: Rarely zero, though. It could only require one.

Insel: It appears that affinity maturation of antibody responses in the young infant require repetitive exposure of antigens as occurs with repeat immunizations in the first year of life. In fact, even measurable antibody titres to diphtheria toxoid requires not uncommonly two immunizations. The finding of affinity maturation of insulin antibodies in young infants suggests either persistent or repetitive antigenic exposure early in life, and before age three years. Diabetes in children appears to be increasing in incidence with the largest increase in children in the first five years of life. We must explain this phenomenon, and by the way, the answer is not routine childhood immunizations.

Leiter: Ezio, what is your estimate for how much of the antibody in the first year is maternal.

Bonifacio: What I just described is excluding maternal antibody.

von Herrath: Let's come back to this viral receptor issue. Viruses occur as quasi species. They mutate a lot and adapt fast. In the LCMV system we have a strain that does persists and one that doesn't, with just one amino acid difference that translates into a more than tenfold difference in receptor binding. This changes the whole immune kinetics following infection. The fact that you could have a population of enteroviruses that cause in certain individuals persistent activation in the islets is still a possibility, if here are viral variants that differ in terms of islet tropism or receptor binding.

Roep: We published a case (Vreugdenhil et al 2000) on echovirus infection, which is also an enterovirus. This was acute, without a sign of autoimmunity. Roivainen has shown that from every serotype of enterovirus there are β cell tropic viruses.

Peakman: She also showed that they differ for killing of β cells. It is not just in tropism.

von Herrath: This could probably explain a lot of the observations. There are enteroviruses that are infecting children, there are probably quasi species that vary in terms of β cell tropism and their killing ability. All this variation could explain a lot. The thing to look for would be whether people who are at risk for type 1 diabetes have any mutation in one of the crucial receptors for enteroviruses or whether they are infected with viral strains that exhibit differences in tropism for beta cells.

Atkinson: That is tricky to explain as a concept. The risk could be higher if certain relatives have this mutation, but remember, 80% of new cases happen in people where there was no family history. How does one explain that?

Butler: In terms of family history and the genetics, the recent observation from genome-wide scans that overlap between Crohn's disease and type 1 diabetes are of interest. Is Crohn's disease occurring younger and younger, as is diabetes?

Flavell: Yes, this is a general observation for all these autoimmune diseases.

Butler: So is the difference seen with diabetes in Estonia and Finland also seen for Crohn's?

Flavell: I don't know.

Butler: For those who want tissue, Crohn's small bowel is an awful lot easier to get at than the pancreas.

Flavell: What is known about natural antibodies in these countries? I'm referring to the anti-carbohydrate antibodies and so on that may play a role in viral maintenance? Hygeine may have a big effect on this.

Insel: Antibodies in breast milk reflect antigenic exposure at mucosal surfaces, especially at the GI tract and so breast milk antibodies will reflect the epidemiology of GI colonization and infections in the environment. I believe that understanding the relationship between the GI tract, epithelial integrity, microbes and the immune system will be critical for understanding the pathogenesis of type 1 diabetes. It should be noted that the NIH has recently announced that the NIH roadmap project will include studies of the 'biome', understanding the role of microbial flora on health and disease.

Leiter: The mouse models of inflammatory bowel disease (IBD) are a bit different from the NOD mouse. NOD does better in terms of developing diabetes quickly when it is put behind a barrier and the enteric flora are eliminated. With the IBD model, it tends to go the other way. If you maintain the models under high barrier SPF conditions, the IBD goes away. To see IBD, such models need to be recolonized with the 'right' bugs.

Eisenbarth: An important caveat is that with a couple of our major animal models, as far as we can tell an exogenous viral infection isn't needed for type 1 diabetes to develop. This might be true in humans also.

Bonifacio: What else can induce IFNα? It's a stress response.

Roep: I would be interested to see whether hypoglycaemia could do this.

Foulis: IFNα isn't seen in the islets of patients with type 2 diabetes.

Roep: Nor is it seen in pancreatitis, which is another interesting feature.

Eisenbarth: Is the class I MHC seen early on in the NOD mouse?

Kay: It's entirely dependent on insulitis. My feeling from our earlier discussion is that in the mouse model, the class I MHC only goes up when we can see insulitis. We know that the insulitis is patchy in the NOD. Those islets that don't have insulitis also don't have class I MHC up-regulation.

Flavell: Known stimulators are double-stranded RNA, 5′ triphosphate RNA and DNA.

Kay: In the mouse it is much more likely that IFNγ is what is up-regulating class I.

von Herrath: What happened to the work by Vagn Bonnevie Nielsen in Vancouver, who had IFNα signatures that he followed in type 1 diabetes patients and normal controls?

D Hafler: It raises an interesting question about systemic lupus, which is a classic disease with an IFNα signature in the peripheral blood. When one looks at RNA microarray data from peripheral blood lymphocytes, in lupus as opposed to other autoimmune diseases there is a signature of IFNα induction. This has been associated with the IRF-5 variant in lupus patients.

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Cytotoxic T cell mechanisms of β cell destruction in non-obese diabetic mice

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Abstract. $CD8^+$ T cells are the principal cellular mediators of β cell destruction in the NOD mouse. Molecular mediators include perforin and granzymes from the cytotoxic granule, Fas ligand and pro-inflammatory cytokines. Our studies in NOD mice have shown that β cell-specific $CD8^+$ T cells use both the perforin and Fas pathway *in vitro*. Reducing antigen presentation on β cells, for example by reducing class I MHC expression by overexpression of SOCS1, protects β cells *in vivo*. Perforin deficiency effectively reduces diabetes in NOD mice but in NOD8.3 mice other mechanisms compensate. We have been unable to identify a major role for direct toxicity of cytokines in NOD mice. However, in the LCMV glycoprotein model they may be more important. Deficiency of IL1 or TNF or Fas has a protective effect (greatest for TNF deficiency) but this appears to be due to effects of these cytokines on the immune response rather than on the β cell. Combinations of interventions, for example, β cell overexpression of SOCS1 combined with IL1 deficiency may be highly protective. It should be possible to define all the molecular mediators of β cell destruction, and it may be possible to inhibit at least some of these.

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Type 1 diabetes is an autoimmune disease with a long pre-clinical phase identified by circulating markers of immune reactivity against the pancreatic β cell—most useful at a clinical level are autoantibodies that recognize well-defined β cell antigens. β cells are destroyed by immune cells during this preclinical phase culminating in insufficient β cell mass to control blood glucose. While a limited amount of histological material exists from patients following diagnosis there are almost no direct pathological observations of the preclinical phase. What is known comes from the chronology of autoantibodies in humans and from studying animal models of which the non-obese diabetic (NOD) mouse is by far the most intensively studied. In mice it has been possible to study pathogenic autoreactive T cells

and T cell receptor (TCR) transgenic NOD mice derived from autoreactive T cells have been particularly useful. This review will focus on the role of $CD8^+$ T cells in diabetes.

Transfer of diabetes with T cells indicated a possible requirement for $CD8^+$ T cells (also called cytotoxic T lymphocytes or CTL) (Miller et al 1988, Christianson et al 1993) for β cells to be destroyed but it was really the striking phenotype of β2-microglobulin-deficient (β2m-deficient) NOD mice that brought $CD8^+$ T cells to centre stage (Katz et al 1993, Serreze et al 1994, Wicker et al 1994). CTL had not previously (or since) been centrally implicated in other autoimmune diseases and there was widespread scepticism. Several groups showed that in β2m-deficient NOD mice that are major histocompatibility complex (MHC) class I and $CD8^+$ T cell-deficient, there is no insulitis and no progression to diabetes. Furthermore, these mice are resistant to transfer of diabetes by spleen cells taken from pre-diabetic NOD mice. Restoration of β cell class I expression with a β2m transgene restored recognition of β cells by CTL (Kay et al 1996). These experiments suggested a need for MHC class I expression and direct interaction with $CD8^+$ T cells for β cell destruction. NOD mice in which β2m was deleted only from β cells ('β bald' mice) were later developed (Hamilton-Williams et al 2003). $CD8^+$ T cells develop normally in these mice because MHC class I proteins are expressed in the thymus and peripheral lymphoid tissues but interaction between them and β cells cannot occur. The resulting phenotype is relatively normal insulitis but substantially reduced diabetes. These mice confirmed that direct interaction between $CD8^+$ T cells and the β cell is needed for efficient progression to diabetes.

β cell destruction

A substantial part of the role played by $CD8^+$ T cells in the immune system is cytotoxicity and they are the cells most responsible for β cell destruction in the NOD mouse. The evidence for this includes the ability of $CD8^+$ T cell clones and TCR-transgenic T cells to kill β cells *in vitro* and *in vivo* (Wong et al 1996, Verdaguer et al 1997, Graser et al 2000) and the reduction in diabetes observed when class I MHC protein expression on β cells is absent or reduced impairing direct recognition of the β cell by $CD8^+$ T cells. Also consistent with this is the substantial reduction in diabetes with deficiency of the major $CD8^+$ T cell effector protein perforin (Kagi et al 1997, Amrani et al 1999). In both these cases there is some residual diabetes, i.e. the frequency of diabetes in perforin-deficient NOD mice and mice lacking MHC class I on the β cell is slightly under 20% (Hamilton-Williams et al 2003) (compared with approximately 80% in wild-type mice) raising the possibility that progression to diabetes in perforin-deficient and β bald NOD mice may be mediated by $CD4^+$ T cells.

The use of knockout mice has allowed better definition of the effector molecules used by CTL. We and others have back-crossed knockouts originally made in the 129/Sv or C57BL/6 inbred strains on to the NOD genetic background for several generations, taking into account the problems of this approach elegantly described by Leiter (2002). The crux of this is that even after many generations of back-crossing, a substantial congenic interval of 129 or BL/6 genes is found flanking the knockout locus. We have not found that the 'speed congenic' approach by which NOD genes associated with diabetes are selected for in the mice used to breed each generation has been empirically useful in part because it fails to address the congenic interval. In cases where reduced diabetes is observed we then work out whether this is solely due to reduced β cell killing either by introducing an inhibitor into the β cell only (for example using transgenes driven by the insulin promoter) or by transplanting knockout islets into wild-type mice. This was done in the case of Fas in which reduced diabetes was observed by others in Fas-deficient NOD mice (Chervonsky et al 1997). We have studied the fate of Fas-deficient islet transplants (Allison & Strasser 1998) or transgenic mice with dominant inhibitors of Fas signalling expressed in the β cell (Allison et al 2005).

Perforin

Perforin is a major constituent of the cytotoxic granules of CTLs and NK (natural killer) cells. While having structural similarity to complement, it is believed to facilitate entry of granzymes into the target cell and their activation rather than simply penetrating the target cell membrane (Trapani & Smyth 2002). Granzymes are a family of serine proteases that activate pathways within the target cell that result in apoptosis. Granzymes are dependent on perforin for their activity so a knockout of perforin is functionally also a knockout of granzymes.

It was reported that perforin-deficient NOD mice developed insulitis with a normal histological appearance but without highly pathogenic invasive insulitis (Kagi et al 1997) (Table 1). Only 17% developed diabetes and it occurred much later than their wild-type littermates. The frequency of diabetes was independently confirmed (Amrani et al 1999) and suggested a major role for perforin-dependent pathways in β cell destruction and also suggested that CTL are the major cell type involved, as perforin is not as clearly an important effector mechanism in $CD4^+$ T cells. Subsequently it has been shown that perforin deficiency does not reduce diabetes mediated by $CD4^+$ T cells (Amrani et al 2000, Cantor & Haskins 2005) (Table 2). It is surprising that perforin deficiency was so effective in reducing diabetes given evidence for other mechanisms of cell death used by CTL and the likely participation of $CD4^+$ T cells. Redundancy of pathways that might have been expected was not observed *in vivo*, in that deletion of one mechanism produced

TABLE 1 What protects the β cell?

High degree of protection
Perforin deficiency
Reduced β cell class I expression
Little or no protection
Fas deficiency
IL1 receptor deficiency
IFNγ receptor deficiency
iNOS deficiency
Protection but not due to reduced β cell destruction
TNF receptor deficiency

TABLE 2 Effects of genetic mutations on diabetes in NOD mice

	CD8 TCR transgenic NOD	*CD4 TCR transgenic NOD*	*NOD*
IL1R$^{-/-}$	No effect	No effect	Slight delay
TNFR1$^{-/-}$	Partial reduction	Reduction	Protection
IFNγR$^{-/-}$	Delay	Delay	No effect
RIP-dnFADD	Slight acceleration	No effect	Partial reduction
RIP-Bcl2	ND	ND	No effect
RIP-SOCS1	Protection	No effect	Significant protection
RIP-SOCS1/IL1R$^{-/-}$	ND	No effect	Protection
Perforin$^{-/-}$	No effect	No effect	Significant protection

such an impressive effect. This suggests that the perforin-independent mechanisms responsible for β cell killing are not very potent in NOD mice and cannot fully compensate for perforin deficiency and also take longer to cause diabetes.

In contrast, redundancy is observed in NOD8.3 mice (see below) in which absence of either perforin or Fas has no effect. The effector mechanism by which 17% of NOD-perforin$^{-/-}$ mice develop diabetes is also not clear as no evidence sheeting this home to a specific mechanism has been reported. Our preliminary data suggest that Fas is not solely responsible.

Recently our group has studied the interaction between β cells and perforin and granzyme B *in vitro*. There is no doubt that β cells are susceptible to perforin and granzyme B but require higher concentrations of granzyme B and longer time for death to become detectable than either typical cytotoxicity targets such as P815 cells or primary haematopoietic cells (Estella et al 2006). Additionally intact islets are highly resistant to perforin *in vitro* because it becomes inactivated by the outer layers of cells and does not make it to the interior of the islet in active form. As

yet we have not devised a strategy to inhibit perforin action in the β cell only but this is a high priority.

Fas

Since the original description that complete Fas deficiency protected NOD mice from diabetes (Chervonsky et al 1997) there have been several attempts to clarify the mechanism. Initially it was proposed that Fas expression on β cells exposed them to Fas ligand on infiltrating T cells and was a critical mechanism of β cell destruction. Ideally testing the role of an effector pathway such as Fas would be explored by blocking its effect only in the β cell rather than in all cells. This has been achieved by introducing dominant negative mutations of Fas or its downstream signalling pathway, into the β cells of NOD mice leaving the rest of the mouse unaltered (Savinov et al 2003, Allison et al 2005). An alternative is to transplant islets from Fas-deficient mice into wild-type recipients (Allison & Strasser 1998). These studies in transgenic NOD mice have shown partial protection from absence of Fas or Fas signalling but much less protection than seen in NOD-Lpr mice. It is unclear why diabetes is reduced at all in these models—why is it that perforin-dependent killing does not destroy β cells in nearly 20% of the mice? Conditional deletion of Fas from β cells using the Cre-Lox system has been studied in the haemagglutinin model of $CD4^+$ T cell-mediated diabetes and this showed no effect of Fas deficiency on diabetes progression (Apostolou et al 2003). Most probably other effects of Fas deficiency, perhaps including abnormal expression of FasL expression in Lpr mice, were responsible for protection of NOD-Lpr mice from diabetes, not reduction of Fas-mediated cytotoxicity of β cells.

A piece of compelling evidence that supports a role for Fas is that transgenic NOD mice that overexpress FasL in β cells have accelerated diabetes likely to be due to β cell 'fratricide' (Chervonsky et al 1997). This is indirect but strong evidence that β cells from NOD mice express Fas allowing the fratricide to occur. Using flow cytometry we have not been able to directly detect Fas expression on β cells from non-transgenic NOD mice but Fas is detectable on β cells in accelerated models of diabetes including TCR transgenic NOD8.3 mice, NOD4.1 and NODBDC2.5 and following adoptive transfer (Darwiche et al 2003). Either Fas is expressed intermittently on β cells of non-transgenic NOD mice or at levels that are lower than in accelerated models and cannot be detected by our methods but perhaps could be sufficient for β cell killing. This low level of Fas expression on β cells of non-transgenic NOD mice, probably secondary to less inflammation and expression of cytokines than in TCR transgenic NODs, may explain why inhibition of Fas is relatively ineffective, also, in perforin-deficient NODs.

TCR transgenic models provide an opportunity to study effector mechanisms of $CD4^+$ and $CD8^+$ T cells separately. Both $CD8^+$ TCR transgenic cells such as

NOD8.3 and $CD4^+$ TCR transgenic cells such as NOD4.1 have been reported to use only the Fas pathway to kill β cells (Amrani et al 1999, 2000). Surprisingly we have not been able to verify protection from diabetes by blockade or deficiency of Fas on β cells in either of these models (Dudek et al 2006). Inhibition of perforin *in vitro* inhibits cytotoxicty of NOD8.3 T cells whereas Fas-deficient targets are killed by NOD8.3 T cells. Perforin-deficient NOD8.3 T cells, however, cannot kill Fas-deficient β cells. Fas-deficient β cells when transplanted can be destroyed by both 8.3 and 4.1 T cells. Overexpression of dominant negative FADD, an inhibitor of Fas signalling, failed to reduce diabetes in either model. Doubtless more experiments will be added to these and others that have tried to clarify the role of Fas. In our hands it plays second fiddle in CTL to perforin and granzymes and is not involved in β cell killing by $CD4^+$ T cells.

Cytokines

Cytokines have been extensively studied as potential mediators of β cell damage in type 1 and more recently type 2 diabetes because of the recognition that combinations of cytokines that include an interferon plus a cytokine such as interleukin (IL)1 or tumour necrosis factor (TNF) that activates NF-κB and MAP kinases are able to kill β cells *in vitro*. Their *in vivo* significance in β cell destruction is less certain. Local cytokine production can be inferred from increased expression of cytokine-responsive genes in the islet such as MHC class I (induced by interferons alone) which is easily detectable (Thomas et al 1998) or Fas (in mice requires IL1 or TNF with interferon [IFN]γ for maximum expression) which is more difficult (Kanagawa et al 2000, Serreze et al 2000, Darwiche et al 2003). Additionally cytokines can be more directly measured. Our data based on RT-PCR of isolated islets indicate expression of IFNγ and weaker evidence for IL1 and TNF.

Cytokines secreted by T cells may act on the β cell and cause or increase β cell destruction. Mechanisms could include increasing β cell recognition by T cells through increased expression of cytokine-responsive genes such as class I MHC proteins, chemokines or cytokines such as IL15 or through up-regulation of cell death receptors such as Fas or through direct cytotoxicity. The best-studied mechanism of direct toxicity, at least *in vitro*, is induction of iNOS expression and subsequent production of nitric oxide (NO) although other free radicals and other mechanisms have also been described. While we have looked hard for evidence of direct toxicity in the NOD mouse *in vivo* we have not found this (Table 3). It is difficult to entirely rule out direct cytotoxicity of cytokines as an important mechanism but our data favour increased recognition of β cells by the immune system as the most convincing effect. What evidence is there against direct toxicity *in vivo*? This includes normal progression to diabetes in NOD mice in which IFNγ (Hultgren et al 1996) or either chain of its receptor is deficient (Kanagawa et al

TABLE 3 Evidence that β cell destruction is not due to the direct toxic effects of cytokines

Absence of cytokine-induced β cell damage in perforin knockout NOD mice
Failure of cytokine and iNOS knockouts to reduce diabetes in NOD mice
Mice with high circulating levels of cytokines do not develop diabetes
Diabetes induced by $CD4^+$ T cells is not prevented by blocking cytokines

2000, Serreze et al 2000) or non-functional on the β cell (Thomas et al 1998) or in NOD mice in which iNOS is deleted. Similarly there is no more than a slight reduction in diabetes in IL1 receptor 1 (IL1R1) knockout NOD mice (Thomas et al 2004) and in TNFR1-deficient NOD mice it is likely that protection is due to immunoregulation rather than reduced β cell destruction. The protection from diabetes with normal insulitis seen in perforin$^{-/-}$ mice suggests that the cytokines produced locally are not effective at killing—although local cytokine production has not been accurately measured in these mice. Also in mice with high circulating concentrations of cytokines due to systemic inflammation, for example due to loss of regulators of inflammation such as suppressor of cytokine signalling 1 (SOCS1) (Chen et al 2004), or locally in cytokine transgenic mice, for the most part there is no evidence of β cell loss. An exception to this are RIP-IFNγ mice in which local expression of IFNγ triggered an influx of inflammatory cells including lymphocytes that destroyed β cells by uncertain mechanisms (Sarvetnick et al 1988).

A small effect of IL1R deficiency was observed in that diabetes in BDC2.5 mice was slowed a little and there was slight reduction in non-TCR transgenic NOD mice. However, when IL1R$^{-/-}$ islets were grafted into other mice they were equally susceptible as wild-type islets (Thomas et al 2004). When IL1R antagonist protein was administered protection was again seen (Sandberg et al 1997). These data suggest that any effect of IL1 antagonism may be on immune cells and that IL1R deficiency on β cells is not protective. The effect IL1 antagonism has on the immune system may be to reduce proliferation of effector T cells and relatively increased regulatory T cells (O'Sullivan et al 2006).

Why are β cells not more susceptible to cytokines *in vivo*? One explanation may be that networks of regulators of inhibitors of cytokines exist *in vivo* specifically to limit the damaging effects of cytokines. Exposure of β cells to TNF primarily triggers expression of cell-protective mechanisms rather than activation of apoptosis (Irawaty et al 2002, Kim et al 2005, 2007, Liuwantara et al 2006). When a dominant inhibitor of NF-κB was expressed in islets, progression to diabetes became more rapid indicating that the net effect of NF-κB activation is protective (Kim et al 2007). Genes involved in this protection may include cFLIP and A20 (Liuwantara et al 2006). Similarly we found that NIT cells with defective NF-κB signalling through the TNF receptor were more susceptible rather than less

susceptible to TNF-mediated killing. They were, however, protected from killing by IL1 and IFNγ *in vitro* because the reduced signalling limits the induction of iNOS (Thomas et al 2006). Together these data indicate that the net effect of NF-κB signalling is protection from the mechanisms of cell death in NOD mice and that β cells are protected from TNF, that would otherwise be cytotoxic, by NF-κB-mediated gene expression.

Redundancy of cytokines in β cell destruction may be one reason their individual deficiency has had little effect on progression to diabetes. To examine this we overexpressed SOCS1 in the β cells of NOD mice (Chong et al 2002, 2003, 2004). SOCS1 blocks many cytokines including IFNγ and IFNα but there is also evidence for at least partial blockade of signals through TNF, IL1 and Toll-like receptors. RIP-SOCS1 NOD mice have reduced MHC class I on the β cells, no up-regulation of Fas when this would normally be seen, and relatively normal insulitis. A partial reduction in diabetes was seen in RIP-SOCS1 NOD mice and complete protection when SOCS1 was overexpressed in the β cells of NOD8.3 mice indicating that NOD8.3 mice are highly dependent on the action of cytokines on β cells. In these mice the 8.3 T cells have normal levels of activation markers because of normal activation in the draining node. Nevertheless *in vitro* studies showed that T cells recognized RIP-SOCS1 islets less as judged by reduced proliferation, IFNγ secretion and granzyme B up-regulation, all markers of T-cell activation. Therefore our interpretation was that the reduced T-cell recognition of β cells, at least partly due to reduced MHC class I expression, was the mechanism by which SOCS1 protects from diabetes (Dudek et al 2006).

By understanding at a molecular level the pathways involved in β cell destruction it is hoped that highly targeted forms of immunotherapy could be developed that would not have the same side-effects as generalized immunosuppression. An example of this might be development of perforin inhibitors that by at least partially blocking perforin could have a substantial impact on diabetes progression. Although complete perforin deficiency has deleterious effects in humans, there is evidence that partial deficiency may be more benign and potentially effective. Clearly the redundancy of effector pathways remains a challenge. It is possible that this will be solved by combinations of therapies that are individually effective for the various pathways. We have preliminary data about the effectiveness of some combinations. A very effective way of decreasing β cell killing appears to be reduction of recognition of β cells by $CD8^+$ T cells, for example by decreasing MHC class I expression. It is difficult to imagine how gene therapy approaches such as overexpression of SOCS1 could be applied except in a transplantation setting and the hazards of reduction of β cell antiviral defences remain unclear. Even in transplantation, transduction of a high percentage of β cells remains extremely challenging. Therefore the path to application will be an important problem to address as the understanding of β cell apoptosis in diabetes unfolds.

Acknowledgements

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DISCUSSION

Foulis: I'm delighted that what you are suggesting is that this kind of cytomix is not a significant mechanism in β cell death. I must admit that I never bought into that idea because of diseases such as chronic pancreatitis and cystic fibrosis. If you want to see a lot of cytokines in a pancreas, you don't look at type 1 diabetes, you look at chronic pancreatitis. The β cells don't get killed in that disease, even though they are sitting in a soup of inflammatory cells, macrophages and T cells.

Kay: If we look at the histology of some of these pancreatitis models we can see enormous exocrine inflammation. We know that there are a lot of cytokines present, and we can even see effects on the β cells such as class I up-regulation, but we don't see β cell destruction. Although we can raise the possibility that knocking out combinations of cytokines would be much more potent than the individual cytokine knockouts, we have failed to find evidence that this is an important mechanism *in vivo*. The literature is still quite dominated by those sorts of studies, where islets exposed to cytokines are being studied. It has taken many years to try to unwind that focus. I think the focus should much more be on mechanisms of CTL killing, such as the perforin/granzyme pathway, than it is on the potential toxicity of high concentrations of cytokines.

Herold: How can your studies resolve the problem Matthias von Herrath raised about the chronicity? These are all acute assays. This shouldn't last for years. Is it that you kill all the β cells in some of the islets each time, and it just takes time to get to all of the islets or do you kill some of the β cells in all of the islets? When we have looked at human CD8s in the peripheral blood, they don't have the phenotype of regular memory T cells. If anything, they seem to be a lot more naïve.

Roep: There is something odd about the autoreactive CD8s versus the virus-specific CD8s in terms of how they recycle T cell receptors, especially after stimulation.

Peakman: I agree. Have you ever generated mouse CTLs that are virus specific? It is a nice comparison to have; it might unravel why the process is slow. The other

thing is that all these assays are done with ratios of 10:1 or 20:1, and it is just not the same as you would have in the islet.

Kay: These *in vitro* assays give us a limited amount of data because we need a readout after a few hours, where the process itself would normally occur over a long period. It is the same with the type 2 diabetes situation where you are looking at the effect of hyperglycaemia on β cells. This is something that might occur over years in a person, but we try to mimic it in an experimental setting. As well as these *in vitro* assays, the other important read-out is looking at what the intervention does *in vivo*. Both things need to be looked at to get the maximum information. I don't think we understand the source of the chronicity from these studies. One thing I've always found puzzling is that in the partial phenotype seen with Fas-dominant negative expression on the β cell, why aren't those cells just killed by perforin? What is happening here in terms of different pathways acting on individual β cells? Given the redundancy of the pathways, it is surprising that we can see anything by knocking out an individual pathway.

Roep: What about the possibility that the β cell destruction is an acute process? Even if you have four antibodies, the extent of the insulitis is usually limited, so it could be that there is a short window when destruction is occurring. There isn't any evidence that the destruction process itself is slow.

von Herrath: We should calculate this. We have the data on how long it takes to kill a β cell, how long β cells live and how many islets are present. We know how fast islet cells replicate under attack, and we know on average how long the disease takes to develop. It might be that you are right: if you calculate how many islets have to be affected at a given time, it is only a relatively small percentage.

Foulis: At presentation, 23% of the insulin-containing islets are affected by insulitis (Foulis et al 1986). Yet we know that the C peptide secretion will persist for years. This would suggest that this insulitis is relatively ineffective in destroying β cells.

Roep: The spreading may be ineffective. It is the cross-sectional way we look into things, but we don't know about the chronicity. Christian Boitard did an experiment involving partial pancreatectomy in NOD mice, leaving them with only half of the β cell mass. The expectation would be that if this is a chronic process, they would end up getting diabetes much sooner than the ones who had the full pancreas. This was not the case. The manifestation was at exactly the same time: this argues that once the process is underway, it could occur very fast.

Eisenbarth: We have data that when the immune system wants to destroy β cells, it can do it quickly. The IPEX children have type 1 diabetes in the first couple of days of life, then we have Sutherland's twin-to-twin transplants where it took about two weeks for them to get diabetes. My guess is that the immune system is being held in check in some way.

Roep: Yes, the same thing is seen with islet transplantation. There are cases where there is destruction of 100 million β cells in a couple of weeks.

Kay: Although this isn't such a good example because you are dealing with frequency of alloreactivity.

Roep: Even in cases without alloreactivity, this recurrence can be seen.

Atkinson: Ed Leiter and I are collaborating with an investigator from Connecticut, who has developed real-time continuous glucose monitors for studies of rodents. She has tested these in mice and looked at the glucose responses in NOD mice at different ages (Klueh et al 2006). You can follow the glucose excursions for days. NOD mice at young ages have five-day glucose patterns that look pretty normal; meaning, they look similar to those in control strains of mice. Then, as the NOD mice age, their oscillations and the noise in glucose determinations grow dramatically. By 12 weeks of age, those that are still 'non-diabetic' will hit glucose lows of near 40 and go as high as 300, whereas age-matched control mice don't see these excursions.

Roep: Couldn't this be the number of islets involved in the control of glucose?

Atkinson: We're currently trying to correlate the degree of the noise related to glucose excursions to the number of islets that remain in the pancreas.

Peakman: β cells don't work on their own: islets work as a functional unit, so if you knock out one or two you are disturbing the whole function. You can have impairment without too much destruction.

Leiter: There are two issues of dogma in the NOD mouse. One is that β cells are dying by apoptotic cell death at the hands of the immune system. I have done a lot of electron microscopy over the years on NOD islets *in situ*. I see a loss of membrane permeability from the outside in. The nucleus always looks quite intact in these cells that apparently are losing osmotic control. The only thing I can relate to for sure as apoptosis is this new rat model of type 1 diabetes from Hanover, Germany (Lenzen et al 2001). They show the kind of nuclear fragmentation that Peter Butler would look for in the classical pathology textbook of what apoptosis should look like at the ultrastructural level. *In situ*, while I'm sure there are some effector clones such as NY8.3, they probably do kill by apoptosis. With cytomix and everything else in the milieu, the milieu certainly counts. It isn't just perforin and Fas around those β cells. Could it be a little more complicated than just apoptosis and β cell death? The other dogma is that the immune system sees its antigen in the pancreatic lymph node, activates, and thence trickles back into the islet where it will see the up-regulated class I on the β cells. I was interested to see that Alan Foulis showed strongly expressed class I in prediabetic cases, and what appeared to be the vascular endothelium in the pancreas. The insulin B-chain peptide reactive G9C8 T cell clone may be able to extravasate through the high vascular endothelium which surely is expressing lots of MHC class I and directly

into the islets. We need to keep a broad perspective when we extrapolate to the human.

Kay: Yes, we need to think about the β cell in a feet-up, feet-down kind of way. The end result is that the β cells are killed, but we don't know how this happens. There is a lot of evidence in the mouse that the dogma relating to the pancreatic lymph node is right. T cell activation, as a first guess, likely occurs in the draining lymph node, and maybe the endothelium does contribute. It would be wrong to dismiss the pancreatic lymph node as an important site of initiation.

Flavell: Alan, you said that at presentation, only 23% of the islets were infiltrated. Is that correct?

Foulis: Yes, I'm quoting the figures from about 50 patients. On presentation, a third of the islets contain insulin. Of those that do, 23% have insulitis. There is quite a lot of insulitis yet it seems that the rate of β cell destruction must be slow.

Roep: This means that that proportion of islets is under attack at that stage. Since we don't have longitudinal data, it could be that the attack is completed a week or two later. This could still fit with the continued production of C peptide because 77% of the β cell positive islets are still making insulin.

Santamaria: We all make the assumption that inflammation or insulitis as seen under the microscope is a nasty thing, like an ulcer. Insulitic lesions are composed of some pathogenic T cells as well as some regulatory T cells, and even regulatory autoreactive $CD8^+$ cells. Under the microscope they all look the same, but I think β cell destruction is a slow process. Some high affinity autoreactive T cells will kill their targets and then will die. The idea that one CTL will kill a β cell and then jump to another islet is fallacious. T cells get activated, migrate to the tissue, kill their target, then they get reactivated and probably die. With each new wave of CTLs recruited to the tissue there is recruitment of lower affinity β cell-reactive $CD8^+$ T cells, which have regulatory (anti-diabetogenic) properties. There are brakes on the system. Insulitic lesions are not war machines: they are inefficient attackers.

Kay: Can you comment on Kevin's question earlier on about the phenotype of the 8.3-like cells, in terms of whether they look like conventional viral-specific CD8s?

Santamaria: The 8.3 is an intermediate affinity T cell receptor. It is highly pathogenic. In the mouse, when these T cells are activated they do not survive as memory T cells. In fact, we find very few memory T cells in the 8.3 NOD mouse. In NOD mice expressing an 8.3-like, but low affinity T cell receptor (10-fold lower affinity as measured by biacore) we can find lots of memory T cells. In these animals there is an accumulation of memory autoreactive T cells that have regulatory properties. These cells are suppressive towards autoreactive CD8 cells. If you look for memory as an indicator of T cell activation in the context

of diabetes, I think you will find the opposite to what you expect from viral infections. Viral infections are hit and run events where the virus activates T cells, the virus is cleared and now T cells are allowed to become memory cells. But in chronic autoimmunity, such as in diabetes, where autoantigen is constantly being exposed to those T cells, the T cells that have been activated may not have the chance to survive as memory T cells because they undergo reactivation-induced cell death, whereas a cell with a lower-affinity T cell receptor may benefit from chronic antigenic stimulation. The message here is we cannot assume that autoreactivity implies pathogenicity. I think there are a whole range of autoreactive T cell receptors: some will be pathogenic, and some will do nothing and some will be anti-diabetogenic.

von Herrath: I wanted to return to the topic of the degree of insulitis. I am confused. Did you say 23% or 30%, and what did Danny Pipeleers have in his studies?

Pipeleers: In reviewing the literature we noticed that insulitis was described in all recently diagnosed cases of children under age 7 years but became less frequent with increasing age at onset (Pipeleers & Ling 1992). In cases with insulitis, the number of islets presenting this feature was low. With the majority of type 1 diabetic patients being diagnosed after the age of 15 years, it can be concluded that insulitis is so far not a common feature of the pancreas in recent-onset type 1 diabetic patients.

Lew: Frank Carbone from the University of Melbourne thinks that effector CD8s don't move. When he transplants using a herpes simplex system, the donor mouse is labelled Ly5.1, for example. If he transplants another HSV ganglion on the other pole with Ly5.2, he finds that the 5.1 stays on one pole and the 5.2 stays on the other. They never cross. Whether this is true in diabetes, or moving from one islet to another, is unclear. I suspect CD8 effectors are sessile and remain in a spot.

Santamaria: There is a difference between memory and effectors. I don't know whether they move or not; I just think they don't survive as memory cells.

von Herrath: That is an important point. In the face of persisting antigen, the high affinity effector memory cells will do some killing and then they will apoptose. They might run out of steam, and not keep on going for so long. The lower avidity cells, in contrast, might keep on going longer. This issue is unknown. If you get a flu infection with memory effectors in the lung, it does not usually happen that half of these guys just leave. If the antigen is there they will stay in the lung and do their job; if they have too much to do they will die by activation-induced cell death at some point. Concerning the pancreas these kinetics are not known. It is difficult to understand how these cells would leave an affected islet and go to another.

D Hafler: There's a recent paper on asymmetric division, in which there is one cell developing one function and the other another (Chang et al 2007). Could this

sort of mechanism be playing a role in these types of diseases, so you have chronic stimulation and for every effector cell generated a different type of regulatory cell is made, which might be controlling things? I'd always thought that if you are a memory cell that is what you are, but if expansion is taking place in lymph node and islet, perhaps something funky is going on where there is asymmetric division like this.

von Herrath: You would have to observe how long it takes one single islet to die. This is basic information we lack.

Roep: The example George put forward, with the twin grafts, was a very fast effect. This is a whole organ and cells need to move around and find the islets, yet in a couple of weeks it was all over with.

Kay: There must have been memory cell involvement there.

D Hafler: They could have been primed ready to go.

Santamaria: It is a dynamic system. It is not that you either have memory or you don't. There will always be some memory in the population. I didn't mean to suggest that pathogenic T cells will never become memory T cells. I see this as a spectrum. It is a balance. In the case of non-immunosuppressed human pancreas isograft transplants it was four weeks, which is rapid in the absence of immunosuppression. The same thing happens in NOD mice: islets transplanted into a diabetic NOD mouse will be wiped out in days. So there is memory of pathogenic autoimmunity. In a naïve mouse it takes time to activate and recruit naïve autoreactive T cells in numbers that are sufficiently high enough to kill enough β cells.

D Hafler: Is the fact that the organ is destroyed so quickly when it is transplanted because when the islets run out, there is a build-up of cells with no place to go, and so when a new organ comes in it is wiped out fast? Or is it because there is an architecture of the islets that is much more fragile in the transplanted organ?

Santamaria: Both are possible. In the NOD mouse, we take these islets and put them in the kidney capsule, so they are all clumped together.

Roep: What if you do a whole pancreas transplantation into the NOD mouse?

Santamaria: I don't think anyone has done this.

Flavell: If you transfer T cells out of the NOD mouse into a RAG mouse, it will destroy the islets in a couple of days. There is no problem getting there: I think it is straightforward regulation. If a NOD mouse is immunized with KLH, there is a perfectly good response. The difference is that there is this mechanism opposing what is happening in this first phase. This has been eliminated somehow, so when you graft, the graft is blown away immediately.

Leiter: The IPEX (FoxP3-deficient) children are a tremendous example of this. 80% of children with IPEX get diabetes. This means we are all set up to get type 1 diabetes if we don't have regulation.

Roep: The conclusion is that the destruction process can be very fast. Regulation can slow down the process.

Kay: These assays depend on getting rid of this activation.

von Herrath: Do you think the regulation occurs in the islets or systemically?

Roep: Jeff Bluestone says it is in the lymph nodes.

von Herrath: He also says that at the end of the process it is in the islets.

Roep: He says that there are plenty of Tregs in the islets at the time of diabetes.

Herold: It depends on the important site for generation of the autoagressive response. The important regulation might be outside rather than inside the islets.

D Hafler: It is the same for RA and MS: there is a high number of $CD4^+CD25^+$ in the tissue. We don't know why, but there are a lot there.

von Herrath: Hasn't Vijay Kuchroo shown that in the EAE model they run out of steam in respect to their ability to control effector cells directly in the brain?

Roep: There are active lesions without infiltrate in MS. Is there hyperexpression of class I?

D Hafler: I was wondering about this earlier. Normal-appearing white matter has alterations by spectroscopy and pathology. I don't know if class I was involved.

Leiter: Whatever the memory is in the insulitic infiltrate in the NOD mouse and BB rat, when an animal has been diabetic for a week or more, it will be β cell deficient. Pseudo islets seem devoid of most of the lymphocytes. If we do an islet syngraft, there is rejection in a week. There is clearly residual memory for the effector arm, but the regulatory arm is apparently far outweighed at this point in the pathogenic process.

Roep: Once the balance is tipped, everything is lost.

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Type 1 diabetes: chronic progressive autoimmune disease

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Abstract. A wealth of data in animal models indicates that type 1A diabetes results from T cell-mediated specific destruction of islet β cells. There is evidence for the NOD mouse model that insulin is the primary autoantigen and a specific insulin peptide B:9-23 is central to pathogenesis. It is also now possible to predict the development of type 1A (immune mediated) diabetes for the great majority of individuals with a combination of genetic, immunological and metabolic parameters. Such prediction is possible because of the chronic nature of the autoimmunity and loss of β cell function that precedes the disease. Given the ability to predict type 1A diabetes trials at all stages of the disorder to prevent β cell destruction are now possible.

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Figure 1 illustrates stages in the development of type 1A diabetes beginning with genetic susceptibility and ending with loss of β cell mass (Eisenbarth 1986). With our current inability to directly assess β cell mass over time in individuals, the shape of the curve related to β cell mass is unknown with some investigators favouring multiple exacerbations resulting in progressive β cell loss. There is a limited amount of pancreatic histological data and almost none for 'prediabetic' individuals. We have initiated a program to obtain pancreas from organ donors expressing anti-islet autoantibodies, and hopefully with time this lack of histological data will be rectified (Gianani et al 2006). At onset of diabetes the islet lesions are heterogeneous with some islets normal, others invaded by lymphocytes and still others pseudoatrophic with only non-β cells remaining in islets in the absence of insulitis (Foulis & Clark 1994). It is likely that this heterogeneity of lesions for

This paper was presented at the symposium by George Eisenbarth, to whom correspondence should be addressed.

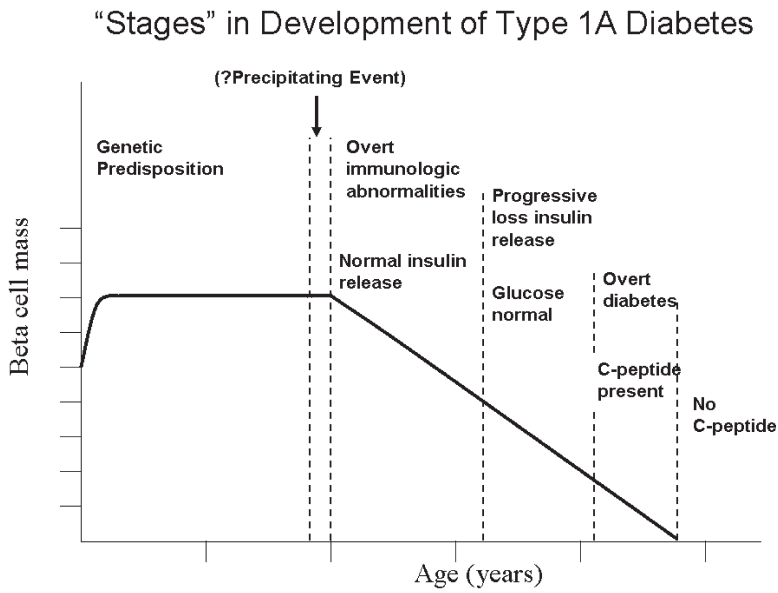

FIG. 1. Stages in the development of type 1A diabetes. Modified from Eisenbarth (1986).

both human and animal models underlies the chronic progressive nature of β cell destruction. Recent reports indicate some preservation of islet β cells in long-term patients (Meier et al 2005), but in general β cell mass is dramatically decreased (>99%) and it will be important to identify exceptions to such dramatic destruction as it may provide evidence of β cell replication or perhaps more likely alternative forms of diabetes.

Each of the stages in Fig. 1 contributes to the prediction of progression to diabetes except for identification of triggering environmental factors that remain poorly defined. This ability to predict is perhaps the best evidence of the chronic progressive nature of the disease.

NOD mouse

The NOD mouse is the most extensively studied animal model of type 1 diabetes. Mice that develop diabetes progress through a series of 'checkpoints' (Andre et al 1996). In NOD mice there is usually a period of approximately three to four weeks preceding the development of insulitis and insulin autoantibodies, followed by primarily peri-islet insulitis and then progressive loss of β cell mass with invasion of islets by T lymphocytes. At the onset of diabetes there is heterogeneity of islet lesions and degranulated β cells are present. With reversal of diabetes with immunotherapy these β cells regranulate and can be stained for insulin (Sherry et al

2006). In the NOD mice insulin appears to be a primary autoantigen. Deleting responses to insulin by inducing tolerance prevents diabetes and eliminates response to Islet Glucose-Related Phosphatase (IGRP) (Krishnamurthy et al 2006). A knockout of the insulin 1 gene prevents 90% of diabetes, while knocking out insulin 2 gene accelerates diabetes. Both insulin 1 and insulin 2 genes are expressed in islets but only insulin 2 is expressed in the thymus. When both insulin genes are knocked out and a mutated insulin interrupting a key insulin epitope (insulin B chain peptide 9–23) diabetes is prevented (Nakayama et al 2005). During the prediabetic phase, similar to human, NOD mice express high levels of insulin autoantibodies, but of note insulin autoantibodies frequently disappear by the time of diabetes onset and some NOD mice progress to diabetes without expressing insulin autoantibodies. In addition NOR mice that rarely develop diabetes but have insulitis and do not progress to diabetes express insulin autoantibodies with a similar time course. Thus islet autoantibodies only partially reflect T cell-mediated anti-islet autoimmunity even for a primary autoantigen such as insulin.

Genetic susceptibility

The genetic susceptibility to type 1 diabetes is determined by genes related to immune function with the potential exception of the insulin gene (Fig. 2) (Todd et al 2007). Even for the insulin gene the polymorphism associated with diabetes risk has been correlated with greater expression of small amounts of messenger RNA for insulin in the thymus (Pugliese 2002). The major determinants of diabetes risk are genes within and/or linked to the major histocompatibility complex (MHC). It is estimated that class II MHC alleles account for 41% of the identified loci determining familial aggregation of the disorder while the insulin gene and PTPN22 (the next most important loci) and all the newer implicated loci account for only 7% additional familial aggregation (Todd et al 2007). This leaves much to be explained and also suggests that most non-MHC genes have a minor influence on the familial aggregation of type 1 diabetes and will not contribute significantly to genetic prediction. We have evidence that major determinants related to diabetes susceptibility are yet to be discovered within and linked to the MHC independent of DR and DQ HLA alleles (Aly et al 2006b). In particular polymorphisms of DP alleles importantly modify risk both for the general population and for siblings with the highest risk DR3/4-DQ2/8 genotype. DR3/4-DQ2/8 siblings who are HLA identical to a diabetic proband have a risk as high as 80% for activation of anti-islet autoimmunity and 60% for diabetes. If such DR3/4-DQ2/8 siblings share only one haplotype identical by descent with their proband, risk is only 20%. One locus we are studying is at the UBD region approximately 3 million base pairs telomeric of DR, but likely there will be a

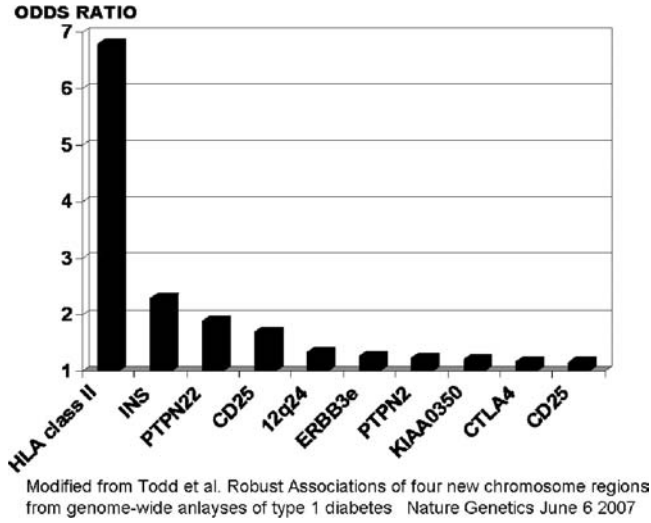

FIG. 2. Odds ratios of a series of genes associated with type 1 diabetes. HLA class II, insulin and PTPN22 known prior to recent whole genome analysis and provide by far the greatest contribution to current known loci contribution to familial aggregation of type 1 diabetes (>90%).

series of new important MHC linked loci, many with effect sizes greater than those reported for current non-MHC loci. The existence of common extended haplotypes where all single nucleotide polymorphisms and all HLA alleles are identical for as much as 9 million base pairs will both hinder (because of extensive linkage disequilibrium making localizing genetic influences more difficult) and potentially aid (allowing major segments of MHC to be fixed and thus ruling out influences of HLA alleles for new loci) discovering these other loci (Aly et al 2006a, Alper et al 2006).

With long-term follow up the majority of discordant identical twins of patients with type 1 diabetes eventually express ant-islet autoantibodies and progress to diabetes, but anti-islet autoantibodies can first appear 30 years after the first twin develops diabetes (Redondo et al 2001). Thus genetic susceptibility appears to persist for life and progression to diabetes is usually preceded by a long prodrome of anti-islet autoantibody expression measured in years.

Diabetes-associated anti-islet autoantibodies

It is now possible with fluid phase radioassays to measure anti-islet autoantibodies reacting with four islet autoantigens (GAD65, IA-2, insulin and ZnT8). (Fig. 3) Expression of ≥2 of the autoantibodies is associated with extremely high risk and are rarely transient (Barker 2006). In the DAISY study which is currently 'biased'

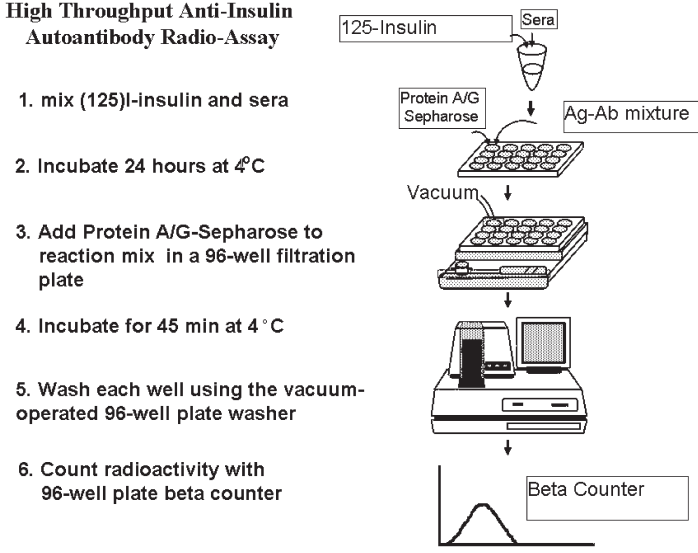

FIG. 3. General format for high-throughput assays for anti-islet autoantibodies utilizing fluid phase filtration separation of antibody bound labelled autoantigen from free autoantigen. In insulin autoantibody assay I^{125}-labelled insulin is utilized, but for other autoantigens such as GAD65 and IA-2, *in vitro* transcription and translation of cDNA is utilized to produce labelled autoantigen. From June 2007 web book Immunology of Type 1 diabetes, ed. George S. Eisenbarth, *www.barbaradaviscenter.org*, with permission.

toward young children (because of following children from birth) 95% of pre-diabetic children express anti-insulin autoantibodies while at onset approximately 50% continue to express insulin autoantibodies. This is similar to the NOD mouse where insulin autoantibodies can be transient in a mouse progressing to diabetes. Nevertheless the same individuals who in their prediabetic phase may have lost expression of insulin autoantibodies usually have acquired other autoantibodies and most often express ≥2 of the four autoantibodies during their pre-diabetic phase. In general populations such as cadaveric donors only 1/300 individuals express ≥2 of the autoantibodies (Gianani et al 2006). When an individual expressed only a single autoantibody less than 30% progress to diabetes with long-term follow up and often single autoantibodies are transient or of lower affinity. In the BABYDIAB study almost no children expressing a single autoantibody progress to diabetes (Schlosser et al 2005). Thus the term 'diabetes associated anti-islet autoantibodies' with the expectation that not all 'positive' autoantibodies are equivalent and only a subset of autoantibodies are associated with high risk. We would suggest that single autoantibodies often represent 'false' positives for assays with 99% specificity but in a disease that only occurs in 1/300 individuals and such 'false' positives are naturally much less common when

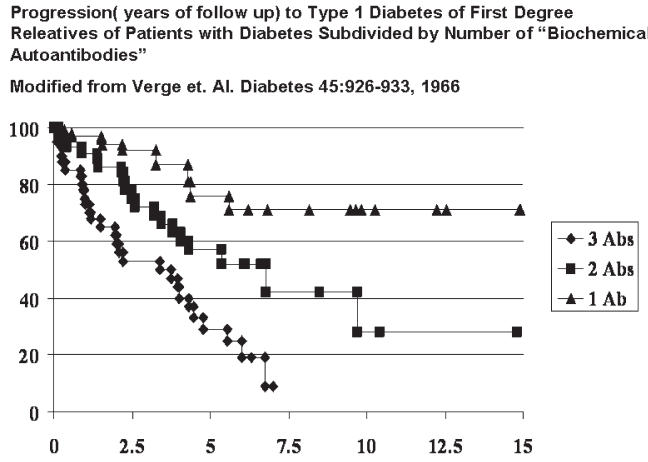

FIG. 4. Relatives of patients with type 1 diabetes subdivided by the number of islet autoantibodies expressed of GAD65, insulin or IA-2(ICA512) versus the percentage not diabetic (Y axis) with follow up in years (X axis).

multiple autoantibodies are present, with each assay having 99% specificity. Almost all individuals progress to diabetes with long-term follow up who express multiple diabetes-associated autoantibodies (Verge et al 1996, Bingley & Gale 2006, Redondo et al 2006) associated with progressive metabolic deterioration and eventual hyperglycaemia (Fig. 4).

Metabolic progression

Data for progressive metabolic deterioration preceding diabetes by years came from studies of identical twins and in particular intravenous glucose tolerance testing of monozygotic twins with a twin-mate with type 1 diabetes (Srikanta et al 1983). The intravenous glucose tolerance test consists of giving a non-physiological stimulus, namely intravenous glucose and measuring first phase insulin secretion as the sum of insulin at 1 and 3 minutes post the glucose bolus. Newer studies of many individuals progressing to diabetes make it clear that it is possible to follow metabolic deterioration with simpler indices. For example progressive increases of HbA1c in the normal range precede development of type 1 diabetes (over years) in the majority of childhood progressors of the DAISY study (Stene et al 2006). Oral glucose tolerance abnormalities precede diabetes and C-peptide secretion on the oral glucose tolerance test is a useful parameter (Sosenko et al 2007, Barker et al 2007). In analysis of Trialnet both intravenous glucose tolerance test abnormalities and oral glucose tolerance test

abnormalities when combined had the highest sensitivity of detecting abnormal metabolism (Barker et al 2007).

There are suggestions that type 2 diabetes is related to type 1 diabetes and in particular insulin resistance contributes to development of type 1 diabetes (Wilkin 2006, Fourlanos et al 2004). The genetic data would indicate that the two disorders are distinct (Todd et al 2007) though insulin resistance (e.g. extreme obesity) has been associated with 'earlier' development of diabetes in individuals with higher fasting insulin, though still with loss of first-phase insulin secretion on intravenous glucose tolerance testing.

C-peptide is cleaved from proinsulin in secretory granules and secreted in a 1:1 ratio with insulin. Thus secretion of C-peptide is utilized as a measure of insulin secretion (Palmer et al 2004). At the onset of diabetes significant C-peptide production is present. The Diabetes Care and Complications Trial (DCCT) has clearly demonstrated the importance of maintenance of even small amounts of C-peptide secretion for prevention of hypoglycaemia with insulin therapy and prevention of secondary diabetes complications such as retinopathy and renal damage (Palmer et al 2004). Thus the importance of trials at the onset of diabetes to maintain islet β cell mass and insulin secretion. In most trials there is a clear inverse relationship between C-peptide secretion and HbA1c. In the honeymoon phase of type 1 diabetes, the ease of insulin therapy and excellent HbA1c is related to recovery of some insulin secretion. Most patients with type 1A diabetes maintain some insulin secretion for years following diabetes onset, and there are reports of some secretion in patients more than 50 years after the onset of diabetes. There are also recent reports of the presence of islet β cells within pancreas of long-term patients with diabetes (Meier et al 2005). The presence of a small number of such cells may be important if they could be expanded. To date, we lack evidence in humans of expansion of these cells even with immunosuppression after pancreatic transplantation that also restores patients to 'euglycaemia', but this is an area of active investigation.

It is likely that anti-islet autoimmunity contributes not only to the development of type 1A diabetes, but also to the common loss of insulin independence in patients following islet transplants and less common loss of function in patients following a pancreas transplant. The presence of anti-GAD65 or IA-2 autoantibodies prior to islet transplantation was associated with loss of insulin independence (Shapiro et al 2006). Patients with the highest risk HLA DR3/4–DQ2/8 genotype more often lose pancreatic transplant function (Stavros et al 2007), and presence of anti-islet T cells correlated with loss of β cell function (Roep 2003). Assays for T cell anti-islet autoimmunity remain poorly standardized, though there is considerable effort in testing, validating and improving such assays so that they can function both in prediction and provide a surrogate marker for trials of prevention of immunological β cell destruction.

Conclusion

Given the ability to predict the development of type 1A diabetes there are now trials underway at all stages of the disease, from children with genetic susceptibility to islet transplantation after almost all β cells have been destroyed. The chronic nature of the destruction after activation of anti-islet autoimmunity provides a window of opportunity for diabetes prevention though it may prove easier to prevent activation of anti-islet autoimmunity than to turn this process off. The earlier an intervention, the lower the certainty that diabetes is developing. The highest reported genetic risk for DR3/4-DQ2/8 siblings sharing both HLA haplotypes identical by descent with their sibling proband is approximately 80% for persistent anti-islet autoantibodies and 60% for progression to diabetes by age 15 (Aly et al 2006b). In that only 15% of patients have a first degree relative with type 1 diabetes and only 30–50% of patients with type 1 diabetes have the DR3/4-DQ2/8 genotype, defining high genetic risk in the absence of a relative is essential. We have evidence that major non-DR and DQ loci in and linked to the MHC remain to be defined that should allow similar genetic prediction in non-relatives.

Once anti-islet autoimmunity is present as evidenced by expression of multiple anti-islet autoantibodies prediction is much more certain and we believe it will be important for the field to define non-progressors both to determine their frequency and to define parameters associated that may naturally limit progression to diabetes. The current ability to predict type 1 diabetes has led to trials for prevention and organizations such as Trialnet and the Immune Tolerance Network are actively enrolling patients, and in North America patients with new onset diabetes and relatives of patients can be evaluated for diabetes risk or trials by calling a central number (1-800-HALT-DM1).

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DISCUSSION

Herold: Could you clarify the rates of progression of diabetes? These details are important for prediction. You showed cumulative insulin autoantibody levels by age. Does this actually happen? We almost need a three-dimensional graph to show progression of diabetes. Do you always acquire more specificities as you progress? The data we have about progression identifies people with multiple antibodies when they are first seen. We don't have the same life table data of seeing one to two to three antibodies developing in a prospective way.

Eisenbarth: We can draw the table of when people first acquire multiple antibodies. Almost all the children we have were first tested at nine months of age. We have the data. If you get one antibody you are very likely to get the next one or two pretty quickly.

Herold: If what you are saying is true, the risk of progression would be almost 100%. Once someone gets one antibody, they will progress.

Eisenbarth: One of the problems with the original data is that they were cross-sectional. The antibody data I showed were coming in from different ages; we weren't following children from birth. If you are 30 years old and have one antibody, you are unlikely to get another antibody. But a six month old child with one antibody will be likely to get the next one or two, if they are progressing to diabetes.

Bonifacio: The insulin antibodies that come up by themselves are coming up at an early age. Almost invariably, these people progress. Sometimes it takes a few years before they get onto the next set of antibodies, other times it will be quicker. If they don't progress, we find that those insulin antibodies are lower affinity, or there is something peculiar about them. They are just not the same as a typical diabetes ones. The antibodies that develop later progress to multiple antibodies

less frequently. If you develop insulin antibodies at the beginning you progress, and some of these children already have multiple antibodies. If the antibodies appear later, we see less progression. We see single GADs that just go on and on as single GADs. We also see diabetes in some of these. The single antibody diabetes cases are rare for us.

Eisenbarth: As we look at individuals we can now measure four different antibodies over time. Let's say that insulin antibodies come up first: they can go away, and others can come up and go away, too. This is a complication. 95% of the children we have followed to diabetes have insulin autoantibodies at some point, but at onset only 50% have these autoantibodies. It is good that we have the multiple assays. The cross-sectional studies are taking children at any age and we have no idea what happened at the earlier time points with these children. Some of them with one antibody could have had three earlier on.

Roep: What is the proportion of patients at diagnosis that have a single antibody?

Eisenbarth: About 15% have no antibodies, and about 60–70% have multiple antibodies.

Flavell: What is going on with the T cell responses?

Eisenbarth: I have no idea.

Kay: How could you distinguish between a false positive and something that is biologically meaningful about an autoimmune response that develops but does not progress?

Eisenbarth: Rarely, it is easy. One example: a person had an antibody to iodinated insulin. This was a false positive, because we use iodinated insulin in our assay. The endpoint is did the person develop diabetes or not? If we can get an assay that better predicts who develops diabetes, we might want to throw away certain results, such as low affinity anti-insulin antibodies. Until we get into the pancreas and insulitis our assays will only partially likely correlate with predictive antibodies.

Insel: Could the loss of antibody responses be due to killing of the B lymphocytes by T cells?

D Hafler: Sure. That would be interesting to look at because of the affinity maturation and the recognition of particular peptides of insulin.

Eisenbarth: I don't think it is that common for antibodies to come back up.

Bonifacio: We certainly can see insulin antibodies go down and come back just before onset. But what we see is hypervariable with relation to diabetes. In one child, we saw all three antibodies rise more than 10-fold three months before diabetes onset. Then all but one disappeared prior to diabetes onset.

Kay: In Peter's Achenbach studies are affinity and titre related at all?

Bonifacio: Most things are related to titre, from what we can tell, except for affinity. We can have high titre low affinity antibodies. These low affinity antibodies

are peculiar. They range from cold IgM antibodies to IgA antibodies, and there are some that react better with chicken insulin than human insulin, for example. The insulin antibodies that are associated with diabetes progression are homogeneous in what they see. They are all high affinity and react with proinsulin. They seem to have a similar pattern when they are competed against different insulins.

D Hafler: What is the epitope?

Bonifacio: It is hard to tell, but the antibodies are dependent on having the right amino acids in the A chain 8–13 regions.

Santamaria: This raises the question about your insulin 1 and 2 knockouts. When you transfer islets from an early SCID into a double knockout with a transgene, is it possible that these animals make autoantibodies so fast because they are not tolerant to insulin 1 and 2?

Eisenbarth: There is one amino acid difference in the insulin, and they come out in a two week period.

Santamaria: This doesn't mean that would be the case in a mouse that only expresses the wild-type insulin, because that amino acid difference may affect the ability of the autoantibodies to bind. The autoantibodies are raised against numerous epitopes on insulin, presumably.

Bonifacio: It is pretty homogeneous. I would say there aren't a lot of epitopes.

Santamaria: Can your transgene elicit the same type of antibodies, in terms of affinity and titre, to wild-type insulin. In your novel knockout deficient mice there is no B cell tolerance, and presumably it would be easier to raise autoantibodies in these mice.

Eisenbarth: In a Balb/c mouse we can take these peptides and do the same thing. If we take a normal Balb/c mouse and immunize with B9–23, within two weeks insulin autoantibodies develop. They can't be absorbed by the peptide. No other peptide of insulin does this for us. We can also take a sequence with the B16 alanine, and in mice it can raise antibodies.

Roep: Now that all these general population cohorts are progressing, do you see the same sequence of events in terms of appearance of antibodies as you see in the ascertainment in children of diabetic parents?

Eisenbarth: I don't think I can tell the difference between someone who is a relative of someone with type 1 diabetes and someone from the general population, except in the magnitude of risk.

Bonifacio: Olli Simell in Finland now says that the data are similar.

Roep: When I asked him he said it was a mixed bag.

Bonifacio: He says it is predominantly insulin and it can be others. But we also say it can be others. We sometimes see small children come up with GAD first.

Roep: This brings me to the HLA haplotype sharing: I predict it is HLA, not MHC. Susceptibility is limited to HLA class 1 and 2.

Eisenbarth: We have done conditional logistic regression. We are now looking at this UBD locus. The best distinction we can make is that the UBD locus adds to disease prediction above HLA alleles. It turns out that there are extended haplotypes of 9 million base pairs for some individuals. If you ask for someone to be DR3 A1 BA, 80% of those chromosomes are identical for every SNP. For some individuals they are for 9 million base pairs; for other individuals for 4 million base pairs are identical on extended conserved haplotypes. If we take these 8.1 haplotypes and do a genome screen using SNPs, we get a good signal increasing risk with certain SNPs. We think there is another locus in the MHC for the 8.1s.

D Hafler: Could it be outside the MHC somewhere else on chromosome 6 that is also being inherited?

Eisenbarth: Yes. This includes the whole population, not just sibs. There is another locus probably out at PRSS. Within 50 cM there could be an influence. There is enough potential for recombination if you get far enough away from DRDQ that the estimate that only 41% of the familial aggregation of type 1 diabetes is due to the MHC might not be right.

D Hafler: This is different from MS where the odds ratio of MHCs is about 2.

Kay: Does the decline in the insulin autoantibodies that is sometimes seen bother you in terms of autoantigen-specific therapy? Do you think that insulin might be a crucial initiating antigen but not a good candidate for therapy?

Eisenbarth: I wouldn't want to extrapolate from what the antibodies do. We have data from insulin knockout mice that transfer disease. If we take islets from the insulin knockouts and transplant them into the NOD mouse they will be wiped out. Islets are susceptible even though they don't have this sequence. But if we take a pancreas with the double knockout islets from a SCD and transfer to new-onset NOD then we see a big difference. More than half the mice don't get diabetes at all, whereas mice with normal sequences will always get diabetes relatively quickly.

D Hafler: We have been doing some work on cloning directly from the draining lymph nodes of diabetics in terms of insulin reactivity. We continue to see a fair degree of insulin reactivity among the clones we generate by single cell cloning from these nodes reacting to the insulin 1–15 chain. We have not seen the same degree of clonal expansion that we saw in the two long-term diabetics. Also, one of the main concerns and criticisms that we and others have of the work is that the amount of peptide required to get a response is quite high. When the insulin peptide is reduced, releasing sulfhydryl bonds, the reactivity goes up quite significantly. So now we are getting much more at lower doses (100 μg/ml peptide). I don't know whether this has any relationship to the disease, but by the same method we have not seen any reactivity to GAD.

von Herrath: When I look at these islet antibodies, as they increase in time and some of them go away, if this is compared with viral responses the nature of these

responses when they affect different antigens is maybe cyclical. This could argue for viewing T1D as a relapsing remitting syndrome, where a cycle of islet attack driven by one antigen or epitope is followed by immune regulation and recovery, until the autoaggressive response is reinitiated by T cells recognizing a different antigen or epitope. Such processes are not linear, which is the response type seen when lots of epitopes are targeted at the same time.

Eisenbarth: My comment for David Hafler is that I don't think MS is relapsing remitting that much.

D Hafler: I agree: I think it is relapsing, but it is not remitting. It is like a stroke—there is an embolism that goes to the brain, a lesion and oedema, and when the oedema disappears most stroke patients get better, but we don't call this remission. I think MS is similar to type 1 diabetes where there is a constant influx of lymphocytes into the organ in a patchwork type distribution. They set up shop and begin to cause inflammation.

von Herrath: But in MS there are single lesions that disappear.

D Hafler: Pathologically, there is always some damage.

Bonifacio: George, you said that there were different rates of diabetes development. What is determining this?

Eisenbarth: I don't know. In part, the younger you develop the antibodies, the faster the rate, on average. This correlates with having this DR34DQ8 genotype. Rates for MZ twins can differ. If one twin develops diabetes after age 25, the risk of the other might only be 10%. Before age 10, it is a very high risk.

Roep: Have you looked at HLA in those twins?

Eisenbarth: It is very high.

Flavell: What is known about the age of development of Tregs in humans?

D Hafler: If we look in cord blood we can find them.

Atkinson: It's a hard question to answer because when people store their cord, they are doing it for a purpose. In newborns, Tregs are different from adult Tregs. You start out life with potent Tregs.

Roep: There is an increase in the number of regulatory T cells with age in humans.

Atkinson: That increase with age is in the number of these intermediate population Tregs.

Current and past prevention and intervention trials in type 1 diabetes

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Abstract. Halting the autoimmune attack on β cells by redirecting or dampening the immune system remains one of the foremost therapeutic goals in type 1 diabetes (T1D). Progress in the field has been slow due to important ethical considerations. Namely, side effects from excessive immunosuppression cannot be tolerated because of the reasonable life expectancy with insulin substitution therapy. Nevertheless, we have now learned a significant amount from past prevention and intervention trials, which allows us to plan and design better interventions and preventions for the future. This article will summarize the existing experience and explain the prioritization of future approaches based on JDRF's analysis including novel combination therapies for T1D.

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Type 1 diabetes (T1D) is one of the most common autoimmune diseases affecting almost 20 million people worldwide (Steck & Rewers 2004). During pathogenesis, insulin-producing pancreatic β cells are progressively destroyed by autoreactive $CD4^+$ and $CD8^+$ T cells. Clinical manifestation characterized by an increase in blood sugar levels occurs after approximately 80% of the insulin production capacity has been lost (Atkinson & Eisenbarth 2001). In the past two decades, immunemodulatory approaches to prevent or cure T1D have been developed and tested with some encouraging recent results. Development of a cure for T1D has been particularly difficult, because insulin-substitution affords a reasonable life quality and expectancy and the disease frequently affects young adults and children. Therefore, the ethical window for any treatment is rather small and long-term side effects have to be avoided. On the other hand, insulin cannot prevent all of the late complications of diabetes, and the life expectancy is usually reduced by 10–15 years.

It is known that systemic immunosuppression, for example with cyclosporin, can halt β cell destruction (Carel et al 1996). However, the protection only lasted as long as the drug was present; long-term immunological tolerance to β cell

antigens (Ags) was not achieved and extended therapy was not feasible due to side-effects. In contrast, one much more promising intervention tested clinically during the past five years has been the application of non-Fc binding anti-CD3ε antibody (Ab), engineered as a $F(ab')_2$ fragment of hamster anti-CD3ε (145-2C11) for preclinical studies or as a fully humanized IgG1 (hOKT3γ1[Ala-Ala]) for human trials (Chatenoud & Bluestone 2007). Although its mechanism of action is not yet fully understood, a decrease in the number of auto-aggressive T cells together with an expansion of a $CD4^+$ regulatory T cell (Treg) population expressing the α-chain of the interleukin (IL)2 receptor (CD25) relying on transforming growth factor (TGF)β have both been demonstrated following short-course treatment with anti-CD3 in NOD mice (Chatenoud 2005). Thus, such Tregs might explain why mice treated after recent onset were completely protected for life after anti-CD3 treatment and that, in two independent trials in humans, C-peptide was preserved for 18 months following a short-course treatment (Herold et al 2002, Keymeulen et al 2005). However, C-peptide began to decline after 18 months indicating that permanent tolerance to β-cell antigens had not been achieved. Therefore, efficacy needs to be enhanced. Safety concerns will prevent increasing the human anti-CD3 dose, since temporary EBV reactivation was seen in most individuals in the recent European anti-CD3 trial, and other options will need to be explored. One promising avenue is the β cell antigen-specific induction of Tregs.

Immunization with islet autoantigens, by various means and routes, can induce islet antigen-specific Tregs and prevent T1D (Homann et al 1999). These 'autoreactive' Tregs can act as 'bystander suppressors' and inhibit site-specifically heterologous autoreactive immune responses. For example, transferred insulin B-induced Tregs selectively proliferated in the peripheral lymph nodes (PLNs), where their cognate Ag is being presented by antigen presenting cells (APCs) during diabetes development. There, they were capable of dampening auto-aggressive CD8 responses (Homann et al 1999). This suppressive effect was associated with IL4 and IL10 production by the Tregs. Thus, antigen-specific induction of Tregs can result in long-lasting tolerance to β-cell antigens mediated by local immune modulation in the PLNs, which makes this intervention safe with low potential for side-effects. However, numerous tests in animal models show that the efficacy is limited and prevention of diabetes is only seen when the immunization is given during the pre-diabetic phase. Therefore, antigen-specific interventions will likely need 'help' to be used successfully in humans, especially in recent-onset diabetics (Staeva-Vieira et al 2007).

Prevention trials

To identify suitable subjects at risk, genetic testing combined with screening for islet specific autoantibodies needs to be performed. The resulting cohort of patients

exhibits various degrees of T1D risk depending on the number of autoantibodies to islet antigens and their genetic predisposition. Prevention trials are hard to conduct, expensive and allow for not much more than 2–3 arms because of timeline and recruitment issues. In addition, safety considerations are of particular importance, given that individuals who may never develop disease may be treated.

Completed prevention trials

Antigen specific. Several trials using autoantigens to induce regulatory islet specific T cells following mucosal immunization have been completed (Table 1) (Staeva-Vieira et al 2007). The results are disappointing, despite the fact, that all of these interventions had proven to be successful in preventing T1D when given to prediabetic NOD or other T1D animal models. The reasons for these apparent failures could be manifold. Currently, there are no reliable means of tracking autoantigen-induced Tregs in the blood and therefore we cannot directly determine the immunological reasons for these failures. In addition, the dose of oral or nasal insulin used may have been too low. Finally, the mucosal antigens may only be effective when administered early during pathogenesis and individuals enrolled may have already progressed too far on their way to develop T1D.

Antigen non-specific. Several systemic immune modulatory approaches have been tested for the prevention of T1D, based on promising data in the NOD mouse model (Shoda et al 2005). Unfortunately, none of them have brought success,

TABLE 1

Agent	*Route*	*Development Stage*	*Year Completed*	*Efficacy*	*Principle Investigator*
Insulin	Parenteral	Pilot	1993	Positive trend	Jackson, Eisenbarth
Insulin	Parenteral/s.c.	Pilot	1998	Positive trend	Ziegler
Insulin (DPT1)	Parenteral	Phase II	2002	None	DPT1 Study Group (TrialNet)
Insulin (DPT1)	Oral	Phase II	2005	No overall Subgroup*	DPT1 Study Group (TrialNet)
Insulin (INIT I)	Intranasal	Phase I	2004	No T1D accel. Immune changes	Harrison/ Colman
Insulin (DIPP)	Intranasal	Phase I	2007	No effect	Simell

TABLE 2

Agent	*Route*	*Dev Stage*	*Year*	*Outcome*	*Investigator*
Ketotifen (histamine antagonist)	Oral	Pilot	1994	No Effect	Bohmer
Cyclosporine	Oral	Pilot	1996	Delay; No Prevention	Carel
Nicotinamide	Oral				
DENIS		Efficacy	1998	No Effect	Kolb
ENDIT		Efficacy	2004	No Effect	Gale
Various combinations		Pilots	'94–'05	No Add Eff.	Dahlquist/ Bonifacio/ Parent
Bacille Calmette-Guerin (BCG)	i.d.	Pilots	'95–'05	No Effect	Dahlquist/ Bonifacio/ Parent
Dietary Gluten Elimination	Oral	Pilot	2002	No Effect	Ziegler

which might be due to the fact that NOD mice and humans exhibit significant differences in their immune reactivity profiles. It is in general much easier to completely abrogate diabetes in the NOD model, and over 300 strategies have been documented to be successful to date. Maybe it is not surprising that none of them have translated to humans so far. Probably more stringent experimental settings are needed and more than one animal model should be tested to ensure translatability. Notably, vaccination with BCG (Bacillus Calmette Guerin, tuberculosis vaccine), although highly efficient in preventing T1D in the NOD, showed no effect on human T1D. These observations are disappointing, because all of the interventions listed in the adjacent table have essentially no side effects and would have been well-suited for long-term application (Table 2).

Ongoing/planned prevention trials

Antigen specific. Despite these failures, new prevention trials have been planned (Table 3). Noteworthy is the repetition of the oral insulin (DPT1) trial in individuals at high risk for developing the disease, where a small effect had been noted in the previous trial upon post-trial segregated data analysis (Skyler et al 2005). The pre-point trial attempts to address two main concerns of previously unsuccessful oral insulin prevention trials: it will be conducted very early during pathogenesis in children at risk and the oral insulin dose will be, in some of the arms, up to 10-fold higher than in the DPT1 trial.

TABLE 3

Agent	*Route*	*Development Stage*	*Investigator*
Insulin (INIT II)	Intranasal	Phase II	Harrison/Colman (DVDC)
Insulin (DPT1 repeat)	Oral	Phase II	DPT1 Study Group TrialNet
Pre-POINT	Oral & Intranasal	Phase I/II	Bonifacio (JDRF)

TABLE 4

Agent	*Route*	*Dev Stage*	*Year*	*Investigator*
COMPLETED STUDIES				
DiaPep277	s.c.	Phase II	'99–'06	Cohen
NBI-6024 (Insulin APL)	s.c.	Phase I	2006	Gottlieb
Ins B chain & IFA	s.c.	Phase I		Orban (ITN)
ONGOIGN STUDIES				
PI peptide Vaccine	i.d.	Phase I		Peakman/Dayan (DVDC)
AlumGAD65	s.c.	Phase II		Diamyd
PI DNA vaccine (BHT-3021)	i.m.	Phase I		Bayhill Therapeutics

New onset trials

Completed vs. ongoing/planned

Antigen specific. Interventions after diagnosis, in 'recent-onset' T1D, have proven to be successful, when only lesser amounts of remaining β cell mass are available. Therefore, the bar is set higher and the aggressive immune response destroying islets has to be dampened more rapidly and strongly. Only a few antigen specific interventions have shown success in recent-onset diabetes in animal models (as listed in the adjacent table), and these were tested in humans. Trials with heat-shock protein peptides (hsp277) (Raz et al 2001, Lazar et al 2007) and altered insulin B chain ligands (Nurocrine, NBI-6024) (Alleva et al 2006) have brought no success. In these cases, no animal data supported the application after onset of hyperglycaemia. Insulin B chain administered together with IFA has shown good promise in preventing NOD T1D, and has shown indication for Treg generation in peripheral T cells administered to human diabetic patients (Orban, unpublished). Efficacy is unknown and will now be assessed after successful completion of a phase I safety trial (Table 4).

TABLE 5

Agent	*Route*	*Dev Stage*	*Investigator*
Hydrolyzed cow's milk (TRIGR)	Oral	Phase II	Akerblom
Vitamin D	Oral	Phase I	Taback (CDA)
Docosahexaenoic acid (DHA) NIP Diabetes Pilot	Oral	Pilot	Chase/Clare-Salzler (TrialNet)
hOKT3g1(Ala-Ala) (anti-CD3)	i.v.	Pilot	Herold (TrialNet)

Antigen non-specific. Many systemically acting immunological compounds have been tested in recent-onset T1D (Tables 5, 6). Among these, anti-CD3 antibodies have provided the most promising data, where C-peptide decline was halted for over 2 years in two independent trials in Europe and the USA. Thereafter autoimmunity recurred and loss of β cell mass paralleled that in the untreated group. ATG (anti-thymocyte globulin), has shown similar short term efficacy in animal models (Simon et al 2007), but did not exhibit the same positive effect on regulatory T cells (Bresson & von Herrath, unpublished). Bresson and von Herrath have proposed that the ability to induce Tregs will distinguish those systemically acting immunosuppressants that can be beneficial long-term from those which will only provide short-term relief.

A promising new compound that can induce regulatory function is anti-CD20, which has been shown to induce regulatory B cells in humanized mouse models (Hu et al 2007). In addition, immature dendritic cells might promote long-term immune regulation. Similarly, autologous cord blood transfusion might augment the number of Tregs.

Combination trials

In order to reduce side effects and maximize therapeutic efficacy, some have proposed combining several compounds (systemically acting or antigen specific). If the single drugs have different modes of action, additive effects can be expected. If in addition they converge on promoting Tregs, Tregs could be augmented significantly and true synergy could occur, as this has been described for the combination of anti-CD3 and mucosal islet antigen administration (Bresson et al 2006) and for the combination of rapamycin and IL2 (Rabinovitch et al 2002), which can both augment Tregs. Other combinations that make logical sense are systemic immune modulators and compounds that enhance β cell function or generation, for example GLP1 agonists such as exenathide (Table 7).

TABLE 6

Agent	*Dev Stage*	*Year*	*Efficacy*	*Investigator*
Cyclosporine	Multiple trials	'84–'96	Remission Side effects	Stiller, Bach, Filippo
ATGAM + prednisone	Pilot	1985	Ins requirements Side effects	Eisenbarth
Nicotinamide	Pilot	1991	No effect	Chase
BCG	Pilots	'94–'99	No effect	Lafferty Elliott, Chase
Diazoxide	Pilot	'96–'04	No effect	Karlsson, Ortqvist
IFNα	Pilot	2001	possible effect	Brod
hOKT3g1(Ala-Ala) (anti-CD3)	Phase I	2002	Remission out to 18 months	Herold (ITN)
ChaglyCD3(TRX4) (anti-CD3)	Phase II	2005	Remission out to 18 months	Chatenoud (JDRF)
AHST, ATG, Cyclophosphamide	Phase I/II	2007		Burt/Voltarelli
hOKT3g1(Ala-Ala)	Phase II	Herold		ITN/TrialNet
hOKT3g1(Ala-Ala) 4–12 mo after diagnosis	Phase II	Herold		NIDDK/JDRF
Probeta hOKT3g1(Ala-Ala)	Phase II/III			MacroGenics
ChAglyCD3(TRX4)	Phase II/III			Tolerx
ATG (Fresenius)	Phase I	Saudek		
***ATG** (Genzyme)	Phase II	Gitelman		ITN
Anti-CD20 (Rituximab)	Phase II	Pescovitz		TrialNet/ITN
Transfusion of autologous cord blood	Phase I	Haller/ Schatz/ Atkinson		JDRF
Autologous gene-engineered DCs	Phase I	Trucco		NIDDK
***Anakinra (IL1-RA)**	Phase I/II	Mandrup-Poulsen		JDRF
***immature mDCs**	Phase I	Clare-Salzler		

*Soon to be launched

Immunotherapeutic pipeline prioritization

In the spring of 2007, JDRF convened an expert panel to discuss prioritized potential immunotherapies for use in T1D. The major considerations in the prioritization process are summarized in the following table. Safety, preliminary clinical data and availability of the drug were the main decisive issues (Table 8).

TABLE 7

Agent	*Dev Stage*	*Investigator/Company*
MMF/DZB	Phase II	Gottlieb (TrialNet)
E1-I.N.T. (EGF-1 and Gastrin analogs)	Phase II	Transition Therapeutics
IL2 and rapamycin	Phase I	Rabinovitch/Greenbaum (ITN/TrialNet)
Anti-CD3 and intranasal insulin	Phase II	Herold/von Herrath/Bluestone (DVDC)
Anti-CD3 and exenatide	Phase II	Herold/Greenbaum (TrialNet)

TABLE 8 Considerations in Prioritization Process

1. **Rationale**
   - Compelling scientific rationale for use of therapy in T1D
   - Sufficient preliminary clinical data
   - Requirement for additional pre-clinical data
   - Consider combination therapies with another drug
2. **Application**
   - Availability of drug
   - Source of drug; any IP issues
   - Advantages to drug/therapy compared to other therapies
   - Major challenges
3. **Safety**
   - Availability of sufficient safety data
   - Existence of regulatory approval
   - Any major SAE

Potential next steps for T1D immunotherapies

Table 9 depicts the prioritization list generated by the JDRF-convened expert panel during the meeting of spring 2007. The compounds listed as 'ready for clinical translation' have fewer concerns for side effects. Interestingly, the Immune Tolerance Network has generated a very similar list, but included ECDI-mediated tolerance and the 'power mix' (Zheng et al 2003; anti-IL2 and anti-IL15). In conjunction with the JDRF combination therapies will be explored. Compounds suitable for combination are listed in Table 10. These will ideally consist of the following components: (1) systemically acting compounds that suppress aggressive autoimmunity and ideally preserve or augment Tregs; (2) compounds that can promote β cell regeneration or preserve islet cell function; and (3) agents that induce or enhance islet-specific Treg activity.

TABLE 9 Immunotherapy Prioritization Outcome Top Five Monotherapies in new onset T1D

Ready for clinical translation
- Alpha-1 antitrypsin (AAT)
- Anakinra (IL1-RA)
- Anti-CD22 (needs more safety data)
- Polyclonal Tregs (wait for safety data from GVHD)

Need additional pre-clinical development
- Cord blood
- Fixed cell therapy (ECDI, multiple Ags)
- Antigen specific Treg, designer Treg, DC cell therapy
- Anti-CD45RO/RB—evaluate transplantation data first
- Anti-LFA1 (Raptiva)–toxicology data; lack of enthusiasm due to safety profile; transplantation data
- Anti-CD40 (need more safety data)
- Anti-CD137
- NKG2D
- TRX1 (anti-CD4)

TABLE 10 Immunotherapy Prioritization Outcome Top Five Combination Therapies for T1D

1. **Reducing (memory)-effector response, promote long term tolerance**
   - Anti-CD3 (#1 efficacy, decrease dose ideally)
   - ATG (enhances Treg function in the NOD, wait for mono-trial)
   - Anti-CD20 (wait for monotherapy outcome)
   - Rapamycin (has not been in recent-onset trial, wait for IL2 Rapa trial)
   - Targeting the CD40 pathway
2. **Beta cell preservation—replication**
   - Exenatide
   - Gastrin, GLP-1, E1-INT
   - Anakinra (IL1-RA)
   - Intensive insulin therapy
   - Inhaled insulin
   - TZD
3. **Antigen-specific(?) immune regulation**
   - Insulin (oral, nasal)
   - Treg cell therapy
   - DC therapy (Ag pulsed)
   - Fixed cell therapy (ECDI, Ag specific)
   - AAT
   - Vitamin D3

Future directions

The research community needs to focus on developing β cell antigen-specific immunotherapeutics and novel approaches for their delivery to induce durable and safe immunoregulation that when combined with β cell regeneration therapeutics will 'cure' established type 1 diabetes. Second, developing therapeutic approaches that prevent progression of immune-mediated insulitis to insulin-dependent diabetes in at-risk populations will be crucial. Last, devising safe therapeutic and preventive candidates and approaches for universal use in infancy to prevent type 1 is of high importance.

To achieve these goals, strategies in trial design have to start on a broad base with several compounds simultaneously ('reverse pyramid'), which should be combined early on in the disease process, if safety allows. Lead investigators for approaches on the prioritized list have to be identified, and FDA liaisons have to be created early on.

Many challenges remain. Effective translation of dosing and scheduling regiments from mouse to humans has to be established. The ability of animal models to accurately predict therapeutic outcomes in patients has to be carefully assessed and improved—perhaps with the use of humanized mice. Biomarkers for clinical response and efficacy have to be established. Better understanding of the immunopathogenesis of human T1D needs to be gained. The risk–benefit between safety and efficacy has to be carefully evaluated. Most importantly, interactions between Investigators, Industry, and Funders need to be leveraged in order to hasten the identification of effective T1D immunotherapies and expedite their development. For prevention trials in particular, T1D may need to be redefined to acknowledge that double islet antibody positive individuals with impaired glucose tolerance have 'diabetes' that requires early intervention. Sensitive, specific, inexpensive, and nononerous sampling approaches (e.g. saliva) will need to be developed in order to detect β-cell specific antibodies and allow population-based, cost effective detection of type 1 diabetes.

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DISCUSSION

Pipeleers: I fully back the focus on translation. Our team has been fortunate to receive JDRF funding for clinical trials which otherwise would not have been possible. This might not be specific for Belgium but quite general in Europe.

I understand from your presentation that the current JDRF road map has been greatly influenced by structures in the USA. Does it also take into account the structures, obstacles and opportunities in Europe? Will it imply that clinical trials will be more and more designed centrally in the USA or will European centres continue to have the chance to conduct trials according to their ideas?

Insel: The answer is yes to both questions. We are worldwide in our orientation. JDRF spent time in Europe recently looking at clinical trial infrastructures, registries and regulatory issues, to try to understand the European side of things. We are interested in conducting trials outside the USA where there may be advantages because of cost, and better defined populations and ready access to these populations. We are very interested in investigator-initiated trials. In the last 6 months, much of our activity has been focused on three new trials that were investigator initiated. We have been setting up clinical trial optimization committees to work on the clinical trial protocols that include clinical trial specialists, statisticians, and ethicists that work with the principal investigators (PIs). Some of the investigator-initiated trials are using drugs already on the market approved for other diseases. There will continue to be opportunities for investigators to come forward with clinical trials. The third point is that we are also interested in small, proof-of-concept clinical trials. This is where we see our specific niche. We think we can bring value and take risk out of product development by conducting early proof-of-concept clinical trials. If successful, it lowers the barrier for others to take on product development.

Staeva: Also I want to point out that it is not a rigid process; we are open to new ideas. One of the funding mechanisms at JDRF will continue to welcome unsolicited, innovative proposals from investigators.

D Hafler: I'd like to emphasis the importance of training, particularly with the lack of medical scientists coming through the pipeline. Physician scientists are a dying breed.

Insel: I wholeheartedly agree. We are concerned about this, but we are not sure how to fix it.

Butler: One of the themes that comes across is cross-fertilization. This sort of meeting is a great opportunity in this regard. Those of us who are not immunologists are terrified of the nomenclature, even though we are interested in the work. One opportunity might be to have a forum where immunologists train other scientists in a workshop. The emphasis would probably be on postdocs. Also, as a clinician I see spectacular things happening with rheumatoid arthritis, Crohn's disease and ulcerative colitis, but why isn't the same sort of progress being made with type 1 diabetes? Perhaps we need to meet with them to see what we could learn from what they have been doing.

D Hafler: This is exactly what the Federation of Clinical Immunology meeting does.

Staeva: JDRF has recently released an RFA (request for applications) on common mechanisms in autoimmune diseases. The goal of the RFA is to foster interactions between people in various autoimmune disease areas, in order to understand type 1 diabetes better. Regarding the interactions between β cell biologists and immunologists in type 1 diabetes, we at JDRF are starting to think about a potential

initiative that would foster collaborations between these two groups. This will likely be jump started with a workshop.

Kay: This is partly a circular problem related to the progress of the field. In the time we have been practising medicine disciplines such as rheumatology and renal medicine have become highly focused on immunology, whereas in endocrinology people are still giving thyroxine for hypothyroidism and insulin to regulate blood glucose: there is an entirely metabolic focus. Even though endocrine autoimmunity has been a focus for a long time, it hasn't got into the consciousness of people. We battle with this in the transplant area, where there is a big gap between the regular endocrine treating community of people looking after patients with type 1 diabetes and people who are strong proponents of islet transplantation. It is a problem for the field.

Peakman: What is the timescale for your drug pipeline? Do you envisage 10 trials running concurrently?

Staeva: We will probably try to focus on 3 or 4. We can't do all 20. There is no set number. It will be partly determined by resources—both financial and access to patients, which is a bottleneck.

Peakman: I wouldn't have thought of access to patients as a bottleneck: there are hundreds of thousands of them.

Insel: Several companies are planning to launch phase III trials that will enrol hundreds of patients, including trials with anti-CD3 and GAD. We are attuned to the issue of competition for new onset patients.

Butler: To add to your list of partially positive trials, one which was positive was diazoxide.

Insel: Karlsson and colleagues from Sweden demonstrated that a short course of diazoxide in new onset type 1 diabetes temporarily preserved residual functional β cells.

Butler: There was a study published a few years ago (Björk et al 1996), which was one of the most impressive preservations of C peptide in type 1 diabetes. This has been done and it worked. It may not have prevented diabetes, but it preserved β cell function better than most of the ongoing efforts. Novo Nordisk recently produced a new diazoxide-like compound that doesn't make your blood pressure drop or make you hairy.

Insel: There are some companies in the USA also working on this.

Bonifacio: You touched on the alternative hypotheses to autoimmunity. The first one you focused on, stress and modified β cell protein, I always find attractive except when I think about the transplant model and twin model, where you put in islets without this defect and which are producing proteins that are not modified post-translationally, and these are being knocked out. Any model has to include some sort of autoimmune component, unless you are proposing that the bit of pancreas that is being transplanted is now super-stressed and not working properly.

Insel: β cells that have been isolated and then transplanted are stressed and susceptible to apoptosis. Newly regenerated β cells may also be especially susceptible to β cell stress.

Bonifacio: Not when the whole organ is transplanted.

Roep: If you do auto transplantations into the non-autoimmune background the prospects are far greater.

Bonifacio: The other issue was the α1 antitrypsin and inflammation. I thought this deserved a bit of discussion here.

Atkinson: α1-antitrypsin is a serine proteinase that is also an acute phase reactant. It is clearly anti-inflammatory, yet a whole series of additional effects have been associated with it. There is a human clinical disorder called α1 antitrypsin deficiency. These individuals make a mutated form of the molecule, and they are treated by α1 antitrypsin derived from blood donors. The use of blood-derived product is required since this can't be made recombinantly in an effective therapeutic means such as insulin or factor IX can. α1 antitrypsin is available, safe and has low toxicity. So the question becomes one of, 'Why use it in diabetes?' There is a series of papers (Song et al 2004, Lu et al 2006) on overexpression of α1 antitrypsin in NOD mice preventing the disease. Charles Dinarello also has data in allotransplant models showing that α1 improves transplant outcomes (Lewis et al 2005).

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CD8 and cytotoxic T cells in type 1 diabetes

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Abstract. The intra-islet cellular infiltrate found at post mortem in patients with new-onset Type 1 diabetes mellitus (T1DM) comprises both $CD4^+$ and $CD8^+$ cells, yet very few studies have investigated CD8 T cell responses to islet autoantigens. We therefore examined the response of peripheral blood CD8 T cells from new-onset T1DM patients and control subjects possessing HLA-A*0201 genes to potential CD8 T cell epitopes contained in a panel of peptides derived from proinsulin, glutamic acid decarboxylase, islet-specific glucose-6-phosphatase catalytic subunit-related protein and islet amyloid polypeptide, each putatively presented by the HLA class I molecule, HLA-A2.1 (A*0201) using a variety of techniques including *in vitro* culture with peptide, enzyme-linked immunospot (ELISPOT) assay and HLA tetramers. We find CD8 T cells present using these techniques, some of which have cytotoxic activity. The demonstration that rare islet autoreactive CD8 T cells are detectable in blood should promote mechanistic studies on these cells, as well as advancing T cell assay development.

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It has become widely accepted that type 1 diabetes is an autoimmune disease that is characterized by the activation of T cells recognizing islet autoantigens (Roep 2003). Although it is often assumed that these autoreactive T cells are the mediators of islet β cell damage, direct evidence for this is only available from rodent models of autoimmune diabetes in which adoptive transfer experiments are feasible (Utsugi et al 1996, Wong et al 1999). In human, the assumption rests predominantly on observations such as the T cell-dominated islet infiltrate found at diagnosis (Bottazzo et al 1985); the transiently beneficial effect of T cell inhibitory therapies such as cyclosporine (Bougneres et al 1990) and monoclonal anti-CD3 antibody (Herold et al 2002, Keymeulen et al 2005); and case reports of the emergence of type 1 diabetes in recipients of bone marrow grafts from diabetic donors (Herold et al 2002, Keymeulen et al 2005, Lampeter et al 1993, 1998). There is, therefore, a continued need for the careful characterization of islet

autoreactive T cells in human, with the aim of matching their phenotype and function to those expected of pathogenic effectors, as well as enabling the re-enactment of pathogenic scenarios *in vitro*, or, in the future, in humanized murine models.

With this in mind, it is notable that the majority of human studies to date have focused on the analysis of CD4 T cell responses. A recent review documented 156 well characterized CD4 T cell epitopes derived from human islet autoantigens, compared with as few as 20 for CD8 T cells (Di Lorenzo et al 2007). Very few previous studies have attempted to directly assess CD8 T cell islet autoreactivity *ex vivo* (Pinkse et al 2005, Toma et al 2005). This imbalance is presumably a reflection of the greater ease with which CD4 T cell responses against whole antigens or overlapping peptide sets can be examined by detection of proliferation, cytokine production, or cloning. In contrast, the detection of CD8 T cell responses to whole antigens or overlapping peptide sets is technically challenging, with the requirement for large peptide sets (each peptide needing to be offset by only one residue) or cross presentation of whole antigen, as well as robust methods for detection of responses. However, the potential impact of studies of human CD8 T cells is underlined by equivalent studies in mice, which have identified islet autoreactive CD8 T cell clones that are capable of the rapid, unaided destruction of islet β cells *in vivo* (Utsugi et al 1996, Wong et al 1999). Moreover, the direct *ex vivo* enumeration of such pathogenic CD8 T cells in the blood of young non-obese diabetic mice can be predictive of disease (Trudeau et al 2003). It is therefore timely that there have been recent advances in the prediction and identification of CD8 T cell epitopes in human, through the use of HLA binding algorithms (Rammensee et al 1999), cell-free assay systems (Pinkse et al 2005), and mice transgenic for human HLA class I molecules. The focusing of these studies onto proinsulin as an important candidate autoantigen, and HLA-A2.1 as a dominant human restriction element (Hassainya et al 2005, Pinkse et al 2005, Toma et al 2005), have provided a platform on which the systematic analysis of CD8 T cell responses in type 1 diabetes can be commenced.

We therefore initiated a study of CD8 T cell responses in patients with recent-onset type 1 diabetes. Based on our previous experience with analysis of CD4 T cell autoreactivity to islet multiple epitopes (Arif et al 2004), we used subjects with selected HLA genotypes (*A*0201*) to screen a large number of putative epitopes, drawn from more than one autoantigen, in order to identify a discrete peptide panel that could be discriminative of disease. This could facilitate the cloning of islet-specific CD8 T cells in order to perform mechanistic studies, and might also be useful in the development of T cell surrogate markers for monitoring therapeutic trials. As a primary read-out we adapted *in vitro* culture technologies, interferon (IFN)γ enzyme-linked immunospot (ELISPOT) assay (which remains the most sensitive approach to the detection of rare antigen-reactive cells), HLA binding

assays, HLA-tetramers and cytotoxicity assays. In addition, alongside this, we were able to perform a preliminary evaluation of the emerging technology of HLA class I tetramers for use in the detection of islet autoreactive CD8 T cells.

Results

Using selected insulin peptides and HLA-A2 tetramer technology, we were able to identify CD8 T cells that were positively stained at low, intermediate and high intensity, comparable to those seen against viral peptides in the peripheral blood (Fig. 1). Studying a number of patients with T1D and control subjects, there is a clear trend for higher levels of staining in patients (Fig. 2).

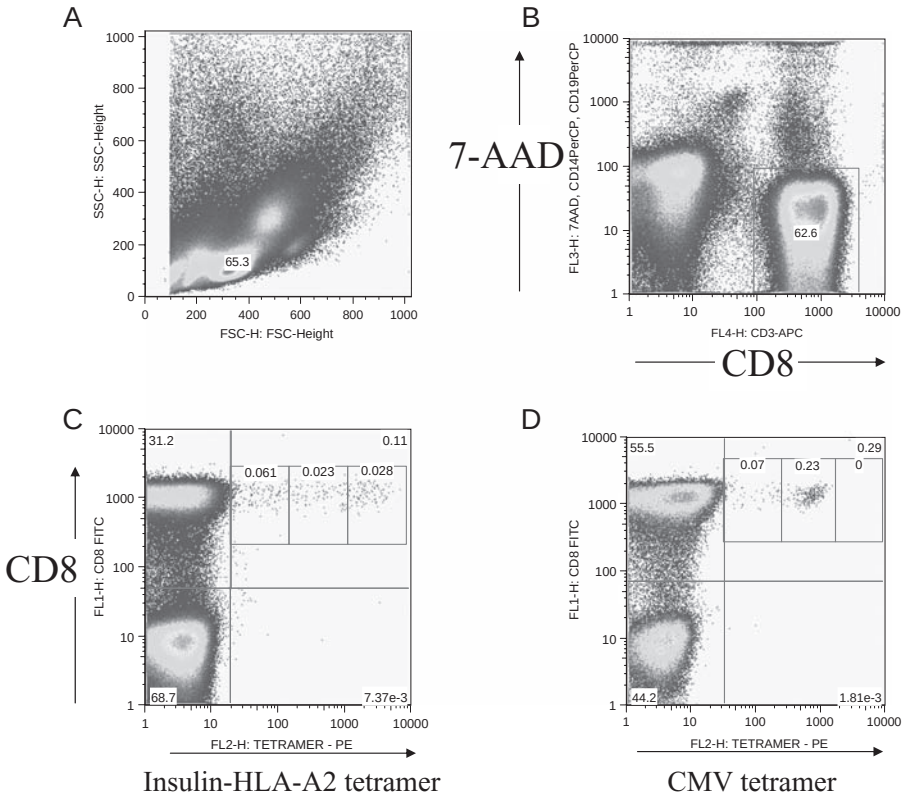

FIG. 1. Peripheral blood lymphocytes are identified by forward and side scatter (A) and then by live/dead markers and CD8 staining (B). The live $CD8^+$ T cells are then stained with either the HLA-A2 tetramer loaded with insulin peptide (C) or the CMV peptide (D), showing comparable staining to both.

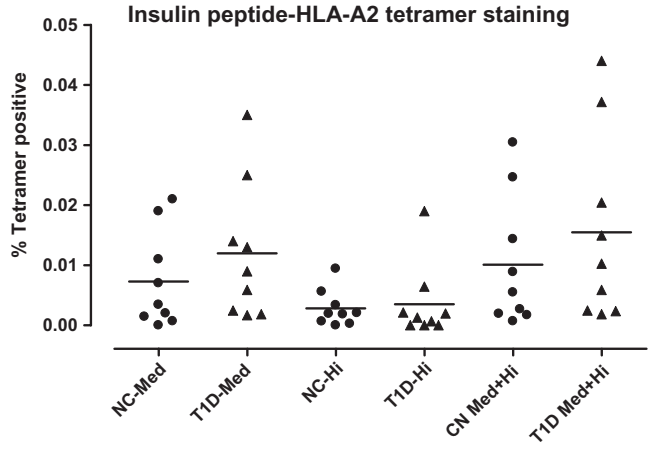

FIG. 2. Percentage of $CD8^+$ T cells positive for insulin-tetramer staining in patients with T1D or normal controls (NC), at high (hi) and intermediate (med) levels.

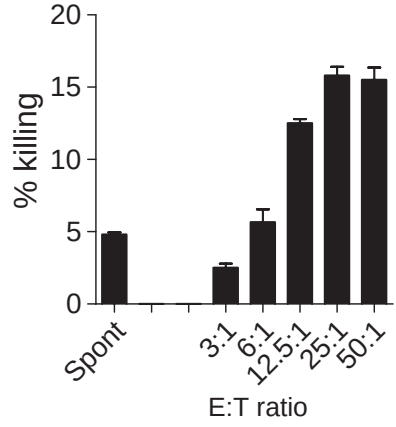

FIG. 3. Cytotoxicity of a CD8 T cell clone reactive against insulin and generated from a T1D patient against cells loaded with insulin, showing specific killing. Graph shows different effector:target ratios and spontaneous release as measures of CTL killing of the target cell line which expresses insulin.

Using flow-based sorting we were able to enrich and clone CD8 T cells from some of the tetramer staining cells and examine their functional phenotype. Cytotoxicity assays were carried out against a carrier cell line transfected with the requisite insulin gene sequence. Some of the clones obtained were cytotoxic for these insulin-synthesizing cells (Fig. 3).

Discussion

There has been a notable recent surge of interest in the identification of epitopes derived from islet cell antigens that are potentially recognized by autoreactive CD8 T cells in type 1 diabetes patients (Hassainya et al 2005, Pinkse et al 2005, Toma et al 2005). This has been fuelled both by a continued emphasis on the study of disease mechanisms in type 1 diabetes, and a pressing need for biomarkers of T cell-mediated destruction of islet β cells or disease remission (Alizadeh et al 2006, Peakman & Roep 2006). For many reasons, the analysis of CD8 T cell responses has lagged behind that of CD4 T cells, but this imbalance has begun to be redressed in the form of recent publications identifying putative candidate CD8 epitopes of β cell autoantigens. Much of the recent work has represented a concerted effort to focus on identifying CD8 T cell epitopes in proinsulin (Hassainya et al 2005, Toma et al 2005), considered by some to be the most disease-relevant autoantigen. However, there are also other studies within recent years that have highlighted potentially important single epitopes from GAD65 (Panina-Bordignon et al 1995), IAPP (Panagiotopoulos et al 2003) and IGRP (Takaki et al 2006). The present study is the first to attempt approaches using differing technologies in concert.

The frequency of islet peptide-specific responder CD8 T cells that we detect in peripheral blood is relatively low compared to antiviral T cells, at a median of 2 per million peripheral blood mononuclear cells (PBMCs), an order of magnitude below the typical antiviral response. Only three previous studies have attempted to directly assess CD8 T cell autoreactivity *ex vivo* (Panagiotopoulos et al 2003, Pinkse et al 2005, Toma et al 2005). Proinsulin B10–18 has been shown to be a target of autoreactive CD8 T cells in two previous reports, one focusing on recurrent autoimmunity in type 1 diabetes patients receiving islet transplants (Pinkse et al 2005), and the other, like our own, on recent-onset type 1 diabetes subjects (Toma et al 2005). We speculate that our data reflect the true frequency of responder cells, a concept supported by the findings with HLA tetramers. HLA tetramer technology has transformed the ability to identify and study antigen-specific clones of T cells in the peripheral blood. In autoimmune disease, there has been limited success in defining autoreactive T cells using this approach and to date, in type 1 diabetes, it has only been applied using class II HLA tetramers to detect CD4 T cells responses, although, notably, with this modality there has been no reported success with direct *ex vivo* staining. In the present piloting study we show direct *ex vivo* staining of small populations of CD8 T cells with an HLA-A2.1 tetramer loaded with the proinsulin B10–18 epitope. The detection of these rare cells by flow cytometry was greatly facilitated by the exclusion of potential false-positive events related to B cells, monocytes and dead cells. The finding of a significant correlation between tetramer staining and ELISPOT responses provides strong

support for the contention that the tetramer-stained cells are peptide-specific and autoreactive.

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DISCUSSION

Herold: So the clone will not kill cells from the HLA-2.1 transgenic mouse?

Peakman: We assume not, although we haven't done the experiment.

Roep: We know that the insulin-specific CD8 does kill HLA-2.1 transgenic mouse islet, so we have a positive control.

Herold: My understanding is that in the reconstituted mice that Dave Serezze's group has studied, they have had more difficulty seeing destruction of the murine A2.1-positive islet than human islet. Do you have any thoughts on why this might be the case?

Roep: That could be explained by the relatively low expression of the transgene. If you compare mouse and human islets, there is a log difference. This transgenic has much less of the HLA-A2 and supposedly less of the autoantigenic peptide.

Lew: I have always thought of the 'honeymoon period' as an endocrinological effect, but from your data it could be that it is an immunological effect. If you are giving insulin, you reduce blood glucose, and the cell itself makes less insulin and this makes it less of a target for anti-insulin responses.

Peakman: You can speculate this. If it were true it would argue that the one thing you need to get right quickly, and control from day 1, if you want to preserve β cell mass, is blood glucose.

Pipeleers: What was the incubation time in the cytotoxicity studies?

Peakman: They are all 4 hour assays.

Pipeleers: You are most probably looking at necrosis, because this is a short period for picking up cell death. Usually, with streptozocin, several hours are needed to see the cell disintegrate. How do you measure the cytotoxicity?

Peakman: It is done with the equivalent of a chromium-release assay.

Pipeleers: How do you know that it is β cell specific?

Peakman: We don't. This is an experiment we are trying to do now.

von Herrath: Are you using dissociated islets for these killing assays?

Peakman: Yes.

Roep: You have different end points, don't you? You can look at CD107, which is a lytic marker. On the effector side you could look at this as well.

Peakman: Yes.

Pipeleers: If you were to kill β cells specifically, and wash away debris after 4 h before culturing for another 12 h, you should end up with a preparation that is enriched in non-β cells. Immunostaining can provide an answer also taking into account that an isolated human islet preparation contains a high proportion of other cells.

Peakman: I'm pretty sure it is nowhere near pure. If we add peptide we get lysis up to 80–90%.

Roep: The other test would be to get pure β cells, rather than β cells inside whole islets. The exposure of β cell to the T cells in your case is also inferior.

Pipeleers: I would suggest that you FACS-enrich them before a cytotoxicity assay. Whatever the measurement you use to count dead cells, it is much easier to work with a FACS-enriched single cell preparation.

Flavell: Do you think that the peptides released from β cells sensitize the α cells and so on?

Peakman: No, I think it is β cell specific.

Flavell: If you are killing more than that, perhaps it is because the dead cells are releasing peptide.

Leiter: What is the frequency of this clonotype in both the non-diabetic and diabetic people you have looked at, in blood?

Peakman: In terms of primed effectors that will respond in 24 h in Elispot, it is 1 in 30 000 in patients. We don't see responses in controls.

Leiter: I was thinking of clonotype detection with the tetramer.

Peakman: We are just getting going with this.

Bonifacio: I want to comment on generation of the clone in this manner. When I was looking at T cells and thinking about all this, the tumour immunology guys suggested a positive control for me, which involved taking my peptide that is going to bind to an A2, take anyone's HLA A2 blood and stimulating a few times. You can generate them like that. After the stimulation we get an effector population because these cells have undergone stimulation. Is this real?

Peakman: We tried to do it from three controls and haven't been able to take it any further than the enrichment stage.

Roep: We have tried to prime with dendritic cells. In patients this is very difficult, but we have succeeded to prime controls. They don't lyse β cells.

Peakman: We've got in with a tumour immunology group who are trying to help us make more of these clones from other patients. Their experience is abysmal.

Roep: There is a problem in the ascertainment of the peptide epitopes. We might be flawed going for high affinity, which is what most people do, looking for high affinity binding of peptide to HLA2. One of the results we have from the epitope discovery program is that the majority of the peptide epitopes that have proven to

be epitopes, distinguishing patients from controls, have a low affinity of binding to HLA2.

von Herrath: This affinity issue is interesting. If you have clearly lower affinities or avidities compared with viral reactivities, we are entering a range where cross-reactivities are maybe more common for these cells. The lower avidity cells can be 'unpleasant' in the sense of propagating autoimmunity. For example, if you have preexisting autoimmunity, they tend to hang around longer and can be amplifying autoimmune processes, because they don't apoptose as fast. It makes sense if there is thymic selection that these low avidity cells would be present as the predominant autoreactive T cell populations.

Roep: We have a clone against GAD, which is class II and which is cross-reactive with cytomegalovirus peptide. If you then look into the peptide binding affinity for the GAD peptide it is low. The viral peptide has a high affinity for binding to HLA-DR3.

von Herrath: You might find the same for this clone: it could react with other peptides.

Peakman: Yes, we have to look at this.

General discussion I

J Hafler: I'd like to mention some recent work by my current supervisors, John Todd and Linda Wicker. It might be interesting to bring up the past scans that we have done following up on the Wellcome Trust Case Control Consortium (WTCCC) data. As we move forward, looking at the six or seven different regions identified in the scan, and then on our follow-up, the non-synonymous single nucleotide polymorphism (SNP) scan, along with 4000 cases and 5000 controls we did as follow-up, I can mention the genes we identified and give some background. In the WTCC there were seven diseases originally looked at: bipolar disorder, hypertension, type 1 diabetes, type 2 diabetes, rheumatoid arthritis (RA), Crohn's and coronary artery disease. This was 2000 cases and 3000 controls. With that we identified seven regions in type 1 diabetes and then did a follow-up with a non-synonymous scan, which originally looked at as many non-synonymous SNPs that could be arrayed and which assays could be designed for. This ended up at around 13000. In the other Affymetrix chip we used there were 4000 cases and 5000 controls. From this we came up with four unequivocal regions. We don't know for certain whether we have the best SNPs. A key question will be at what point do we start looking at function? The regions that came up were one on chromosome 4 (4q27), three on chromosome 12 (12q24, 12p13, 12q13), one on chromosome 16 and two on chromosome 18. Chromosome 18 had PTPN2 and CD226 (which came up in the non-synonymous scan). Chromosome 16 had this KIAA0530, which is interesting because it was in the multiple sclerosis (MS) scan. But also being in this group, there were two genes near it, including SOX1, which is close by in that linkage disequilibrium (LD) block. What are the next steps? At what point do we have the genes? What is the functional work that can be done?

Leiter: In the type 2 diabetes genome-wide scans, many of linkages were the more common SNPs in the population, rather than a rare variant. In the type 1 diabetes scan you are describing it might make sense to prioritise the SNPs that are rarer.

J Hafler: We did see a lot of common variants. This was prevalent through the different scans.

Leiter: You may have your best results with function looking at the rarer ones.

von Herrath: I'm not a good immunogeneticist, but in a situation like this wouldn't it be logical to start at this point to go after candidate genes and look at them in great detail? We know that SOX plays quite strong roles in the diabetes models. IL21 is another.

Eisenbarth: If you look at the actual numbers, the differences in allele associations between cases and controls are something like 45% versus 50%, or 50%

versus 48%. For the MHC the strongest associations may be 80% versus 16%. The low association makes it harder to distinguish case from control for prediction. I imagine the regions will be sequenced in more detail. A major question is whether there is a common pathway. How sure are we that we have identified immune-mediated genes? It is nice that the type 2 genes didn't come out in type 1 diabetes. I have never bought that hypothesis that type 2 diabetes has very much to do with type 1 diabetes. This is a contentious area. Is there a good possibility that there is a common pathway for these seven genes that you could figure out there function? Together you could then predict an immunological pathway.

Butler: Certainly, SOX1 is important in insulin signalling, for example. There could be variation with some elements of decreased insulin sensitivity in the context of partial, perhaps transient β cell insult that becomes more established because of less signalling.

Eisenbarth: TCF7L2 had no effect for type 1 diabetes.

Roep: I sense from these new data that with an odds ratio of close to 1 it will be impossible to make any functional sense of direct associations. Even with CTLA4, the initial functional interpretation proved spurious in retrospect. With CTLA4 you could imagine a couple of experiments that could be done, that make sense. Still, it was impossible to explain the genetic disposition, the polymorphism, with functionality. I'm personally discouraged going into this kind of study.

Atkinson: We have been working with John Todd and Linda Wicker to look at the CD25 explanation. We also have a study looking at whether there is an effector T cell defect, or a regulatory T cell defect, or both. We are starting to look at the stability of CD25 on those two cell populations, and we are interested in proteases that may influence this process, including matrix metalloproteases (MMPs). In terms of why were are interested in the stability of surface CD25, one of my former fellows took a look at soluble serum CD25 and found that there are big differences in the levels in all our serum. John and Linda sent us a few thousand samples blind, and we measured serum CD25, sending the results back coded. They found that these two polymorphisms caused a twofold difference in the mean level of CD25. This will soon be published (Lowe et al 2007).

D Hafler: Lisa Maier in our lab has looked at the CTLA4 polymorphism, with the A–G, and has examined early signalling events in terms of ZAP70 phosphorylation. There are highly significant differences in signalling with the polymorphism. It is unclear what the next-level mechanism is. If you have the diabetes-like polymorphism your naïve cells enter cell cycle more easily. The CD58 polymorphism results in differences in induction of FOXP3. If you have the allelic variant associated with MS, which is odds ratio 1.2, there is distinctly less FOXP3 induced with LFA3 engagement. Many of these responses may not be linear, but rather sigmoidal. One may be on a steep curve and small differences in an allelic variant may magnify differences.

Roep: We are looking into polymorphisms in cytokines and chemokines. Also, in the study we did on honeymoon, often we saw that functionality could be demonstrated in the control population but was overruled by disease.

D Hafler: Looking at controls, it's clear that these variants each have a minor effect. It is the addition of many of these variants together that creates the risk. Finding the common pathway is going to be critical.

Santamaria: There are difficulties in trying to come up with immunological explanations for the genetic associations especially with diabetes, but also any autoimmune disease. One is that linkage to a particular chromosomal region doesn't imply that a particular gene is going to have a deleterious mutation. You could be doing studies with CD25, for example, and find some associations. But are these associations mechanistic? To try to define how a particular region contributes to susceptibility at the level of the organism is a fundamental challenge in human immunology. These studies can only realistically be done in mouse currently.

Insel: When we look at these new chromosomal regions, the genes with the highest LOD scores are insulin and HLA. Are we satisfied that we understand how the insulin gene is contributing to human type 1 diabetes? What should we be doing with respect to loci where we may even have a better understanding?

Peakman: The tools are getting better so maybe we should have another look. The data on insulin are rather weak.

Eisenbarth: Insulin has a good hypothesis. There are interesting data from the AIRE knockout that controls thymic insulin expression. It is not such a simple story, but it is a developing story. It is also a reasonable hypothesis that the insulin VNTR is controlling the message within the thymus.

von Herrath: With insulin, a crucial question is whether this is one single pathway. This has profound implications for how you would treat the disease. If there is antigenic spread and other antigens being targeted, it is a completely different situation than having one Jα and one sequence driving the disease. Dissecting this could be done in a targeted fashion. If there are multiple genes contributing to the disease, it will probably be difficult to therapeutically modulate all of them. Would the idea then be at the end to use these as markers to subclassify the disease? If we could subgroup the patient populations prior to therapy it could be powerful.

D Hafler: It will be critical to spend the next few years trying to define the allelic variants. If you have a SNP on particular haplotype block and there is no LD, you can often nail the gene. CD25 has been much more complicated. Interestingly, for the CD25 variant it has become clear that the variant is different.

Roep: The strongest locus is HLA, and in all honesty we don't know why HLA DQ2 and DQ8 in combination is giving such high risk, while we don't know why DQ6 is protecting. There are so many questions.

D Hafler: The good thing about MHC is that by making a dense map of MHC, which has now been done, the field can drive it down deeper and see what variants might be there. We first have to define structurally better what the MHC hits really are.

Peakman: There is less you can do about MHC, but there is more that you might be able to do about peripheral T cell function. We should go for these because they might be more 'druggable'.

Bonifacio: The interaction might be a good thing to look for. We should look at pathways. One of the ways we could do this is mathematically: if you look at the combination of the SNPs that are susceptible, you get a lot more. These regions where there is interaction should be studied.

D Hafler: The tools for doing this are just evolving, and the epistatic interactions have not revealed anything as of yet.

Roep: It's a neural network type of thing.

Foulis: We are talking about whether there is a genetic risk associated with alleles of class II MHC, and how this could explain diabetes. There was a vogue in the 1980s for looking for aberrant expression of class II MHC on cells in autoimmunity. I'm convinced that this is a property of type 1 diabetes, where there is aberrant expression of class II MHC on β cells in most cases of diabetes, a finding unique to type 1 diabetes (Foulis & Farquharson 1986). This idea has gone out of fashion.

Eisenbarth: It is a controversial area.

Pipeleers: We had this discussion 30 years ago (In't Veld & Pipeleers 1988). Our standpoints haven't changed. Electron microscopy is needed to rule out that class II positive cells are macrophages that have ingested fragments of the β cells.

Foulis: I ruled this out with double staining for CD68. We can't do electron microscopy on formalin-fixed autopsy tissues.

Flavell: We have to reduce it to real genes and then have function. What this means is that one needs real models of human disease. We need to get human genes into mouse, and once one can manipulate human genes in such an environment we can get some broad brush answers.

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General discussion II

D Hafler: I want to give two short messages. The first is to follow up on the paper we published about two years ago. Our lab is interested in multiple sclerosis (MS) and we developed tools to analyse CSF in brain tissue where we have very few cells. This technology involves single-cell cloning where one uses flow cytometry with a dilution of one cell per well, and using phytohaemagglutinin (PHA), and IL2 to get high cloning efficiency with fresh peripheral blood. This gives us an opportunity to sample a tissue where there are not a lot of cells, grow up the cells without bias and to interrogate those cells to look, in particular, for antigen reactivity. We used this technique to examine draining lymph nodes from a series of individuals with type 1 diabetes. We took lymphocytes from pancreatic lymph nodes or islets stained with CD4 and sorted these cells at one cell per well with PHA titres and anti-FAS antibody. We cultured for about two weeks, expanded the clones as needed and then characterized them by CD4/CD8 expression, TCR analysis, looking at α,β chain and examining antigen reactivity. In the original work we looked at control tissues, pre-diabetics and long-term diabetics. In the work we reported we found a surprising degree of clonal expansion for two of the three subjects who had long-term diabetes for 20 years for a particular T cell receptor β chain. 10 of the 20 clones we generated had the identical TCR β chain sequence, and of these, half had the identical α chain sequence. Similarly, in the second diabetic subject we saw this tremendous degree of β chain and α chain clonal expansion. A third subject who had disease for 1.5 years had a pattern that looked somewhat like the pancreatic tissue lymph nodes from the controls where there is no high degree of clonal expansion, just a few pairs of clones. In the two diabetics we have cloned from subsequently we have seen clonal expansion more like this. They are also short-term diabetics. The surprise when we examined these clones against a panel of antigens, GAD, pro-MBP and pro-insulin, was that we found that a number of clonally expanded T cells reacted to the insulin A 1–15 chain, although the reactivity was low and required a fair amount of peptide in order to induce a response. With anti-DR antibody we blocked the response. On the basis of work by Len Harrison's group, we know that reducing agents increased the reactivity of this insulin chain which has three sulfhydryl bonds. By reducing the peptide we find more robust responses. At 100 μM peptide we can see reasonable responses. These are not clones that were generated against the antigen. When we do this we get very highly reactive T cells to MBP or GAD. We have always had a hard time generating insulin responses in normal individuals or diabetics, perhaps because insulin is in the circulation. Next we wanted to see whether we could induce a response using whole insulin or pro-insulin, using monocyte-derived

dendritic cells. Both can be processed and presented to the clone, using IFNγ secretion as a readout. We have now examined a subject three years after onset of diabetes, and one can see that in the pancreatic lymph node one of them had no clones and another had four out of 16 clones that were insulin α1–15 chain reactive. We have now made hundreds of peptides and we intend to screen all the clones we are generating out of the pancreatic lymph node against a range of different antigens.

I want to end with a brief discussion about Bart Roep's point on degeneracy. Clearly, T cell clones have a very low K_d of around 10^{-5}. They are highly degenerate and cross-reactive. TCRs can be multispecific: they are almost always cross-reactive using combinatorial libraries. A number of TCRs have been crystallized against self-antigens. It has been found that as opposed to the TCRs that have been generated against non-self antigens, the three crystal structures generated to date against self antigens suggest a somewhat different recognition, with most of the free energy binding of the TCR with MHC is in fact with the MHC, and less energy is coming from the peptide. This raises the issue as to whether or not autoreactivity may be somewhat different on the structural basis. There was somewhat of an accidental experiment recently published (Cai & Hafler 2007). We were trying to develop better methods for working with autoreactive T cells. We did CFSE loading of T cells and stimulated them with APCs, which basically have endogenous self antigen MHC. We took the CD4 populations entering the cell cycle (CFSE low), as opposed to non-proliferating CD4 cells and did single-cell cloning. We used self antigens and microbial antigens to examine antigen reactivity. The accident was that we were trying to do this with antigen where we added antigen rather than just endogenous self-peptide to generate T cell measurement. The more interesting observation was that the control, which had no antigen, generated autoreactive T cells. A number of the clones appear to have a surprising degree of reactivity even though they have never seen antigen other than what they saw in the culture. And this was MHC restricted. In summary, about 0.4% of CD4 cells entering the cell cycle respond to self peptide and MHC. The CFSE high cells or CD4 cells generated with foreign antigens never exhibit this degree of cross-reactivity. T cell clones stimulated by endogenous self peptide seem to have more cross-reactivity with degenerate T cells. Our hypothesis is that the free energy of binding of these clones generated which are self MHC, may be predominantly from the MHC or MHC backbone. This raises a question. We have looked at the T cell clone generated from the draining lymph nodes and they don't have this degree of cross-reactivity that we have seen so I don't think the clones we have generated are like those we generated with self-MHC, but it raises the issue of what self-reactivity really means. The MLR may represent T cells that have not undergone negative selection. This work may give some insight into what this autologous mixed lymphocyte reaction (AMLR) may be representing.

Roep: In the NOD, wasn't there an AMLR, phenomenon?

Leiter: That was our first paper. The AMLR in NOD/Lt mice is weak compared to related strains (Serreze & Leiter 1988).

D Hafler: It was low in MS.

Insel: Is there anything unique about the TCRs of these clones?

D Hafler: No. A number of them are oligoclonal, generated by the AMLR reaction.

Roep: The natural processing of the insulin A-chain peptides was an important part of your case. But the response to pro-insulin tended to be higher. Does this imply that the naturally processed peptide epitope is a pro-insulin peptide? Did you do extensions of that peptide?

D Hafler: I'm not sure. That's a good point: we should do extensions, to make sure we have the right peptide.

von Herrath: For me, the theme with these low avidity cells is that the cells are too 'wimpy' to precipitate any major autoimmune attacks from scratch. But it makes them probably more dangerous, if you already have a problem in an area, and then these cells are called in. If there is a preceding problem for another reason, with MHC up-regulation and IFN involvement for example, when these cells enter the islets because of their avidity they need more antigen but they also live longer when they are stimulated. The relative resistance to activation-induced cell death would make them more dangerous when they enter an autoimmune lesion.

D Hafler: It is just hard for me to believe that such wimpy clones in terms of proliferative response can be causing disease. But then when we see the work by George Eisenbarth, then maybe we shouldn't be looking at this proliferative response as an indication of how pathogenic a T cell might be.

Insel: What is the cytokine profile? Have you tried to generate these from cord blood?

D Hafler: Once you generate long-term clones all bets are off in terms of cytokines. They make IL13. We have not looked in cord blood for insulin-reactive T cells.

Insel: What about looking for cells that have just entered the cell cycle?

D Hafler: That would be interesting, but we haven't done that.

Herold: Can you give the details of the cloning procedure? Is the first *in vitro* stimulation done with or without BSA?

D Hafler: We use human sera. We use human AB sera for cloning from the pancreatic lymph node. It doesn't really matter, because we are adding PHA. If we want to make a clone against the MBP or insulin, we will take APCs or PMNs, add the antigen and clone out T cells by the usual techniques. Here, by adding PHA, we are trying to get every clone to grow. So it doesn't matter if there is insulin there or not. When we test the clones later we do it with and without antigen, so there probably is some insulin there in the background.

Roep: Can I get back to the promiscuity of the TCRs. We did some studies with peptide libraries that you briefly refer to, and calculated that the chance of a clone recognizing another epitope is one in a million, which is quite high. When you then do the calculation, the chance that one autoreactive T cell recognizes another autoreactive antigen is almost nil. This was confirmed by Don Mason in his statistics.

D Hafler: If we look at Roland Martin's data, he was able to take a few MBP clones and show that they also reacted to myelin. There is experimental evidence that one can easily find a MBP clone that will go up to another myelin antigen.

Roep: Are you sure it is an actual TCR triggering that is responsible for that?

D Hafler: We have a single clone and it enters into cell cycle. When you do these calculations there are certain assumptions one has to make. What assumptions did you make to come to that one in a million number?

Roep: We did titrations and made several DR3-dedicated peptide binding libraries, and so on. We were able to pull out new epitopes that were quite different and could identify their natural equivalents. Bert Hiemstra and Don Mason pointed out that one T cell receptor can recognize one in a million peptides, and any peptide can be recognized by one in a million T cells.

Lew: Did you ever find a second α chain in any of the clones?

D Hafler: Yes.

Lew: Were there more second α chains in your cross-reactive clones?

D Hafler: I'll have to go back and look. I would be surprised if the second α chain were responsible. I think what we are looking at is predominantly MHC reactivity. It is hard to imagine these randomly generated clones recognizing five different peptides.

Lew: You are probably right. However the Mark Davis *Nature* paper (Krogsgaard et al 2005) says that endogenous peptide/MHC complexes (which would be so common throughout the whole system) promote T cell activation in co-operation with the 'cognate' peptide. Thus, a second α chain not only may increase the chance of cross-reactivity but also may lower the threshold for activation.

D Hafler: I think we need a crystal structure to understand what we are seeing here. There are things we still don't understand, for sure!

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Genetic and therapeutic control of diabetogenic CD8$^+$ T cells

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Abstract. CD8$^+$ T cells are important contributors to the initiation and progression of type 1 diabetes (T1D). A very significant fraction of islet-associated CD8 T cells in NOD mice recognize epitopes of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), a non-essential endoplasmic reticulum-resident protein of unclear function. IGRP is also a target of CD8 T cell responses in human T1D patients. In NOD mice, most IGRP-reactive CD8 T cells target the IGRP$_{206–214}$ epitope and are diabetogenic. We have shown that the pathogenic activity of this T cell subset is controlled by genetic elements associated with diabetes susceptibility and resistance. One of these elements (*Il2*) has been recently implicated in susceptibility to several human autoimmune disorders, including T1D. In mice, *Il2* polymorphisms control a negative feedback mechanism initiated by activated, IL2-producing autoreactive T cells in the pancreatic lymph nodes that increases the regulatory activity of CD4$^+$CD25$^+$ T cells. Not all IGRP-reactive CD8 T cell clones are pathogenic, however, and we have evidence that some of these clonotypes are actually anti-diabetogenic. We had previously shown that administration of altered peptide ligands (APL) targeting IGRP$_{206–214}$-reactive CD8 T cells resulted in diabetes protection only at doses that did not delete low-avidity clones, suggesting a protective role for these clonotypes. I discuss evidence showing that transgenic expression of a low-avidity IGRP$_{206–214}$-reactive T cell receptor (TCR) efficiently prevents the development of insulitis and diabetes in NOD (non-obese diabetic) mice and that these cells do so by killing autoantigen-loaded antigen presenting cells in the pancreas-draining lymph nodes. These results illustrate a novel mechanism for regulation of immune responses to self-antigens and expose a new target for therapeutic intervention. Here I briefly summarize work done by us and others indicating that a prevalent subset of autoreactive CD8 T-cells in the NOD mouse are major (albeit likely dispensable) players in the pathogenesis of spontaneous autoimmune diabetes in the NOD mouse; that these T cells are targets of genetic elements affording autoimmune disease susceptibility and resistance; that they can either be diabetogenic or anti-diabetogenic according to their avidity for peptide/MHC; and that they can serve as useful targets for therapeutic intervention.

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Type 1 diabetes (T1D) and autoreactive CD8$^+$ T cells in mice

Non-obese diabetic (NOD) mice develop a form of T1D, closely resembling human T1D, that results from selective destruction of pancreatic β cells by T cells recognizing several different autoantigens (Lieberman & DiLorenzo 2003). Although initiation of T1D clearly requires the contribution of CD4$^+$ cells, there is compelling evidence that T1D is CD8$^+$ T cell dependent (Liblau et al 2002). A few years ago, we discovered that a significant fraction of islet-associated CD8$^+$ cells in NOD mice use CDR3-invariant Vα17$^+$ Jα42$^+$ TCRs, which we refer to as '8.3-TCR-like' (Santamaria et al 1995, Verdaguer et al 1996, 1997, DiLorenzo et al 1998). These cells, which recognize the mimotope NRP-A7 (defined using combinatorial peptide libraries) in the context of the MHC molecule K^d (Anderson et al 1999), are already a significant component of the earliest NOD islet CD8$^+$ infiltrates (DiLorenzo et al 1998, Anderson et al 1999), are diabetogenic (Verdaguer et al 1996, 1997), and target a peptide from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (Lieberman et al 2003), a non-essential protein of unclear function. The CD8$^+$ cells that recognize this peptide ($IGRP_{206–214}$, similar to NRP-A7) are unusually frequent in the circulation (>1/200 CD8$^+$ cells) (Lieberman et al 2003, Trudeau et al 2003). Notably, progression of insulitis to diabetes in NOD mice is invariably accompanied by cyclic expansion of the circulating $IGRP_{206–214}$-reactive CD8$^+$ pool (Trudeau et al 2003), and by avidity maturation of its islet-associated counterpart (Amrani et al 2000).

We have provided evidence that avidity maturation of the 8.3-like CD8$^+$ T cell response is regulated by tolerance and competition. That is, comparison of the fates of CD8$^+$ cells expressing high and low affinity $IGRP_{206–214}$-reactive TCRs (in transgenic mice) has revealed that the former are kept in check by deletional tolerance (Han et al 2005b). Islet inflammation, however, fuels expansion of the few high-avidity clones that survive deletion, at the expense of their low-avidity counterparts, promoting avidity maturation.

More recently, we have shown that islet-associated CD8$^+$ cells in NOD mice recognize multiple IGRP epitopes, indicating that IGRP is a dominant autoantigen for CD8$^+$ cells, at least in murine T1D (Han et al 2005a). NOD islet-associated CD8$^+$ cells, particularly those found early on in the disease process also recognize an insulin epitope (Ins B15–23) (Wong et al 1999).

Autoreactive CD8$^+$ T cells and their antigenic targets in human T1D

Association studies have suggested that certain HLA class I alleles (i.e. HLA-A*0201) afford susceptibility to human T1D (Fennessy et al 1994). Pathology studies have shown that the insulitis lesions of newly diagnosed patients consist mostly of (HLA class I-restricted) CD8$^+$ T cells (Bottazzo et al 1985), which are

also the predominant T cell population in patients treated by transplantation with pancreas isografts (from identical twins) or allografts (from related donors) (Sibley et al 1985, Santamaria et al 1992).

Insulin is a key target of the antibody and $CD4^+$ response in both human and murine T1D (Wong et al 1999, Kent et al 2005, Nakayama et al 2005, Toma et al 2005). The human insulin B chain epitope $hInsB_{10–18}$ is presented by HLA-A*0201 to autoreactive $CD8^+$ cells both in islet transplant recipients (Pinkse et al 2005) and in the course of spontaneous disease (Toma et al 2005).

IGRP, encoded in chromosome 2 (Martin et al 2001), has been recently identified as a β cell autoantigen of potential relevance in human T1D (Takaki et al 2006). Two HLA-A*0201-binding human IGRP epitopes ($hIGRP_{228–236}$ and $hIGRP_{265–273}$) are recognized by islet-associated $CD8^+$ cells from MHC class I-deficient NOD mice expressing an HLA-A*0201 transgene (Takaki et al 2006). Notably, the islet-associated $CD8^+$ cells of these 'humanized' mice were cytotoxic to HLA-$A*0201^+$ human islets (Takaki et al 2006). Most importantly, hIGRP-reactive $CD8^+$ cells have now been identified by several groups (Standifer et al 2006, Mallone et al 2007) in PBMCs of T1D patients.

Genetic control of diabetogenic $CD8^+$ T cells via IL2

Multifactorial diseases with high population prevalence develop as a result of interactions between multiple genetic and environmental factors. The inherited contribution to the familial clustering of common diseases is due to many loci across the genome with common and rare alleles with low penetrance, which is shaped by the genetic background of the individual, the general population background and environmental exposures. Familial clustering of autoimmune diseases is strong and can in general be classified by the nature of their association with polymorphisms in the HLA genes of the major histocompatibility complex (MHC). Since the early 1990s, several loci have been mapped by genetic linkage and association analyses in humans and in rodent models of autoimmune disease, including T1D. In human T1D, four loci, in addition to the HLA region, had been identified: the genes encoding insulin, the negative immunoregulatory molecules CTLA4 and LYP, and, most recently, the α chain of the interleukin 2 receptor (CD25) (Maier & Wicker 2005). In the spontaneous mouse and rat models of T1D, aside from MHC effects, variation of the CTLA4 gene has also been implicated, as has variation in another gene encoding a negative regulator of T cell activation, CBLB (Maier & Wicker 2005). Recent genome-wide studies in human T1D have confirmed the contribution of previously identified loci and have identified new ones, including a region in chromosome 4q27, containing the *Il2* and *Il21* loci (The Wellcome Trust Case Control Consortium 2007, Todd et al 2007). This region has also been recently implicated in

susceptibility to Graves' (Todd et al 2007) and celiac diseases (van Heel et al 2007).

Insulin-dependent diabetes 3 (*Idd3*), on mouse chromosome 3, has a major effect on susceptibility to T1D, experimental autoimmune encephalomyelitis and autoimmune ovarian dysgenesis induced by neonatal thymectomy. Using a positional cloning strategy, it was shown that the *Idd3* region spans 780 kb and includes the *Il2* gene (Maier & Wicker 2005). We have recently demonstrated that the NOD haplotype of *Il2* single nucleotide polymorphisms predisposes to organ-specific autoimmune disease by reducing IL2 production from antigen-specific ($IGRP_{206–214}$-reactive) $CD8^+$ T cells. This impairs a feedback mechanism initiated by activated, IL2-producing autoreactive T cells in the pancreatic lymph nodes that increases the regulatory activity of $CD4^+CD25^+$ T cells (Yamanouchi et al 2007). Since activated $CD4^+CD25^+$ regulatory T cells interact with dendritic cells (DCs) (Tang et al 2006), suppress DC maturation (Serra et al 2003), and inhibit the cross-presentation of β cell autoantigens to autoreactive T cells (Serra et al 2003), impaired IL2 production by autoreactive T cells impairs the homeostatic control of autoreactive T cells, enabling autoimmunity. This work has demonstrated, for the first time, how an autoimmunity gene modulates disease susceptibility at the level of the organism, and has mechanistic implications for the recently reported associations of the *IL2–IL21* gene interval and *IL2RA* in human autoimmune diseases.

Prevention of T1D by expansion of low avidity autoreactive $CD8^+$ T cells: a new paradigm

Spontaneous organ-specific autoimmune disorders, including T1D, result from complex immune responses against numerous epitopes in multiple antigens that arise spontaneously in a stochastic sequence. This complexity is compounded by the fact that lymphocyte clones recognizing identical epitopes engage antigen/MHC molecules within a broad range of avidities, the strength of which correlates with pathogenic potential (Amrani et al 2000, Liblau et al 2002). Consequently, the outcome of any antigen-based immunization strategy for the prevention of autoimmunity (to induce tolerance) is likely to be influenced by the choice of autoantigen(s), dose, periodicity of treatment, and route and form of administration, limiting the applicability of this approach to humans.

Administration of soluble peptides (without adjuvant) is an effective way of inducing antigen-specific T cell tolerance (Aichele et al 1994, Toes et al 1996). Previously, we showed that treatment of pre-diabetic NOD mice with soluble NRP-A7 (an $IGRP_{206–214}$ mimic) blunted avidity maturation of the $IGRP_{206–214}$-reactive $CD8^+$ subset by selectively deleting clonotypes expressing TCRs with the highest affinity for peptide/MHC (Amrani et al 2000). These observations raised

the possibility that NRP-A7's anti-T1D activity was mediated also by fostering occupation of the 'high avidity clonotype niche' (emptied by NRP-A7 treatment) by 'low avidity' (and potentially anti-diabetogenic) clones. To test this hypothesis, we identified altered peptide ligands (APLs) with partial, full or super agonistic activity for $IGRP_{206–214}$-reactive $CD8^+$ T-cells and compared their anti-T1D activity over a wide dose-range.

Chronic treatment with moderate doses of an intermediate affinity APL (NRP-A7) or high doses of a low affinity APL (NRP-I4) afforded T1D protection. This was associated with local accumulation of *low avidity* $IGRP_{206–214}$-reactive $CD8^+$ cells at the expense of their high avidity counterparts, which were deleted. Unexpectedly, chronic treatment with high doses of a high affinity APL (NRP-V7) or the natural ligand ($IGRP_{206–214}$) only afforded marginal protection. Strikingly, the islets of these mice contained almost no $IGRP_{206–214}$-reactive $CD8^+$ cells, but increased populations of $CD8^+$ cells recognizing other IGRP epitopes. This led us to conclude that peptide therapy in autoimmunity may be most effective when it fosters occupation of the target organ lymphocyte niche by non-pathogenic, low avidity clones (Han et al 2005a), a prediction supported by mathematical modelling (Maree et al 2006). Importantly, this hypothesis is also consistent with the observation that $CD8^+$ cells expressing a low-affinity $IGRP_{206–214}$-reactive TCR (Han et al 2005b) are anti-diabetogenic. Ongoing studies of these mice, suggesting a major contribution of memory phenotype T cells to diabetes resistance via killing of autoantigen-loaded DCs in the pancreatic lymph nodes, should allow a detailed examination of the mechanisms by which low-avidity autoreactive clonotypes inhibit pathogenic autoimmunity and, possibly, the design of novel immunointerventional strategies exploiting this phenomenon.

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DISCUSSION

von Herrath: The concept of controlling immunity by killing cells is very attractive. If you think about this, the high avidity cells should also be able to kill dendritic cells (DCs). This killing happens all the time. We see this in viral infections where the driving force of the immune response can be eliminated by cytotoxic T lymphocyte (CTL)-mediated killing of CD8α DCs that occurs rapidly. Then there is a reduced immune response to the virus, and the virus persists. I find this concept very interesting.

In the end, the question, whether CTL eliminate more DCs or more β cells in type 1 diabetes, comes down to an issue of distribution. The high avidity cells would kill more β cells and for some reason the low avidity cells perhaps stay in the periphery. Do you know, whether there is there a difference between these two populations, perhaps in the way that they are activated? Or could it be that you would proportionally find the low avidity cells more in the periphery, and the higher avidity ones are more prone to migrate to solid organs?

Santamaria: When the high avidity T cell clones are naïve they cannot kill. Most of these clones will exist as naïve clones in the periphery. They only become activated once they engage antigen in the pancreatic lymph nodes. Then they circulate and home to the pancreas, at which time they might be able to engage and kill β cells, but I think it is unlikely these cells will go back into the lymph nodes. We know that the low avidity T cells do not fit into the classical effector or central memory subsets but it appears that these cells do predominantly accumulate in the peripheral lymphoid organs. We have looked at chemokine receptors on them so I have some descriptive data. We think the reason these cells can kill DCs is because they tend to home to peripheral lymphoid organs rather than the pancreas. The cells that have just been activated are of high avidity and get trapped in the pancreatic tissue: they can't get out of there alive.

Peakman: The other thing that could control the migration is the concept of the endothelium being important. The insulin-specific clone goes in, and needs the class I MHC on the endothelium and presence of the insulin peptide to do this. Presumably your low avidity T cells wouldn't get a sufficient signal at that point to migrate, whereas the high avidity ones would. This could control them getting to the tissue.

Santamaria: Yes, this is possible. I don't know whether for this particular β cell specificity the endothelium plays a role in homing, because the antigen that is recognized is not an abundantly expressed auto antigen. IGRP which is a highly hydrophobic molecule that will only be cross-presented if membranes are phagocytosed or pinocytosed by DCs or macrophages. I haven't seen anything that suggests these cells will *trans*-migrate through endothelium by recognizing $IGRP_{206–214}$.

Peakman: There are potential gap junction communications that could also facilitate presentation by endothelial cells.

Herold: Is there any evidence for a low affinity population in MHC-matched CD8s?

Santamaria: I don't know. In a normal NOD mouse this population would be very small. They don't bind tetramers very well. It would be nearly impossible to detect. In these TCR-transgenic animals, on the other hand, we see things that are rare in a normal mouse magnified.

Herold: Have you crossed the T cell receptor (TCR) on a diabetes resistant background?

Santamaria: Yes. They don't develop diabetes, and nor does the 8.3-TCR-transgenic B10 G7 mice develop some diabetes early on, but beyond a certain age the animals don't develop diabetes.

Eisenbarth: A fundamental debate is whether there is a primary autoantigen. How do you put this in the context of Tom Kay's studies (Krishnamurthy et al 2006)? Do you get rid of an IGRP response and disease continues?

Santamaria: You have pushed a button here! I have never thought that there is a primary autoantigen in diabetes. That's my opinion. I think there must be something wrong with the animals that express IGRP constitutively in antigen-presenting cells (APCs). IGRP is an ER-resident protein that may cause ER stress when overexpressed. Animals that overexpress human IGRP in β cells have swollen ERs. These animals develop some diabetes but have no insulitis whatsoever. Even if we think that insulin is a driver, then insulin would be engaged very early on, and everything else will follow. β cells will be killed, and new antigens would be presented by DCs.

von Herrath: Didn't you have data on this, where you looked at this over time and found some cyclical changes?

Santamaria: This population is prevalent in the NOD mouse. This doesn't mean that it is essential for diabetes in the NOD mouse. I think that $IGRP_{206–214}$-autoreactive CD8 cells will be dispensable for diabetes development. I don't think any antigenic specificity is essential for diabetes development, but this is just my opinion.

D Hafler: In terms of the pathology of the disease, the epitope spreading still could be critical. If insulin is the driver, it doesn't mean that these other responses to GAD and such are not just as important in terms of pathology of the disease. We have to keep this in mind. My favourite antigen is still GAD! Insulin is a wimpy antigen.

Santamaria: If you take any single specificity in isolation, it would be dispensable. But the epitope spreading and the combination is needed for disease to progress to a point of clinical disease.

D Hafler: One of the questions raised earlier was, is this a viral disease? There were elegant studies by Steve Miller in the EAE model (JCI, Theiler's virus). These clearly showed that you start with a viral infection in the CNS, and then there is epitope spreading, and pathological T cell that turns out to be autoreactive. It could well be an initial viral event leading to autoimmunity. It is hard to argue that T cell reactivity itself doesn't play some role in diabetes, but whether the initiating event is an actual viral infection in the tissue is always a possibility.

Tree: In your killing assays, have you nailed down the mechanism by which the DCs are killed? Have you looked at the effect of putting in different TLR ligands on the susceptibility of the DCs to be killed? This could be a neat way that this regulation could be overcome by infection.

Santamaria: This is work in progress, and we are exploring the mechanisms, looking at the usual suspects.

Tree: Do you think it is any of the usual suspects?

Santamaria: I don't know.

von Herrath: My view on this has been that the regulation of killing in immune responses in general is different to any type of IL10 or other mediated regulations.

In virus infections, you kill virally infected DCs fine. If you only killed the other cells, then the virus would always get stuck in DCs and you would have a big problem afterwards. It becomes a system biology problem at this point, because the kinetics of who dies, in which location, and at which frequency becomes very important in these scenarios.

Santamaria: Evolutionarily speaking, it makes sense that evolution has maintained these cells throughout evolution because they subserve a beneficial function. One benefit of having more of the low-avidity autoreactive T cells is to protect against autoimmunity.

Roep: In your Trudeau paper (Trudeau et al 2003), is there a way that you can discriminate between expansion/contraction and homing? Could you look into proinsulin release in relation to some of these peaks?

Santamaria: This is difficult to do. There is such a small percentage of these cells, that you would have to bleed the whole animal.

Roep: Could it be homing?

Santamaria: Yes. We have some mathematical biologists who are working on this issue. They think these cells may include memory cells.

Kay: Is there any way of turning the 17.4 mice into something that looks like the 17.6 mouse? On the Rag knockout background the 17.4 mice have reduced diabetes, and it is clear they need some degree of T cell help. Does this mean that there are more of them in the lymph node and more likely to kill DCs? Is the effect of the CD4 in part to drive them into the islets?

Santamaria: I don't think so. We are looking *in vivo* to see whether we can visualize that killing. The cells that are being killed have to have memory and there has to be a particular set of machinery that will enable them to kill DCs. Otherwise it would be dangerous for the immune system because they would kill APCs every time an antigen is presented. These cells may need to be hit a few times before they are able to kill. I don't think this happens with the 8.3 cells.

Kay: If you took the 17.6 mice and allowed them to go through these cycles of killing the DCs, and then you transferred CFSE-labelled 17.4 cells into those mice, would you lose draining lymph node cross presentation?

Santamaria: We are doing those experiments.

Flavell: You call them memory cells rather than effector cells. What are the markers that distinguish this?

Santamaria: I call them memory cells because they are $CD44^{hi}$ $CD122^{+}$ CD62Lhi $CCR7^{-}$, and they make an enormous amount of IFNγ very fast. This is a phenotype of memory.

Flavell: You mentioned ER stress. Are you referring to this from the mediated point of view of just increasing the antigen, or whether it is promoting ER stress apoptosis?

Santamaria: Some of these autoantigens are highly hydrophobic and won't go through the ER. β cells have the potential to be subject to a lot of ER stress. My thinking is that a β cell that is stressed out would be a cell amenable to inducing an autoimmune response. The cell would break up and the antigens would be shed and so on. We saw some diabetes in a non-diabetes background which was because of ER stress: there was no insulitis. My opinion now is that ER stress will protect from diabetes in the NOD mouse, rather than accelerate diabetes. Don't ask me why I am thinking this way: I don't have an answer.

Butler: Perhaps the difference is the unfolded protein response versus ER stress. The terminology is unfortunate because stress implies death, but all β cells have the unfolded protein response, and that can be increased under certain circumstances. This means that translation is cut down, and all the chaperone proteins are increased: the cells are protecting themselves. The death rate of ER stress, involving translocation of CHOP to the nucleus and Ca^{2+} efflux from ER, is a minority event by comparison.

Santamaria: All the signs of the unfolded protein response are there.

Butler: So then the cells should be dying, but you say that they aren't dying.

Santamaria: They must be dying. The islets of these animals are very small and they stain weakly for insulin. There are many fewer islets in these animals. Do I have evidence of TUNEL-positive staining? No.

Butler: I thought you said that ER was protected.

Santamaria: This is what I feel. If β cell stress alone is able to elicit an immune response, I think I would have seen it in these animals. The fact that I don't see it suggests to me that ER stress either doesn't play a role, or is protective. But I could be wrong.

References

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General discussion III

Combination therapy with anti-CD3 and GAD65

Bresson: I'd like to talk briefly about combination therapy using anti-CD3 in combination with human GAD65 expressed as a DNA vaccine. In the LCMV model we get a good synergy when we combine these two compounds. This was not true in the NOD model, and we now have data showing the importance of the genetic background in response to antigen-specific immunotherapy but this will not be the main focus of my talk today. Here, we use this combination therapy as a tool to get good regulators that are GAD-specific in the RIP-LCMV model. When we looked at the phenotype of these Tregs, they possess a classical regulatory phenotype. The $CD4^+FoxP3^+$ Tregs express CD25 and are CD127 low. They also express CTLA4. It was curious to see that a great proportion of these cells expressed the OX40 molecule, also known as CD134. In the field of diabetes, there are only a few papers on OX40 and they are only focusing on the expression of CD134 on autoreactive T cells that are mainly aggressive cells. Tregs aren't mentioned. Therefore, we wanted to address the following question: Does the OX40 pathway play a role in the bystander suppression observed with these GAD-specific Tregs? I devised a technique to address this question using the siRNA technology. Several questions arose: which RNA should I use and how, and what tests should I do to see whether this pathway plays a role? If we stimulate T cells with anti-CD3 antibody, we get a strong and rapid up-regulation of OX40. I use the AMAXA technology on primary T cells, which in this case are $CD4^+$ T cells purified from mice. I observed that almost 95% of these cells were labelled using an APC-labelled siRNA. This confirms that the AMAXA technology is efficient to transfer siRNA into primary T cells. Then I designed the following study. If we stimulate primary T cell with anti-CD3 we strongly stimulate OX40. But if we then use the siRNA1 we get almost 70% inhibition of the OX40 molecule at the surface of the same cells. Then you end up with two different populations: a control population with a lot of OX40 at the surface, and a population with a lower OX40 expression at the surface. Then I transferred these two T cell population (low and high OX40 expressors) into recipient mice. As control, in a subsequent experiment I also used an OX40L-blocking antibody since there is no commercial mouse OX40-blocking antibody. In both cases, you get a good bystander blockade of diabetes in the recipient mice when the untouched GAD-specific Tregs are transferred, however when OX40/OX40L pathway is blocked with siRNA or blocking antibody the efficacy

is lost. This pathway may therefore be involved in the bystander suppression that we see *in vivo* in these mice. We now have a study going on trying to explain mechanistically what is happening and how OX40 is involved. We are looking at the homing capacity of these cells when OX40 is blocked, as well as the effect on apoptosis. OX40 has been shown to induce memory $CD4^+$ T cells, so blocking this pathway might induce apoptosis. We are also trying to answer whether blocking this pathway will be important for the generation of the Tregs. Overall, this pathway might be important for the suppression function induced by Tregs.

Autologous cord blood transfusion

Atkinson: I'd like to say a little about our work on autologous cord blood transfusion in subjects with type 1 diabetes. To begin, if one was to perform autologous umbilical cord transfusion, what could be the mechanisms that would bring benefit? One notion was that by delivering umbilical cord to individuals with type 1 diabetes, we could stimulate pancreatic regeneration. A second was that this procedure would result in the derivation of new insulin-producing cells from stem cells within the cord. The third, and most favoured by us was that we could restore immune tolerance. As of yet, we still don't know the mechanism entirely and all three remain candidates. In terms of some of the practicalities underlying this procedure, in 1998 a series of private and public cord banks started growing in popularity. With time, more and more people opt to have their children's umbilical cord blood stored: two years ago it was 2.5% of live births, and now it is predicted that the figure is about 4%. It is an expensive procedure, with an upfront cost of US$1700–2100, along with the need for payment of annual maintenance fees. Surprisingly to most, including myself, over 8000 individuals have been administered their cord blood for the purpose of treating approximately 60 different disorders. There are many, many advantages to cord blood, ones that I will review briefly. One of the great therapeutic promises and advantages is that cord blood has greater regenerative capacity than bone marrow. We have talked a lot at this meeting about naivety of cells. Cord blood that has been collected at birth and stored may be naive to any environmental insults that induce type 1 diabetes. The procedure itself is non-invasive. Cord blood Tregs have a high capacity for regulatory activity. The average age of the children who participate in our and other related trials is very young, a facet that relays the procedure's safety. In terms of actual trial experience, our first patient was treated almost three years ago, and was a cooperative effort with the Barbara Davis centre for Diabetes Research. It turned out to be a suboptimal procedure: the target was to have 10^8 nucleated cells per kilo, and given the 11% viability of the stored cord, we were only able to deliver about 4×10^6. This is

far from the 95% viability often touted to be the standard on thawing these commercially stored cells. There were no significant complications with the patient for this procedure. After the procedure the child came in and continued to show good C peptide production. 18 months later she still had over 50% of her initial C peptide. This child also had asthma, and had received two rounds of steroid injection to treat that at 18 months. Her father claimed that this caused a big deterioration in C peptide production, and given what we know about steroids and β cells, this could very well be. There was enough promise from this experience that we approached the FDA to ask for an IND to perform this procedure in 14 patients, which they approved. Individuals were given intravenous infusion of their autologous stored cord blood, and are followed at 3 month intervals. One of the main criticisms going into this trial was that reviewers claimed these patients would be hard to find, but we had over 300 patient inquiries in response to web-based advertising on this. The average duration of disease in the subjects we have treated thus far is about 0.8 years, as it takes time to make all the arrangements, do the staging and get the cord blood retyped. It is important to note that all of our studies use children with an average age of 4 years. We have done better with the number of cells infused, but we still haven't quite got to the levels we wanted. It is very important to note that this was a pilot safety study and there was no control group used. As far as true controls, we are working on this for the future. For now, we developed comparative data from type 1 diabetes patients who came into the clinic at the same time and utilized them as an internal control, matching subjects for age at entry plus initial insulin requirements. At six months, the individuals receiving cord blood have lower daily insulin requirements than the internal control population. The level of control in terms of diabetes management is also improved. Hence, we do see potential for using cord blood and as a result, we are also trying to work with companies to see whether they can store cord blood in multiple aliquots, because this will probably improve an individual's agreement to participate in studies like this.

Leiter: Due to the viability issue, you are putting in some dead and dying cells. You could be getting a syngeneic mixed lymphocyte reaction (MLR).

Atkinson: That is a good question, and if the response of the first child had not been validated by some of these other studies, we might have suspected this. This, being a good clinical response in dead cells rather than to viable cells. However, we have seen promise in both. Time will tell the mechanism.

Leiter: In the new study, are you only putting in cells that are viable at the time of injection?

Atkinson: No, we put in the total cord. The thawed cells are washed to get rid of some of the preservative, and then we add it all in. We take a small aliquot for us to do some mechanistic studies on. What is innovative is that we are

taking children that are developing an immunological memory, and we dump into their body a large batch of naïve cells. We think this is an interesting field to study.

Flavell: What is the frequency of Tregs in the cord preps?

Atkinson: It is about 1–2% of the total T cells.

Flavell: These aren't purified T cells, are they?

Atkinson: The FDA doesn't allow us to do any significant manipulation of the cells, at least not yet. For example, at one stage, we thought of a desire to label them. That was not considered safe. To reemphasize, the safety data we generate from these efforts will hopefully be used to do some more studies. We are working with two groups, one of whom has a GMP facility for doing Tregs isolations and expansions. Conceptually, we'd like to move down a pathway where one would expand these cells from cord blood. It's possible to expand cord blood cells almost 1000-fold and still retain FOXP3 expression and suppressive capacity. The cells have longer telomeres and are much more durable and stable than adult Tregs.

Flavell: Is that with IL2 and TGFβ?

Atkinson: Our collaborators are posing to perform this with artificial APC.

Pipeleers: In your controls, you would expect the C peptide to go down over a period of six months. When you plot individual data for controls and for treated children, is there a difference in the course over that six month period?

Atkinson: Actually, we have data out to nine months. We see two distinct lines, and the rate of loss is greater in the controls. There are a couple of patients who have made it out to 12 months, and there was a big drop between nine months and 12 months in these patients, suggesting that whatever benefit we are providing, if there, is perhaps not durable and will need some other agent co-delivered or a modification in the procedure.

Butler: Just to be a devil's advocate, we know in most placebo interventions in patients with diabetes, if we give them a purple placebo pill to swallow their blood glucose is improved a bit. It is not surprising that the control of blood glucose in your treated group is a little better, and removing glucotoxicity it is not surprising in turn that the C peptide is a little higher.

We recently went through about 40 pancreases of people who had died having had a bone marrow transplant previously, from an adult donor of the opposite gender. We stained pancreas for FISH and insulin, and the study was negative (no β cells derived from bone marrow) (Butler et al 2007).

But we are now following this up in people who had cord blood transfusions. These are mainly for myeloproliferative malignancies. These were trans-gender cord blood transfusions, and we found that these are occasionally positive. We find β cells that are trans-gender in origin. We need to make sure that there isn't mosaicism and so on. I had assumed that this result would be negative! It is small

percentage of the cells but it is a consistent finding. Cord blood may be more pluripotent than adult bone marrow.

Reference

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Towards a curative therapy in type 1 diabetes: remission of autoimmunity, maintenance and augmentation of β cell mass

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Abstract. Recent clinical trials have shown that the loss of insulin production that characterizes progressive type 1 diabetes mellitus can be attenuated by treatment with non-FcR binding anti-CD3 monoclonal antibody (mAb). This approach is a first step towards the ultimate goals of treatment: to improve and maintain insulin production. However, additional interventions will be needed because, with time, there is progressive loss of insulin production after treatment with a single course of anti-CD3 mAb. The basis for the long-term loss of insulin production after immune therapy is not known because animal models have not been informative about the mechanisms, and there are not biomarkers of autoimmunity that can be used to monitor the process. Therefore, strategies for clinical testing might involve both β cell and immunological therapies. Examples of the former include agents such as GLP1 receptor agonists or DPPIV inhibitors which increase β cell insulin content. Preclinical data suggest that co-administration of antigen with anti-CD3 mAb can induce a tolerogenic response to the antigen that may then be administered to maintain tolerance. In addition, other immunological approaches as well as interventions earlier in the disease process may be successful in maintaining greater β cell function for extended periods.

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Type 1 diabetes is a common, increasingly prevalent disease accounting for 5–10% of all diabetes. Type 1 diabetes is characterized by insulin deficiency resulting in hyperglycaemia and ketoacidosis. The aetiology of insulin deficiency is a loss of immune tolerance to pancreatic β cells leading to β cell dysfunction and destruction with disease progression (Atkinson 2005, Steele et al 2004). Both clinical and experimental evidence provide support that type 1 diabetes is a complex mutifactorial autoimmune mediated disease. In newly diagnosed type 1 diabetic patients a

mononuclear cellular infiltrate of macrophages, B lymphocytes and T lymphocytes were observed in biopsies of pancreatic islets (Foulis et al 1991). A recent histopathological analysis of insulitis in patients with new onset type 1 diabetes showed the presence of NK cells and viral inclusions in about half of six patients whereas others showed a predominantly T cell infiltrate, suggesting that the pathogenic processes, at least at the time of diagnosis, may be different among patients (Dotta et al 2007). Six loci have previously been identified that confer susceptibility to the development of type 1 diabetes: the MHC class II genes, the gene encoding insulin, CTLA4, PTPN22 the interleukin 2 receptor α chain, and IFIH1; a more recent study from the Wellcome Trust Case Control Consortium identified four additional regions of association (Wang et al 2005, Todd et al 2007). Many of these genes are involved in the development and maintenance of T cell-mediated tolerance to self antigens. Mouse models of diabetes have been bred that develop autoimmune diabetes in a manner thought to resemble human disease and even restricted by human MHC molecules thereby allowing investigation of the underlying immune mechanisms and identification of therapeutic targets (Delovitch & Singh 1997, Wen et al 2001, Roy et al 2005). These models have shown that the final effector stage of the disease is mediated by T lymphocytes but that other immune cells, including B lymphocytes, play a role in initiation and perhaps amplification of the autoimmune responses (Wicker et al 1994, Serreze & Silveira 2003). Importantly, these animal models have created an opportunity to test new treatments for the disease that affect immune responses. These studies have shown that several T cell-directed treatments can prevent diabetes in NOD mice. Some agents, including anti-CD3 monoclonal antibodies (mAb) are able to reverse diabetes in hyperglycaemic NOD mice (Chatenoud et al 1994, 1997). These preclinical studies have led to clinical trials which have shown promise in arresting the progression of disease soon after onset. However, despite the initial success, the duration of the treatment effect is not indefinite, and hence the long-term benefits of immune therapy have not been fully realized.

A successful treatment strategy to reverse type 1 diabetes and to induce metabolic remission needs three components: remission induction therapy to arrest immune-mediated destruction of the β cells of the pancreatic cells; expansion of β cell mass to ensure insulin independence; and maintenance therapy to continue remission and prevent re-emergence of autoimmunity. Finally, the treatment should be acceptable to patients with type 1 diabetes and produce similar if not better outcomes than current insulin replacement therapy.

Acute effects of immunomodulatory therapy

Immune treatments have been successful in the short term after diagnosis of type 1 diabetes. Broad spectrum chronic immune suppression with agents such as

Cyclosporin A and azathioprine with prednisone have shown clinical effects such as reducing insulin requirements and attenuating the loss of C-peptide (Stiller et al 1984, 1987, Silverstein et al 1988, Bougneres et al 1988). However, the need for continuous administration of these drugs and the risks of chronic immune suppression dampened enthusiasm for their use (Bougneres et al 1990).

An important finding was that unlike these other immune modulators that require continuous administration, anti-CD3 monoclonal antibody could be administered for a brief period to acutely diabetic NOD mice and remission was induced and maintained in a high proportion of mice without the need for continuous immune suppression (Chatenoud et al 1994). Moreover, when bivalent but non-FcR binding anti-CD3 mAb was used the significant cytokine release syndrome that was seen with FcR binding anti-CD3 mAb was prevented and the tolerogenic effects were seen (Herold et al 1992). Based on these findings, we initiated studies with modified (FcR non-binding) anti-human CD3 mAb (hOKT3γ1 [Ala-Ala]) in subjects with new-onset type 1 diabetes.

We showed that a single course of mAb treatment led to maintenance of stimulated C-peptide responses for 1 year after diagnosis and statistically significant improvement in the responses were seen even at 2 years (Herold et al 2002, 2005). Similar clinical findings were reported by Keymeulen et al (2005) who used an aglycosylated anti-CD3 mAb, also non-FcR binding. They reported that insulin usage was significantly improved 18 months after a single course of drug treatment in a double blind trial (Keymeulen et al 2005). Studies in the NOD mouse and in patients indicated that the anti-CD3 mAb treatment induced subpopulations of regulatory T cells although the phenotype of these cells and their actions differ in these reports (Belghith et al 2003, Bisikirska et al 2005, Herold et al 2003).

The improvements in C-peptide responses in treated patients have not persisted indefinitely despite findings in the NOD mouse model that indicated that remission induced with agents such as anti-CD3 mAb was permanent. This difference in outcomes raises a question about the parameters used for definition of disease and the relationship between these descriptors and the disease process. The designation of diabetes in NOD mice is somewhat arbitrary and even differs between publications—generally 200 mg/dl or greater. This is surprisingly high since the mean peak glucose level during an intraperitoneal glucose tolerance test in NOD/scid mice is 164 ± 9 mg/dl. Furthermore, the random glucose level, used for diagnosis, is a relatively imprecise measure of insulin secretory capacity. We recently have shown that NOD mice that have been 'cured' of type 1 diabetes by treatment with anti-CD3 mAb are glucose intolerant despite the return of random glucose levels to near normal (Ablamunits et al 2007). Thus, it is certainly possible that with time, there is loss of insulin secretory capacity in the treated NOD mice, a notion suggested by our recent analysis of β cell area in NOD mice treated with

anti-CD3 mAb (Sherry et al 2006). In humans as well, the diagnosis of type 1 diabetes is not clear cut since the criteria are based on the relationship between the glucose level and the risk of long term complications of the disease, not the pathogenic process. Interestingly, it is very difficult to distinguish individuals with recent onset diabetes from individuals just before the diagnosis of diabetes on the basis of metabolic parameters indicating that the sharp diagnostic distinction does not define the disease process (Sosenko et al 2006, Tsai et al 2006). Nonetheless, when stimulated C-peptides were evaluated over time, there was a loss of the clinical effect beyond the first year after the single course of drug treatment in both human trials (Herold et al 2005, Keymeulen et al 2005).

The barriers to development of a treatment protocol that would result in permanent remission or event reversal of β cell loss in diabetes relate largely to two factors: our lack of complete understanding of the immune mechanism that precipitate and drive the pathogenesis of type 1 diabetes initially, and the basis for β cell failure late in the course of the disease. The processes may not be the same, and therefore therapeutic approaches that may be successful initially may need to be combined or even replaced by other treatments at a later time. Tools are not available for both preclinical studies and studies in patients to study the long term history of the disease. First, animal models of the disease can offer limited insights into the chronic mechanisms. Effective treatments of diabetes are generally permanent—progression of disease after its reversal is not seen. Second, biomarker(s) that can either identify or correlate with the immune process that is associated with the disease remains unavailable. The results from recent workshops to develop and test immune assays that distinguish patients with type 1 diabetes from normal controls showed some promise in making this broad distinction but further studies are needed to determine the specificities and quantitative aspects of these measurements (Seyfert-Margolis et al 2006). In other autoimmune diseases, progress has been made in identifying gene signatures that relate to disease activity—this approach has not been widely tested in type 1 diabetes (Bennett et al 2003).

Thus, both immune and non-immune mediated mechanisms may be involved in the long-term loss of β cell function. We propose two complementary strategies to improve the duration of β cell function after diagnosis involving enhancement of β cellular function and immunological treatment.

TABLE 1 Possible mechanisms of long term loss of β cell function or mass

Immune mediated	*Non-immune mediated*
1. Apoptosis (Mathis et al 2001, Eizirik & Darville 2001)	1. Apoptosis (Bernard et al 1999)
2. Lysis (Pinkse et al 2005)	2. Hypoxia (Moritz et al 2002)

3. Modification of existing antigens presentation of neoantigens (Kent et al 2005, Doyle & Mamula 2005)
4. Reemergent autoagressive lymphocytes (Graca et al 2005)
5. Loss of Tregs (You et al 2005, Kukreja et al 2002)
6. Inflammation (Hotamisligil 2006)

3. Hyperglycemia (Cnop et al 2005)
4. Hypoperfusion (Huang et al 2007)
5. Hyperlipidanemia (Cnop et al 2005)
6. Loss of β cell precursors or growth factors (Rhodes 2005)

β cell strategies to preserve or improve function

One approach is to stimulate β cell function responses or even to augment β cell mass. It is important to consider that the basis for the recovery of β cell function after treatment with immunologics since this understanding will guide the consideration of combinations of agents. For example, recovery of β cell function might be enhanced by agents that are used in other settings whereas proliferation of new β cells would require a cell directed approach. Our recent studies of β cells in NOD mice after treatment with anti-CD3 mAb have shown that the β cells that are found after treatment were largely present even at the time of diagnosis (Sherry et al 2006). The increase in β cell mass following treatment with anti-CD3 mAb is largely accounted for by recovery of β cell function rather than the proliferation of new β cells.

The contribution of metabolic disturbance to the loss of β cell function is not known in the setting of immune therapy trials. In the European anti-CD3 mAb trial haemoglobin A1c levels were maintained at generally excellent levels, but the experience from the DCCT (Diabetes Control and Complications Trial) indicated that glucose control per se may modulate the loss of C-peptide (The Diabetes Control and Complications Trial Research Group 1998). Glucagon-like peptide 1 (GLP1) has been shown to stimulate β cell replication in rodents and to enhance glucose stimulated insulin release in humans and in animal models (Xu et al 1999). Both GLP1 analogue and inhibitor of dipeptidyl transferase IV (DPPIV), the enzyme that metabolizes GLP1 have been approved for treatment of type 2 diabetes. Studies of Ogawa et al (2004) showed that there was significant improvement in recovery of diabetes in NOD mice following treatment with anti-lymphocyte serum when the mice were co-treated with the GLP1 receptor agonist, Exendin 4 (Ogawa et al 2004). In NOD mice, we recently have found that Exendin 4 improved the reversal of diabetes by anti-CD3 mAb but the effect was greatest in mice with modest degrees of hyperglycaemia (≤350 mg/dl) at diagnosis (submitted). The effect of the Exendin 4 treatment was to increase insulin content of the residual islets rather than increasing β cell replication or mass or decreasing β cell apoptosis.

These findings suggest that a combination of metabolic and immunological treatments may be additive through enhancement of the insulin content of residual β cells. It is not clear whether the effect of the GLP1 agonist on β cells is direct or even secondary to an effect on glucose control, but we failed to find a similar improvement in remission of diabetes when diabetic NOD mice were given an insulin pellet at the time of treatment with anti-CD3 mAb.

Immune strategies to preserve tolerance

The effects of the anti-CD3 mAb may wane over time possibly with the replacement of peripheral T cells by new thymic emigrants, particularly in the young subjects who received anti-CD3 mAb in our previous studies. Therefore, one reasonable approach to maintain the response is to test the readministration of anti-CD3 mAb—this strategy is being evaluated in an ongoing trial sponsored by the Immune Tolerance Network (*www.immunetolerance.org*). However, an antigen-specific approach would improve the safety of treatment and therefore permit repeated treatments without the concern of broad immune suppression. In this regard, we have tested whether anti-CD3 treatment will open a therapeutic window (Treg promoting milieu and depletion of autoaggressive T cells) for antigen-specific induction of tolerance resulting in stable remission. In both hyperglycaemic NOD mice and LCMV-induced diabetes in RIP-NP mice we found that combining anti-CD3 mAb with the hpIIp proinsulin II peptide improved the remission rate of diabetes by 28% and 60% above remission with anti-CD3 mAb alone (Bresson et al 2006). The response was best in mice with glucose levels <400 mg/dl at diagnosis, consistent with the European anti-CD3 mAb trial in which the best response to anti-CD3 mAb treatment was in those subjects with the upper half of C-peptide responses at diagnosis and with our observations below concerning the synergy of GLP1 receptor agonists with anti-CD3 mAb (Keymeulen et al 2005). The mechanism of the combination involved reduction in autoaggressive $CD8^+$ T cells and generation of antigen-specific Tregs characterized by cells that produce TGFβ and IL10 and which inhibited the adoptive transfer of diabetogenic spleen cells into RIP-NP recipients (Bresson et al 2006).

Areas for further development

Cellular immunotherapy

The ability of antigen specific Tregs, induced by the combination of antigen and anti-CD3 mAb, or in other experimental settings, isolated on the basis of cell phenotype and expanded *in vitro*, to prevent diabetes caused by a polyclonal T cell response suggests that tolerance might be maintained by repeated administration

of these cells (Tang et al 2004). In this manner, antigen-specific Tregs could be expanded from patients with type 1 diabetes or even after treatment with anti-CD3 mAb, expanded *in vitro*, and re-administered based on clinical evidence of disease recurrence. There are important questions of safety such as the specificity of the regulation and the potential effects of impaired immune surveillance, as well as the stability of the regulatory phenotype *in vivo*.

B cell tolerance

While most attention has focused on developing strategies to prevent T cell responses, the immune effectors in the later stages of the disease, several lines of investigation in the NOD mouse have shown that B cells also are important antigen-presenting cells (Wong et al 2004). Even antigen-reactive Igs have been shown to enhance antigen presentation and precipitate autoimmune diabetes mediated by islet antigen-reactive $CD8^+$ T cells (Harbers et al 2007). An ongoing trial will evaluate the role of anti-human CD20 in new onset patients, but combining a B cell and T cell approach sequentially or in combination might more effectively block recurrence of disease, although the toxicities of B and T cell modulation might require sequential rather than combination treatment.

Disease prevention

A consistent finding among preclinical studies has been the improved efficacy of immune therapy when the impairment in islet function is not extreme. Some but not all clinical studies indicate that the immune response is better with greater initial C-peptide responses, but regardless, the clinical benefits are certainly superior when subjects with greater β cell function are treated. This would suggest that the greatest benefit would be obtained by treatment of subjects before the clinical diagnosis with hyperglycaemia. Interestingly, while there does appear to be evolution of immune responses that might suggest that the immunological responses in subjects with diabetes are not the same as those with prediabetes, there is little evidence that those at the highest risk for type 1 diabetes (i.e. autoantibody positive with impaired glucose tolerance) differ either immunologically or metabolically from those who have crossed the threshold for the diagnosis (Brooks-Worrell et al 2001, Sosenko et al 2006, Tsai et al 2006). Therefore, depending on the risk of the proposed intervention, treatment of subjects before the appearance of hyperglycaemia would be a preferred approach, but can only be done at considerable cost because of the need to screen large number of individuals at risk to identify candidates.

In summary, studies of anti-CD3 mAb have shown promise as an initial therapy for new-onset type 1 diabetes. A combinatorial approach is likely to be needed

to maintain the immunological response and to even improve β cell function. Both immunological and non-immunological approaches might be considered. The effectiveness of both of these strategies is likely be influenced by individual factors as not all subjects will respond to all treatments, but the most important determinant of clinical benefit may be the β cell function that is present at the time of treatment. Intervening at early stages of the disease process represents the ultimate goal—rather than restoring lost cells and mass, preventing its primary loss.

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DISCUSSION

Roep: Kevan Herold, do you think that you can get new β cells after combination therapy with anti-CD3 and exenatide in pancreases of children older than 10? The mice never get older than perhaps one or two years.

Herold: They are not even that old. They are 25–30 weeks. We haven't looked in older mice. The acute mechanism that we believe is responsible for the acute recovery of insulin production is recovery of the existing β cells.

Butler: It is an important issue. Even when you, Kevan, were looking gloomily at your β cell replication rates, I was looking at them enviously. Your rate is three or four orders of magnitude higher than we ever see in adult humans. You see 2%;

we see this perhaps in a one or two year old. After the age of five, if we see 0.1% it is unusual.

Herold: In non-SCIDs it is very low. The high rates are seen in the setting of an inflammatory response.

Butler: That is consistent with organ donor data. There is some cytokine driving replication, which proves it can happen. I didn't think your studies seemed so gloomy because if you can suppress the destruction and leave some replication intact this could work.

Herold: That is assuming that all the β cell death is done. It may not be quite that black and white. It may be that there is still some low level of β cell destruction that continues.

Leiter: You can't equate all BrDU incorporation with β cell replication.

Butler: BrDU has many problems, I agree. This was Ki67.

von Herrath: How common are β cells that make no insulin, but just sit there degranulated? How many of these cells are usually found in humans?

Butler: In humans we don't see them.

Herold: The only way we could find them was by using another marker for β cells.

Butler: We used Vmap2, which we've been asked to use by people who do imaging. We don't find Vmap2-positive cells that aren't β cells. This is predominantly looking at new-onset type 1 diabetes. If we do a cocktail to stain for the other endocrine cells you can account for the endocrine cells in these islets.

Herold: It may depend on the patient.

Butler: These patients had diabetic ketoacidosis, so they had every reason to be degranulated.

Herold: How do you go into a remission? What is the honeymoon?

Butler: That's a good question. I believe that those cells recover. They are not capable of secreting insulin just because they have insulin. Remember, 90% of insulin is never secreted. A very small portion of insulin goes to the plasma membrane to be docked. If it isn't docked the vesicle contents are recycled by crinophagy. The majority of insulin granules in any β cell are not available. The intensity of insulin staining of β cells is a poor surrogate of insulin secretory capacity. In chromaffin cells, the staining and secretion go together, because the pool of hormone substrate is related to what will come out, but not in β cells.

Herold: So you are saying that remission is essentially functional recovery of β cells that were there, and identified by the presence of insulin all along. The way we got into this was that we added BrDU into the water of mice treated with anti-CD3 during the period of recovery from hyperglycaemia. We looked at the proportion of cells that were labelled at the time of remission, which was about 10%. 90% of the cells that were insulin-positive were not BrDU positive. Those cells must have been there before the effects of the anti-CD3 monoclonal antibody.

Then we went back and looked at the time of diagnosis, and found Glut2-positive cells that did not stain with insulin.

Butler: It seems to me these mice differ from humans.

Eisenbarth: Ketoacidosis itself inhibits secretion, not only high glucose.

Butler: We see the same in an 89 year old man who comes to surgery and the long-standing type 1 diabetics. It doesn't seem to be different.

Herold: But how you would take a β cell that is under maximal stimulatory conditions, in this case in a hyperglycaemic host, and make it secrete more? How would this happen?

Butler: You have zero insulin in the docked pool, or you have inhibition of secretion in the case of acidosis. So it could just be taking away the acidosis. New synthesis is zero because your β cells are under maximal stress. You are making no proinsulin, so you are processing no proinsulin and sending no insulin through the Golgi to the dock pool. If you take this patient, put them on insulin and bring down the glucose, this allows the β cells to recover from this stress. The unfolded protein response will be unburdened and proinsulin biosynthesis can proceed.

Pipeleers: I'd like to return to the remark that degranulation makes β cells invisible in the diabetic pancreas. Immunocytochemistry for insulin can be very sensitive so that also poorly granulated cells are detected. Within the rodent and human pancreas there is a gradation between well and poorly granulated β cells. With less sensitive versions of the technique this heterogeneity can be missed. In experimental models it is possible to induce a massive degranulation and still detect insulin-positive cells.

Related to this, Kevan, I like your interpretation that the anti-CD3 may allow regranulation. When we take the data from the European trial we see that the protective effect is preferential in patients with a better insulin releasing capacity at start. But in order to identify these patients we had to perform a clamp. Measuring glucose levels in your mouse model might thus not be sensitive enough to distinguish animals with a higher or lower residual β cell mass. Animals with a beneficial effect of the CD3 may have started with a higher β cell reserve. Don't you think this may be the case?

Herold: Yes, absolutely.

Bonifacio: The timing with anti-CD3 is interesting. If we put islets into NOD mice, at some stage they get destroyed quickly, but the process is slow. With anti-CD3 you need to time it reasonably well and have a certain amount of β cells left, but not too many. What is going on with the timing of anti-CD3 and the mechanism of action?

Herold: There is a rapid immunological effect, within days, that clears out the infiltrate initially, and then cells come back in. I think the functional recovery takes a bit of time.

Bresson: In the RIP-LCMV-GP model, some mice after treatment with anti-CD3 antibody stay diabetic (with a blood glucose value between 250 and 500 mg/dl) for perhaps 15 days, and then they come back to a normoglycaemia (blood glucose value below 250 mg/dl).

Bonifacio: So do mice that are treated with streptozotocin with blood glucoses at 400 mg/dl. Some will eventually come back. If you take them to 600, they rarely come back.

Leiter: It is certainly different in regard to spontaneous diabetes development in the NOD mouse; once diabetes initiates, they may be 300 the first week, then they will be 600, 900 and 1200 mg/dl as time elapses. With streptozotocin, especially multiple 'low' doses, if non-autoimmune prone mice such as B6 are 400 mg/dl initially, they may eventually come down to 150 mg/dl and stay normal. So there is no autoimmune 'memory' to impair expansion of surviving β cells in strains such as B6. Autoimmune diabetes in NOD is very different from streptozotocin diabetes in this regard.

von Herrath: With our anti-CD3 they don't come back.

Immune markers of disease and therapeutic intervention in type 1 diabetes

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Abstract. Type 1 diabetes results from a T cell-mediated autoimmune destruction of the insulin-producing pancreatic β cells in subjects with a genetic predisposition to this disease. Therapies directed against T cells have been shown to halt the disease process and prevent recurrent β cell destruction after islet transplantation. Less is known about the mechanisms by which T cell-targeted therapies modify disease, how the immune system may suppress autoreactivity, and whether (or which) autoantigen(s) are critically involved in disease modulation. Autoreactive T cells have proven to be valuable tools to study pathogenic or diabetes-related processes. Measuring T cell autoreactivity has also provided critical information to determine the fate of islet allografts transplanted to type 1 diabetic patients. Unfortunately, cellular autoimmunity is a difficult study subject, and most activities were aiming at defining disease-associated T cell responses. A perhaps even more important goal will be to define and measure changes in T cell autoimmunity that are associated with disease intervention following immunotherapy, as autoantibodies do not qualify for this purpose. Recently, we have identified immune markers that associate with remission after initiation of insulin therapy ('honeymoon'), and disease suppression with antibody therapy (ATG, daclizumab, anti-CD3) or islet autoantigen. The challenge for the future is to determine which immune factors associate with tolerance to β cell antigens, and to define what measures T cells can provide to suppress autoreactivity.

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Type 1 diabetes: a T cell-mediated autoimmune disease

Type 1 ('insulin-dependent') diabetes mellitus (T1D) is the second most prevalent chronic disease amongst children in the Western world. There is a huge clinical demand for selective intervention therapy, since most T1D patients develop chronic complications despite intensive insulin therapy that combats disease symptoms, not its cause. In the last two decades our understanding of T1D pathogenesis has

increased dramatically, leading to the expectation that the disease is curable by β cell preservation, replacement and neogenesis, provided that the ongoing immune attack is countered. However, no cure exists yet. T1D is an autoimmune disease in which $CD4^+$ and $CD8^+$ T cells infiltrate the islets of Langerhans, resulting in β cell destruction (Roep et al 1990, 1991, 1995, Roep 1996, 2003, Martin et al 2001, Arif et al 2004). Although the precise pathogenetic events are unknown, an extensive body of data in animal models, and more limited studies in human, indicate that $CD4^+$ and $CD8^+$ T cells reactive to islet autoantigens have a key role in the process of β cell destruction, as illustrated by histological analysis of pancreata of newly-diagnosed T1D patients showing T cells present in inflammatory lesions ('insulitis') (Bottazzo et al 1985) and immunosuppressive drugs, including those specifically directed against T cells, delaying disease progression. Therapy with non-activating humanized monoclonal antibody against the T cell surface molecule CD3 at clinical manifestation of the disease suggests preservation of β cells (Herold et al 2002, Keymeulen et al 2005). Finally, T cell-mediated islet autoimmunity in association with diabetes onset and loss of islet-graft function provide evidence that the peripheral blood represents a 'window' through which anti-islet T cell autoreactivity can be observed and studied (Roep et al 1999b, Pinkse et al 2005).

Proinsulin (PI), glutamic acid decarboxylase (GAD65) and islet tyrosine phosphatases (IA-2) have emerged as autoantigens of major disease relevance (Di Lorenzo et al 2007). CD4 T cells specific for these autoantigens exist. Evidence for their clinical relevance includes their isolation from the peripheral blood, the detection of their disease relevant pro-inflammatory phenotype and the beneficial impact of anti-T cell therapies on new-onset disease. The identification of naturally processed peptide epitopes for DR4 led to the first systematic analysis of the Th paradigm in T1D, with the demonstration of proliferating, interferon (IFN)γ-producing (i.e. effector memory) islet autoreactive $CD4^+$ T cells in association with disease development, as well as the identification of interleukin (IL)10-producing, putative regulatory islet autoreactive $CD4^+$ T cells in HLA-DR4-matched non-diabetic control subjects (Arif et al 2004). Indeed, T1D patients producing IL10 in response to islet epitopes manifested the disease more than seven years later than those not producing IL10. This seminal observation is the basis of our current hypothesis that induction of IL10 by islet antigen-specific vaccination under tolerogenic conditions may halt the disease process and preserve β cell function.

Cytotoxic $CD8^+$ T cells also play an essential role in this β cell destruction process. Absence of MHC class I in non-obese diabetic (NOD) mice spontaneously developing diabetes renders these mice T1D-resistant. In humans, 60–70% of T1D patients express HLA-A2. Transgenic expression of HLA-A2 significantly accelerates T1D onset in NOD mice, with HLA-A2-restricted $CD8^+$ T cells appearing in early, prediabetic insulitic lesions (Takaki et al 2006). We and others

employed matrix-assisted algorithms predicting HLA binding, leading to the identification of putative epitopes of β cell proteins (Pinkse et al 2005, Panagiotopoulos et al 2003, Toma et al 2005, Ouyang et al 2006, Standifer et al 2006, Mallone et al 2007), and several epitopes of proinsulin, prepro-islet amyloid polypeptide, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and IA-2 have been identified (Mallone et al 2007, Standifer et al 2006).

There is, therefore, an overwhelming case for autoreactive T cells in the pathological islet processes that cause T1D. The challenge for the coming decade is to refine and harness this knowledge to design effective and safe intervention strategies, to construct monitoring algorithms for use in intervention studies and islet transplantation, and to achieve greater insight into triggers, accelerants and modulators of T cell autoimmunity.

In vivo relevance of islet-specific autoreactive T-cells

We pioneered the cloning and characterization of islet-specific $CD4^+$ T cells from T1D patients (Roep et al 1990) enabling study of distinct parts of the pathogenesis of T1D. T cells directed against GAD65 indicated involvement of islet endothelium in processing and presentation of β cell autoantigens (Greening et al 2003). Autoreactive T cells injected into NOD.scid mice migrated to the islets of Langerhans, resembling early peri-islet insulitis (van Halteren et al 2005). Our study provided the first evidence of *in vivo* accumulation in pancreatic tissue of islet-reactive T cells derived from T1D patients and provides a novel *in vivo* model to assess pathogenicity of human autoreactive T cells and allows study of intervention therapies that may affect this contribution to disease (van Halteren et al 2005). We have employed our autoreactive T cell clones as reagents to design and study potential immunotherapeutic strategies *in vitro* (van Halteren et al 2002, Huurman et al 2006, van de Linde et al 2006). Evidence that human T cell clones possess β cell cytotoxicity *in vivo* is still lacking, but our observations underscore that circulating T cells can qualify as biomarkers with potential relevance to insulitis and T1D pathogenesis (Fig. 1).

In NOD mice a large proportion of islet-infiltrating $CD8^+$ T cells were specific for IGRP. Three novel epitopes of IGRP were uncovered using NOD.β2m^{null}.*HHD* transgenic mice (Takaki et al 2006), and HLA-A2-restricted $CD8^+$ T cells isolated from the islets of these transgenic mice lysed human HLA-A2.1-positive islets. One of the IGRP epitopes of these murine diabetogenic T cell clones is identical to that of our human $CD8^+$ T cell clone that we recently uncovered (Unger et al 2007). We also identified another naturally processed β cell epitope recognized by human autoreactive CTLs: insulin B-chain(10–18) (Pinkse et al 2005). Applying HLA-A2 tetramer technology, we isolated CTLs recognizing these islet epitopes from peripheral blood of T1D patients and cloned them. These clones secrete

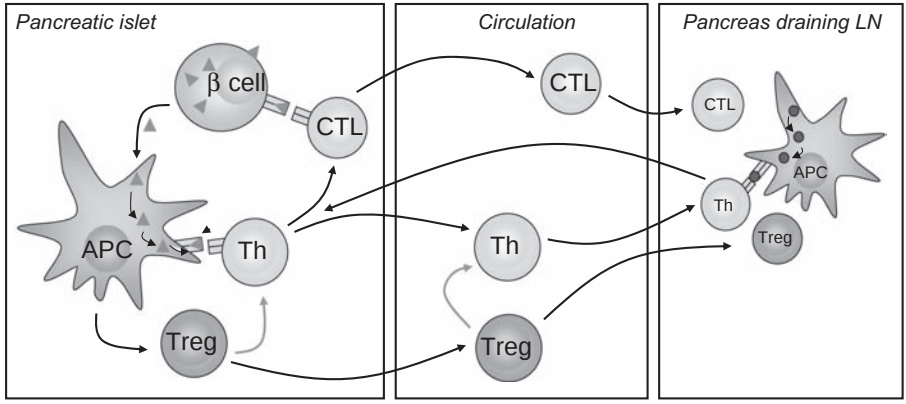

FIG. 1. Recycling of insulitic effector T cells and disease-modifying regulatory T cells offers opportunities for their detection in peripheral blood.

IFNγ and granzyme B upon antigen-specific stimulation *in vitro* and lyse peptide-pulsed HLA-A2$^+$ target cells. Such T cell clones can be studied for their phenotype and function *in vitro* and *in vivo*. A perfect homology of their target epitopes between mice and human implies that these human T cell clones can cross-react with mouse β cells, provided that these are engineered to express HLA class I. Again, such data underscore that circulating T cells can qualify as biomarkers with potential relevance to autoimmune β cell destruction.

Regulatory T cells in T1D

Several different types of regulatory T cell (Treg) have been identified, including CD4$^+$CD8$^+$ T cells and T cells producing large amounts of IL10 and transforming growth factor (TGF)β. The characteristics of Treg populations and outstanding questions regarding their role in the development of T1D are diverse. It appears that in non-diabetic individuals, potentially 'pathogenic' cells are held in check by various Treg-dependent mechanisms. T1D may develop due, at least in part, to a defect (either functional or numerical) in the Treg repertoire. Due to side-effects associated with the long-term use of generalized immunosuppression to delay disease or prevent islet-graft rejection, the induction and maintenance of long-lasting tolerance to islet autoantigens remains a major impetus for T1D research. Moreover, to monitor the efficacy of these trials, assays are urgently required that are able to measure not only diminution in pro-inflammatory 'effector' cells, but also the expansion of islet-specific Tregs to foster new avenues for immunotherapy. We pointed to a generalized dysfunction in regulatory cells in T1D showing defective function of CD4$^+$CD25$^+$ T cells in T1D patients (Lindley et al 2005). Antigen

specificity of regulatory T cells is a requirement to link islet-specific regulation and the development of islet autoimmunity. We first demonstrated islet antigen-specific Treg in non-diabetic individuals, capable of suppressing the proliferation of T-cells recently activated by insulin (Douglas Petersen et al 1999). Our second study, involving limiting dilution analysis (LDA) to investigate insulin reactivity in T1D patients and siblings (Naik et al 2004), also suggests the presence of a regulated insulin response. Finally, we identified HLA-DQ-associated antigen-specific suppression of autoimmunity to insulin (Tree et al 2004). These three studies indicate that islet antigen-specific Treg exist, and can be detected in peripheral blood.

Measuring T cell recognition in T1D

There is a pressing need for robust assays that measure the disappearance or functional silencing of islet autoreactive T cells, or the appearance of regulatory populations. A lack of T cell assays has compromised this effort in several ways. Most obviously, there is no read-out for the target cell (islet autoreactive T cell) for most of the therapies. In addition, it is highly probable that a reliable measure of T cell autoreactivity would be a useful surrogate in intervention studies, allowing them to be conducted to end-point more quickly and with greater economy. We contend that one of the most important limitations on assay design has been the paucity of knowledge regarding epitopes (Di Lorenzo et al 2007). Whole antigen preparations have proved a poor substrate for T cell assay development (Peakman et al 2001). They are invariably contaminated with host cell proteins and of dubious quality, as we demonstrated through Immunology of Diabetes Society T cell Workshops (Roep et al 1999a, Peakman et al 2001). In contrast, synthetic peptides based on known epitope sequences can be reproducibly synthesized chemically to high levels of purity. Epitope discovery has further application to technologies such as HLA tetramers, the deployment of which is dependent upon certain knowledge regarding both epitope and HLA restriction element.

Once reliable and sensitive assays became available, were served by high quality reagents, and began to be performed in laboratories with experience, tentative advances began to be made in the context of trial monitoring, as discussed below. Progress since then has been slow but steady.

Biomarkers

There is a pressing need for biomarkers associated with T1D disease progression. Perhaps even more important is the definition of immune correlates of efficacy of disease intervention studies, where distinction can be made between markers of immunological and clinical efficacy and safety. Since intervention in T1D requires immunotherapy targeting autoreactive T cells, it is conceivable that the

immunological efficacy and safety of immune intervention can be monitored by studying changes in T cell autoreactivity to islets, provided that methods are available that allow sensitive, specific and reproducible measurement of immune responses relevant to disease. We have designed and implemented novel T cell assays or monitoring of three clinical trials (including islet transplantation). This has been highly informative regarding immunological and clinical efficacy and outcome. However, additional and complementary assays are required to study the effector T cell population and to define immunological endpoints of protection.

Immunogenetic biomarkers

A first type of biomarkers that may prove suitable to assess or demonstrate immunological or therapeutic efficacy is provided by the genome. As discussed earlier, HLA constitutes close to 50% of the genetic predisposition to T1D. In appreciation of the role of HLA in thymic education and antigen-presentation, it is mandatory to use HLA-matched patient and control populations to study disease associations and during randomization of patients for clinical trials.

In addition, other genetic polymorphisms may contribute to disease progression and modulation (Fig. 2). We have discovered a functional polymorphism of several immunogenic and metabolic components that are associated with development of T1D (Eerligh et al 2004, Zhernakova et al 2005a, b). Recently, we identified a contribution of innate factors such as RANTES, KIR (van der Slik et al 2003), MICA (Alizadeh et al 2007a) and mannose binding lection (MBL) (Bouwman et al 2005) that could only be found to associate with disease upon stratification

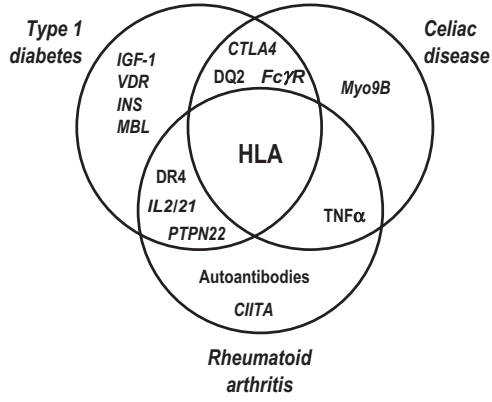

FIG. 2. Genetic markers of type 1 diabetes. Similarities and differences of functional genetic polymorphisms and immune factors contributing to disease predisposition in type 1 diabetes, rheumatoid arthritis and coeliac disease point to shared and specific disease pathways.

for genetic background. Very recently, we discovered a functional genetic polymorphism of IFNγ that associated with disease progression and natural remission (see below) (Alizadeh et al 2006). We propose that other genetic polymorphisms may contribute to immunological and clinical efficacy. For example, several polymorphisms of the human vitamin D receptor (VDR) gene have been identified that are associated with T1D (Koeleman et al 2002), with the FokI polymorphism resulting in VDR proteins with different structures. In transfection experiments, the presence of the shorter F-VDR resulted in higher NF-κB- and NFAT-driven transcription as well as higher IL12p40 promoter-driven transcription (van Etten et al 2007). Concordantly, in human monocytes, dendritic cells and lymphocytes will exert differential activities dependent of their VDR genotype. Consequently, therapy of T1D patients with $1,25(OH)_2$ vitamin D3 may have differential efficacy that is associated with VDR polymorphism (pharmacogenetics). Other immune intervention strategies, involving antibody therapeutics, may result in different efficacy or adverse events as a result of functional genetic polymorphisms of the FcgRII and III genes. Indeed, we recently demonstrated that such polymorphisms contribute to susceptibility to autoimmune diseases that are accompanied by tissue autoantibodies (Alizadeh et al 2007b).

Immune markers of insulitis

An important bottle neck in our efforts to define immunological parameters that may act as surrogates or endpoints for insulitis is the inaccessibility of the target organ in humans. While our studies are limited to peripheral blood, it is conceivable that the conditions in the affected microenvironment are distinct (Fig. 1). Even though it remains important to appreciate this notion, there are several indications supporting an interpretation that certain immune abnormalities mirror the immune responses in inflamed islets. These observations include cytokine levels in serum and islet autoreactive $CD4^+$ and $CD8^+$ T cells that home to pancreatic tissue after injection into immune-incompetent mice (van Halteren et al 2005). Furthermore, recurrent autoimmune β cell destruction of islet allografts was marked by increases in precursor frequencies of circulating islet autoreactive $CD8^+$ T cells (Pinkse et al 2005). The fact that insulitis shows patchy distributions in a given pancreas, implies that immune cells associated with insulitis and β cell destruction recycle and circulate to reach unaffected pancreatic tissue (Fig. 1).

Honeymoon. We studied whether serum cytokine levels and their corresponding functional polymorphic genotypes are associated with partial remission of T1D in an international multi-centre study including patients with newly diagnosed T1D followed for 3 months and characterized for remission status, and matched controls. Serum IFNγ concentrations predicted by genotype and observed serum

levels were discordant in remitters, suggestive of regulation overruling genetic predisposition. Although high-producing genotypes were less frequent in remitters, they were predictive of remission in combination with low serum IFNγ levels. These data imply that remission is partially immune-mediated and involves regulation of IFNγ transcription (Alizadeh et al 2006). Furthermore, all of the 17 immune mediators studied showed remarkable intra-individual stability in their systemic concentrations over time. As a consequence, partial remission was not accompanied by changes in mediator levels except for a moderate decrease of IL1ra concentrations and IL10 concentrations in non-remitters. Baseline levels were associated with the later clinical course in that low levels of IFNγ, IL10 and IL1R1 concentrations were observed in partial remitters. The systemic immunoregulatory state at diagnosis of T1D therefore seems predictive of clinical improvement during the remission phase (Schloot et al 2007).

T cell assays for monitoring peptide therapy. A randomized, double-blind, phase Ib/II clinical trial of DiaPep277 peptide treatment was performed in recent-onset T1D patients with remaining insulin production (Huurman et al 2007). We studied the immunological efficacy of this peptide therapy and correlated this with clinical outcome in 48 C-peptide positive patients assigned subcutaneous injections of DiaPep277 or placebo. All treated patients at each dosage of peptide demonstrated an altered immune response to DiaPep277 while the majority of placebo-treated patients remained non-responsive to treatment, indicating a 100% efficacy of immunization. Cytokine production in response to therapy was dominated by IL10. IL10 production before therapy and development of tolerance defined by autoantigen-specific T cell proliferation were associated with preserved β cell function. Third-party control immune responses were unaffected by therapy, while no potentially adverse immunological side effects were noted. This study underscores that the immunological monitoring that we designed and applied proved instrumental to demonstrate that a peptide drug is immunogenic in type 1 diabetic subjects and has immune modulating properties. Immunological monitoring distinguished therapy from placebo treatment and could determine immunological efficacy. Tolerance to peptide DiaPep277 treatment may serve as an immunological biomarker for clinical efficacy (Huurman et al, submitted).

Very recently, a phase II study was reported at the EASD in Amsterdam on immunization with islet autoantigen (randomized, double-blind, placebo-controlled, multi-centre trial) including 70 T1D children diagnosed within the previous 18 months (M Faresjo, 43rd Annual Meeting EASD, 18–21 September, 2007). Thirty-five patients were randomly assigned to 20 μg of GAD65 (Diamyd™, Diamyd Therapeutics AB, Stockholm, Sweden) and 35 patients to placebo (buffer alone) in a primary injection at day one and a booster of the same concen-

tration four weeks later. The spontaneous secretion of cytokines and chemokines and FoxP3 mRNA expression did not differ between children treated with GAD65 or placebo either before or 15 months after treatment. However, after *in vitro* stimulation with GAD65, patients treated with Diamyd differed significantly from the placebo group, 15 months after the immune intervention. GAD65 induced higher secretion of various cytokines and chemokines in Diamyd-treated patients. Increased FoxP3 mRNA expression by GAD65 stimulation was observed in T1D children treated with Diamyd. Treatment with GAD65 caused a specific immune response to GAD65 that lasted at least 15 months, which indicates that a specific cell population has been induced. The type of immune response invoked concurred with the incentive to modulate the immune response to GAD65 to anti-inflammatory profile. Importantly, we demonstrate that the desired immune correlates can be identified and that such biomarkers of mechanistic efficacy can be measured that associate with therapy with Diamyd. The next challenge is to define which of these associate with clinical efficacy of therapy. In other words, can we define immunological surrogates of clinical efficacy?

T cell assays for monitoring islet transplantation. Islet transplantation provides a unique opportunity to monitor islet destruction within a relatively brief time window. The Leiden laboratory has developed and evaluated technologies that allow detection and characterization of immune components associated with recurrent autoimmune destruction of pancreatic islets (applying proliferation assays, cytokine production) or rejection of islet allografts (definition of precursor frequencies of alloreactive CTLs or $CD4^+$ T cells through automated limiting dilution analysis). These methods have been applied extensively in the longitudinal monitoring of more than 80 T1D patients transplanted with islet allografts under different protocols, as part of JDRF Center for β-Cell Therapy in Europe (Leiden is the Immunological Monitoring Satellite). Although our immunomonitoring is always performed without knowledge of the clinical outcome of the β-cell substitution, our results on T cell auto- and alloreactivity corresponded with β-cell function without exception thus far. The results indicate that immune responses against islet allografts:

(1) are associated with loss of β cell function (Roep et al 1999b);
(2) identify factors associated with (loss of) operational tolerance against the islet allografts in repeatedly transplanted type 1 diabetic patients (van Kampen et al 2005);
(3) are associated with, and distinguish, different immune suppressive protocols (both induction therapy and maintenance immune suppression), allowing improvement of the immune suppressive therapy (Roep et al 1999b, Roep 2003, Keymeulen et al 2006).

We could identify CTL autoreactivity against a β cell epitope, and its association with recurrent β cell destruction after islet transplantation using insulin-specific HLA class I tetramers. Preliminary data show that an elevated insulin $B_{10–18}$ specific CTL precursor frequency in the peripheral blood of islet recipients precedes recurrent autoimmunity and islet allograft destruction (Pinkse et al 2005). Additional biomarkers that have been associated with loss of islet allograft function include increases in mRNA for granzyme REF, increases and/or seroconversion of islet autoantibodies (Bosi et al 2001) and cases showing changes in precursor frequencies of $CD4^+$ T cells against GAD65 (staining with HLA-$DR4^{GAD65}$ tetramer).

Summary

It is our experience that T cell assays may be applied in an appropriate context, with careful attention to study design and choice of reagents and technologies. We are entering an exciting era in which technologies (e.g. ELISPOTs, tetramers) can be combined with advances in epitope knowledge in monitoring strategies that are tailored to specific clinical trials. Several immunological biomarkers have been identified that correlate with disease activity, progression, remission and modulation, and islet allograft survival in type 1 diabetes (Table 1). Additional immune markers of immunological safety (allergic reaction, epitope spreading) and efficacy and clinical safety (no acceleration of disease) and efficacy (clinical benefit) are required. It is anticipated that new markers of immune modulation will come out of the immune monitoring of the different clinical trials in which we participate. It is conceivable that endpoints will between candidate intervention strategies.

TABLE 1 Immunological biomarkers of disease activity, progression, remission and modulation, and islet allograft survival in type 1 diabetes

T1D	*Remission*	*Intervention*	*Islet Tx*
(high affinity) AAb	Loss of T cell responses	IL10	Autoreactive $CD4^+$ T cells
IFNγ	IL10	IFNγ/IL10	IL10 induction
IL10↓	IFNγ ↓	tolerance	Autoantibodies
MBL		FoxP3 Mrna	CD8 alloreactivity
IP10			Pre-allo Abs
RANTES			Granzyme mR NA
T cell autoreactivity			HLA-$DR4^{GAD65}$
Impaired Tregs			Autoreactive $CD8^+$ T cells

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DISCUSSION

Kay: For someone who is looking for a biomarker to include in their clinical trial, what is the bottom line? Is there something that is robust enough?

Roep: I would say that the lowest hanging fruit will be CD8 tetramers. This is something we find quite rewarding, although we need to find more about which epitopes to look for.

Peakman: When you see a tetramer, is there any relationship to whether the islet is an A2 islet?

Roep: Yes. In the first series we always had HLA A2 donor islets, so we couldn't test for this. You only see it if there are HLA A2 islets in there.

Butler: Is there any predictive power from your islet transplant data for pancreas transplant?

Roep: We haven't done this. With pancreas transplant we only did cross-sectional studies. We are a little bit a victim of the success of pancreas transplantation, because the one year survival with insulin independence is 93% and five years it is 86%. We hardly see any reactivity in those patients. We do see correlations with what induction therapies those patients have had. If we compare daclizumab versus ATG we can see different patterns of reactivity. CMV viraemia is seen after ATG, but not daclizumab.

Bonifacio: With respect to rejection, you said that no one would transplant cross-matched positives. So why are we transplanting GAD-containing islets into GAD T cell responsive patients. We must be doing it in pancreas transplantation and it is working.

Roep: I'm saying that in the case of islet transplantation, the immune suppression isn't successful. This could be due to differences in vascularization between islet grafts and pancreas grafts, or the place of implantation. I don't know.

von Herrath: The islets are usually transplanted into an immunologically active site in the liver.

Roep: It must be privileged.

Butler: Part of the motivation behind my question was to get some insight into why islet transplantation is so unsuccessful compared with pancreas transplantation which works quite well. This doesn't seem to matter in the context of pancreas transplantation, yet you showed it matters a lot in islet transplantation.

von Herrath: I would think there are two issues. First, access. Islets in the whole pancreas are in an environment different from the intrahepatic injection, which is immunologically like it normally is for them. Second, there is a higher chance that pancreas or a combined pancreas/kidney transplant will better tolerize for itself.

Peakman: You may have anatomically isolated the islets in pancreas/kidney, because you have lost lymphoid drainage, but in the liver lymphatics are still present.

Roep: In rats we looked at proteasome staining of isolated islets. The rim of those islets is stuffed with immuno-proteasomes in endocrine cells. The way we treat islets is important. We can induce all kinds of ER stress.

Pipeleers: I agree with Matthias that there are many reasons to see the liver as a threat for islet cell transplants. The only way to know is to compare with the outcome of implants in another site.

The better outcome of pancreas transplants can be attributed to many differences such as in the microanatomy of the islets, the total islet mass that is transplanted and the immediate revascularization. In pancreas transplant recipients there is an almost immediate normalization of glycaemia after transplantation; this has so far not been seen in islet cell transplants. When we measure the capacity of an islet cell graft in insulin-independent patients one year after transplant it is 25% of the controls. This number indicates that we don't transplant enough material or that we lose too much after injection.

Bresson: In the Diamyd trial the patients are recruited based on anti-GAD65 antibody. Is there a correlation between the level of GAD65 antibody and the capacity to respond to the therapy?

Roep: That's an interesting question. It was compared because some of these titres went sky high. We selectively looked at these seven patients who had excep-

tionally high autoantibody titres and of these, four had significantly better β cell preservation.

Bresson: You showed that in transplantation, a response against GAD65 is not good in term of graft survival.

Roep: No. If you respond to one antigen alone, it isn't all that bad at all. It is the double reactivity that is bad.

Flavell: Since you mentioned the location of the graft in the liver and potential concerns, has anyone compared liver to kidney capsule in the mouse?

Bonifacio: Yes, the kidney is much better.

Insel: Some people believe the draining lymph nodes that accompany a whole-organ transplant may be beneficial.

Roep: That's an interesting idea.

Re-establishing immune tolerance in type 1 diabetes via regulatory T cells

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Abstract. Type 1 diabetes (T1D) is a disease in which tolerance to self-antigens, such as insulin, is broken leading to expansion of autoreactive T cells that attack pancreatic β cells with consequent loss of insulin production. Regulatory T cells (Tregs) represent a specific T cell subset that plays a key role in inducing and maintaining immunological tolerance to self and non-self antigens. The naturally occurring $CD4^+CD25^+$ Tregs (nTregs) originate from the thymus, constitutively express the transcription factor FOXP3, and suppress immune responses mainly via cell–cell contact. Depletion of nTregs results in systemic autoimmune diseases in mice and, vice versa, transfer of nTregs prevents development of autoimmune diseases. Regulatory T type 1 (Tr1) cells are inducible Tregs generated in the periphery by chronic exposure to antigens in the presence of interleukin (IL)10. Tr1 cells are defined by their unique cytokine production profile (i.e. $IL10^{++}$, $IL5^+$, $TGF\beta^+$, $IL4^-$, $IL2^{low}$, $IFN\gamma^{low}$). Tr1 cells are induced by a specialized subset of tolerogenic dendritic cells and suppress undesired immune responses mainly through production of IL10 and TGFβ. Interestingly, Tr1 cells modulate responses to self-antigens such as insulin- and islet-derived peptides. *In vitro* expansion/induction of Tregs can be therefore envisaged as a therapeutic tool for re-establishing self-tolerance in T1D subjects.

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T1D development

Type 1 diabetes (T1D) is an autoimmune disorder in which autoreactive T cells attack pancreatic β cells, leading to life-long dependency on insulin (Eisenbarth 1986). The mechanisms regulating development and activation of pathogenic T cells remain largely unclear. It is hypothesized that T1D development in human and in non-obese diabetic (NOD) mice, an animal model for human T1D, is dependent on the breakdown of self-tolerance to immunodominant epitopes of autoantigens (Bach & Chatenoud 2001). A number of target autoantigens including insulin, pro-insulin, glutamic acid decarboxylase (GAD65), islet-cell auto-

antibody (ICA or IA-2), heat shock protein (HSP60) and islet-specific glucose 6 phosphatase catalytic subunit related protein (IGRP), are presented to T cells in the context of MHC class I and class II molecules. Circulating autoreactive T cells, which recognize these autoantigens, have been identified in both normal donors and T1D patients and are thought to play a direct role in T1D immunopathogenesis (Arif et al 2004, Atkinson et al 1992, Hawkes et al 2000). Recent evidence that insulin is a primary β cell-specific autoantigen in T1D includes the findings that NOD mice deficient in pro-insulin I and II but maintaining the expression of an altered insulin molecule, fail to develop diabetes (Nakayama et al 2005). Furthermore, insulin-reactive T cell clones have been isolated from the pancreatic lymph nodes of long lasting T1D patients (Kent et al 2005).

T cell tolerance is established centrally in the thymus and further strengthened and maintained through multiple mechanisms of peripheral tolerance. These mechanisms can be broadly divided into three general categories: deletion of autoreactive T cells, failure of autoreactive T cell activation (including the absence of the necessary activating signals or avoidance of antigen presentation), and active immunoregulation involving the action of other cells, such as regulatory T cells (Tregs). It is now evident that a group of Tregs residing within the $CD4^+$ T cell population is involved in maintaining peripheral tolerance and preventing organ-specific autoimmune diseases.

Regulatory T cells

Different types of $CD4^+$ Tregs have been described (reviewed in Shevach 2006), but the most characterized subset is composed of $CD4^+CD25^+$ regulatory T cells (Tregs). Murine and human $CD4^+CD25^+$ Tregs are defined naturally occurring Tregs (nTregs) since they are selected in the thymus and exit into the periphery where they represent about 5–10% of the peripheral $CD4^+$ T cells. nTregs contribute to maintain tolerance by down-regulating undesired immune responses to self and non-self antigens (reviewed in Sakaguchi 2005). nTregs are defined on the basis of constitutive expression of high levels of CD25 (IL2Rα) and the transcriptional repressor factor Foxp3 (FOXP3 in humans). Importantly, both these markers are also expressed by conventional $CD4^+$ non-Tregs upon activation and this highly limits their usage as specific tracers for nTregs. Recently, the IL7R (CD127) and the CD39/CD73 have been proposed as additional markers useful for the characterization and isolation of *bona fide* nTregs, which are $CD127^{low/neg}$ (Liu et al 2006, Seddiki et al 2006) and co-express CD39 and CD73 (Borsellino et al 2007, Deaglio et al 2007). nTregs are characterized by the inability to produce IL2 and the anergic phenotype *in vitro*. This anergy can be broken by the addition of sufficiently potent stimuli such as the cytokines IL2 and IL15. In contrast, nTregs do undergo clonal expansion upon exposure to antigen *in vivo* while retaining their

suppressive properties. Importantly, IL2 signalling is required for nTreg generation, and mice deficient in IL2, CD25 or CD122 suffer from severe lymphoproliferative and autoimmune disorders that can be prevented by transfer of wild-type $CD4^+CD25^+$ Tregs (reviewed in Sakaguchi 2005).

The suppressive targets of nTregs include cells from the adaptive as well as the innate immune system (Fehervari & Sakaguchi 2004). Mixed lymphocyte reactions between Tregs and their potential targets demonstrate that nTregs are capable of suppressing the proliferation and cytokine production of conventional $CD4^+$ and $CD8^+$ T cells. Other studies have also demonstrated that innate immune cells, such as dendritic cells (DCs) and monocytes can be the target of nTreg suppression (Fehervari & Sakaguchi 2004). In order to achieve nTregs suppressive function, TCR and IL2 stimulation is necessary (Piccirillo & Shevach 2004). However, once stimulated, regulation by nTregs is independent of the antigen specificity of the target cells. Histocompatibility between nTregs and their target cells is also not absolutely required for suppression. There is abundant evidence showing that nTregs suppression requires direct cell–cell interaction without the need of cytokines, since supernatants from activated nTregs do not possess detectable suppressive properties (Fehervari & Sakaguchi 2004). One line of evidence suggests that TGFβ expressed on the membrane of nTregs may exert a regulatory effect via membrane-proximal mechanisms and not necessarily act as a soluble factor (Nakamura et al 2001). nTregs mediate their regulatory function by a cell–cell-mediated mechanism involving cytotoxic effector functions associated with the synthesis of perforin, CD18 and granzyme A. This effect is Fas independent. Targets of the cytotoxic effects encompass $CD4^+$ and $CD8^+$ T cells, monocytes, antigen-presenting B cells, and DCs (Grossman et al 2004). An alternative mechanism of nTreg suppression is down-regulation of co-stimulatory molecules on APC, which render them ineffective to promote activation of effector T cells (Teff) (Fehervari & Sakaguchi 2004). Overall, nTregs suppress immunological responses in multiple ways, which may involve negative signals produced by inhibitory nTreg surface molecules, cytotoxic killing, down-regulation of APC function, as well as a number of other yet unknown cell–cell interactions.

Another important category of Tregs is represented by the inducible Tregs, which, on the contrary to nTregs, are generated *in vivo* in the periphery under various tolerogenic conditions. The regulatory T type 1 (Tr1) cells are one of the best-characterized inducible Tregs, and were originally described by our group (Groux et al 1997). Phenotypically Tr1 cells are similar to nTregs since they are anergic *in vitro* and express CTLA4 (Roncarolo et al 2006). However, in contrast to nTregs, Tr1 cells do not express high levels of CD25 or FOXP3, and are characterized by the unique cytokine production profile: they secrete high levels of IL10 and TGFβ, low amounts of IFNγ and IL2, and no IL4. Tr1 cells mediate

suppression through the release of IL10 and TGFβ, which inhibit proliferation and cytokine production of $CD4^+CD25^-$ T, Th1, and Th2 cells. In addition, human Tr1 cells modulate the production of immunoglobulin by B cells (Satoguina et al 2005) and modulate the antigen-presenting capacity of monocytes and DCs (Roncarolo et al 2006). Tr1 cells specific for a variety of antigens, including self-antigens, have been generated both *in vitro* and *in vivo* (Roncarolo et al 2006). IL10 is considered the driving force for Tr1 cell generation, as shown by experiments in which antigen-specific murine Tr1 cells can be induced *in vitro* by repeated TCR stimulation in the presence of high doses of IL10 (Groux et al 1997). IL10 is therefore not only responsible for the regulatory function of murine Tr1 cells, but it is also fundamental for their differentiation. However, for human Tr1 cells it is now evident that IL10 is necessary but probably not sufficient for their differentiation. We demonstrated that, in an *in vitro* system using artificial APC, addition of exogenous IL10 results in a relatively small increase in IL10-producing Tr1 cells (Levings et al 2001), whereas co-addition of IFNα, which further promotes autocrine IL10 production, results in efficient differentiation of human $CD4^+$ Tr1 cells (Levings et al 2001). Interestingly, TGFβ is not required for the induction of human Tr1 cells *in vitro* (Levings et al 2001), although it is involved in the effector function of Tr1 cells and synergizes with IL10 to promote allo-antigen hyporesponsiveness in murine $CD4^+$ T cells (Zeller et al 1999). Additional stimuli have been described to be involved in Tr1 cell induction, including signalling via CD2, the ligand for CD58 (Wakkach et al 2001), and co-signalling via CD46 (Kemper et al 2003). However, it is still unclear whether the CD3/CD46-stimulated T cells are *bona fide* Tr1 cells or if they represent a distinct inducible Treg, which is not anergic and antigen-specific. Furthermore, we recently reported that an anti-human CD45RO/RB monoclonal antibody (mAb) is a potent immunomodulant that induces antigen-specific anergic T cells, which display a Tr1 phenotype and suppress IFNγ production and proliferation of effector T cells via IL10 and TGFβ (Gregori et al 2005).

Not only soluble factors but also specialized APC can generate Ag-specific Tr1 cells. Repetitive stimulation of human naïve cord blood $CD4^+$ T cells with allogeneic immature dendritic cells (iDCs) results in the differentiation of IL10-producing Treg cells (Jonuleit et al 2000), which suppress T cell responses via a cell-contact dependent mechanism. Furthermore, we reported that repeated stimulation of naïve peripheral blood $CD4^+$ T cells with allogeneic iDCs induces the differentiation of human Tr1 cells *in vitro*. In this system, T cells become increasingly hyporesponsive to re-activation with mature DCs and after three rounds of stimulation with iDCs, they are profoundly anergic and are phenotipically and functionally superimposable with Tr1 cells (Levings et al 2005).

Both biological and pharmacological agents can modulate DCs to render them tolerogenic (Adorini et al 2004). Myeloid DCs become tolerogenic by treatment

with immunomodulatory cytokines, such as IL10, either alone or in combination with IFNα (Manavalan et al 2003), G-CSF (Rutella et al 2004), and hepatocyte growth factor (Rutella et al 2006), or with immunosuppressive agents such as vitamin D3, either alone (Penna et al 2005) or in combination with dexamethasone (Pederson et al 2004). The resulting tolerogenic DCs produce high amounts of IL10 but low/negative levels of IL12, and express tolerogenic markers such as ILT3 and/or ILT4. We recently identify a new subset of tolerogenic DCs, termed DC10, which are present *in vivo* and can be differentiated *in vitro* in the presence of exogenous IL10. DC10 are $CD14^+CD11c^+CD11b^+HLA\text{-}DR^+CD83^+$, display a mature myeloid phenotype (i.e. $CD80^+CD86^+$), express high levels of the tolerogenic markers ILT2, ILT3 and ILT4, and the non-classical MHC class I molecule HLA-G, and secrete high levels of IL10 but low amounts of IL12. DC10, either *in vitro* differentiated or *in vivo* isolated, poorly stimulate allogeneic $CD4^+$ T cells, and promote anergic T cells. Importantly, *in vitro* differentiated DC10 efficiently promote the differentiation of allo-specific Tr1 cells, which produce IL10 and have strong suppressive activity after only one round of stimulation (S. Gregori et al, manuscript submitted). These results suggest that DC10 can be used to promote antigen-specific Tr1 cells.

Regulatory T cells and T1D development

Several autoimmune diseases have been documented to be secondary to the loss of nTregs in mice. The seminal studies performed by Sakaguchi and colleagues involved the transfer of $CD4^+CD25^+$ depleted T cells into neonatally thymectomized mice which resulted in the onset of systemic autoimmune diseases such as colitis, gastritis, insulin-dependent autoimmune diabetes, and thyroiditis (Asano et al 1996). The co-transfer of disease-inducing cells with $CD4^+CD25^+$ nTregs resulted in disease prevention. These studies demonstrated that in mice $CD4^+CD25^+$ T cells can regulate the responses of autoreactive T cells *in vivo*.

Most studies concerning the role of Tregs in human autoimmune diseases document either decreased frequencies or defective suppressive functions of Tregs. Of particular interest is the wide variety of human autoimmune diseases in which a defect in Treg function has been demonstrated, raising the interesting possibility that this may be a common denominator causing uncontrolled immune responses to self-Ags (Baecher-Allan et al 2004). T1D is a clear example. It is now accepted that there is no significant difference in the frequency of nTregs in T1D patients and control subjects. In three out of four performed studies it has been demonstrated that T1D subjects have a defective nTreg suppressive ability (Brusko et al 2005, Glisic-Milosavljevic et al 2007, Lindley et al 2005). In contrast, Putnam et al did not find any defective nTreg suppressive function (Putnam et al 2005). Interestingly, it was recently shown that there is a higher level of ongoing apoptosis

in nTregs in recent-onset T1D and in subjects at risk for the disease. This high level of nTreg apoptosis might be a contributing factor to the decreased suppressive potential on these cells in T1D patients (Glisic-Milosavljevic et al 2007). In conclusion, the idea that nTregs play a crucial role in controlling T1D in humans begins to emerge.

A defect in Tr1 cells in T1D patients has also been postulated. T cells from non-diabetic individuals carrying HLA class II molecules associated with the disease (i.e. HLA-DR4) show an IL10 response to islet peptides while T cells from diabetic subjects produce predominantly IFNγ in response to the same antigens (Arif et al 2004). Therefore, islet destruction is characterized by pro-inflammatory autoreactive T cells while the tolerant non-diabetic state is characterized by autoreactive Tr1 cells that secrete the immunoregulatory cytokine IL10.

Regulatory T cells and T1D cure

It is now clear that Tregs represent an essential tool through which the immune system can actively control T1D. Therefore, therapeutic *ex vivo* or *in vivo* induction of Tregs might be highly advantageous in this disease. Much progress has already been made in animal models, which proved that cellular therapy with Tregs is a feasible approach (Roncarolo & Battaglia 2007).

When Steinman and colleagues expanded nTregs from BDC2.5 TCR-Tg mice using NOD-derived DCs, highly suppressive nTregs were obtained which potently blocked diabetes development in an adoptive transfer model. Interestingly, very few NOD-DC-expanded nTregs were needed to suppress disease, while freshly isolated nTregs had little effect on disease incidence and progression (Tarbell et al 2004). Most interesting, the same authors recently demonstrated that DC-expanded nTregs induced long-lasting reversal of hyperglycaemia in 50% of NOD mice in which overt diabetes had developed (Tarbell et al 2007). Similarly, Bluestone and colleagues were able to suppress diabetes in adoptive transfer models using NOD or BDC2.5 TCR-Tg Tregs that were expanded in the presence of anti-CD3, anti-CD28 and IL2 (Tang et al 2004). As expanded nTregs exhibit more potent suppressive activity than un-stimulated nTregs, it cannot therefore be excluded that nTregs had further differentiated after their initial activation. Expansion protocol has been recently considered to increase both nTreg number and suppressive function. Several studies reported that nTregs can be expanded *ex vivo* by potent stimulation via TCR and high doses of IL2 (Godfrey et al 2004, Hoffmann et al 2004). However, it is important to highlight that this cell culture condition is highly advantageous also for Teff that eventually contaminate the purified $CD4^+CD25^+$ cells. Indeed, in humans even the brightest $CD25^+$ T cells contain ~30–50% of *in vivo* recently activated T cells which ultimately outgrow nTregs upon

prolonged culture (Levings et al 2002). We demonstrated that addition of rapamycin to the expansion culture significantly reduces the risk of undesired Teff proliferation since rapamycin selectively allows growth of nTregs while affects proliferation of Teff (Battaglia et al 2006a). Interestingly, rapamycin can efficiently expand nTregs also from peripheral $CD4^+$ T cells isolated from T1D patients (Battaglia et al 2006a).

In vivo induction of Tregs is an alternative therapeutic option. We showed that combined treatment with rapamycin+IL10 blocks T1D development in NOD mice through induction of both nTregs and Tr1 cells. Rapamycin expands the nTregs in the pancreas while IL10 promotes the differentiation of Tr1 cells in the spleen. These two Tregs may act through different mechanisms: $CD4^+CD25^+$ nTregs block T cell proliferation of pancreatic autoaggressive T cells, while Tr1 cells block proliferation and migration of effector T cells to the target organ (Battaglia et al 2006b).

Anti-CD3 mAbs were originally developed as a way of inducing immune suppression by depleting T cells (reviewed in Chatenoud 2003). More recent studies, however, indicate that anti-CD3 mAbs can also modulate immune responses in humans (Keymeulen et al 2005). A single course of treatment with a non-FcR binding anti-CD3 mAb, leads to preservation of insulin production in patients with new-onset type 1 diabetes (Keymeulen et al 2005, Herold et al 2002). The sustained insulin production correlates with improved glucose control and reduced use of insulin. This mAb appears to deliver an activation signal to T cells resulting in disproportionate production of IL10 relative to IFNγ *in vitro* and detectable levels of IL10, IL5, but rarely IFNγ or IL2 in the serum of treated patients. In addition, this treatment induces a population of $IL10^+CCR4^+$ $CD4^+$ T cells *in vivo* (Herold et al 2003). It is therefore possible that anti-CD3 mAbs act as inducers of Tr1 cells *in vivo*. On the other hand, in murine studies, anti-CD3 mAb treatment has been shown to induce *de novo* $CD4^+CD25^+$ Tregs, which suppress through a TGFβ-dependent mechanism (Belghith et al 2003). As an alternative approach, anti-CD3 mAb together with intranasal administration of pro-insulin proved to be effective in reversing recent-onset diabetes in NOD mice with a higher efficiency than with monotherapy with anti-CD3 alone or antigen alone. *In vivo* expansion of nTregs and insulin-specific Tregs producing IL10, TGFβ and IL4 was observed in this model (Bresson et al 2006).

Overall, these findings in animal models and patients indicate that selected immunomodulatory compounds can efficiently induce Tregs either *ex vivo* or directly *in vivo*. New immunomodulatory compounds proposed for the cure of T1D are now being selected also by their ability to promote Treg function and/or expansion/generation *in vivo*. Alternatively, adoptive transfer of *ex vivo* expanded/generated Tregs is currently under evaluation as a valid therapeutic tool for the cure of T1D (Roncarolo & Battaglia 2007). The coming years will be of a great importance

for defining the therapeutic potential of Tregs in the context of T1D and also in many other T cell-mediated diseases.

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DISCUSSION

Tree: It was nice to see the turning of the Th1 cells into a more Tr1 phenotype, but if you believe the data that Hermann Waldmann has presented on transcription factors shared among different T cell subsets, it suggests that turning a Th2 into a Tr1 it is going to be much easier than turning a Th1 into a Tr1. Have you tried taking haemagluttinin-specific Th1 cells from people primed with influenza antigen and seeing whether you can turn these into Tr1s?

Gregori: No, we haven't done yet these experiments but it is true that the differentiation of Tr1 cells from antigen-specific polarized Th1 cells has not been demonstrated so far.

Roep: You want to make insulin-specific Tregs, and you showed that there is linked suppression. Since insulin is a systemic antigen, this would imply that you would get systemic suppression.

Gregori: IL10 is an immunosuppressant cytokine that can have a broad effect. However, it has to be taken into account that antigen-specific Tr1 cells secrete IL10 and TGFβ only after activation with their specific antigen, but once activated they can mediate some levels of bystander suppression. Bystander suppression would be limited to the site where Tr1 cells localize, as no pan immunosuppression has been observed in mouse models or patients with high numbers of circulating Tr1 cells (Battaglia et al 2006, Bacchetta et al 1994).

Roep: But the insulin is systemic.

Gregori: Antigen-specific Tr1 cells are recruited in the target organ where are activated via their specific antigen presented by antigen-presenting cells. In case of insulin-specific Tr1 cells they will be recruited in the pancreas and will suppress effector T cells locally and not systemically.

Roep: Theoretically, I was struggling with the fact that you say there is linked suppression, and you have antigen-specific Tregs. So wherever Tregs see insulin processed and presented by the DCs, they would exert their suppressor function.

D Hafler: If you take DCs out of the blood, they won't trigger insulin-reactive T cells.

Roep: I thought you might want to choose another antigen!

Herold: How would you ensure the stability of the cell phenotype? If you take the rapamycin away, how will you know *in vivo* that these are still Tregs?

Gregori: The overall idea is to inject rapamycin-expanded Tregs and treat patients *in vivo* with rapamycin.

Herold: Have you thought about screening assays that you might use to check your product, to make sure it is what you think it is? Is there an *in vivo* assay?

Gregori: We were thinking to validate our product *in vivo*. We have a model of human islet transplantation in NOD/SCID mice. In these mice injection of allogenic peripheral blood mononuclear cells (PBMCs) promotes islet rejection. We can therefore envisage injecting Treg cells expanded *in vitro* in the presence of rapamycin together with autologous PBMCs to determine whether they can prevent islet allograft rejection in NOD/SCID mice.

D Hafler: In terms of treating a long-term autoimmune disease, do you have any idea of the half-life of the natural Tregs and the infused Tr1 cells? There are some data in humans suggesting that the half-life of differentiated Tregs is short.

Gregori: We have a pilot clinical trial of adoptive transfer of IL10 anergized T cells containing Tr1 cells after bone marrow transplantation. Clinical results in

a few patients tested are promising but we do not have specific markers to directly follow Tr1 cells after infusion. We detect IL10 in the serum (low but detectable levels) of treated patients and we are using different methods to determine the presence of IL10/Tr1 cells *in vivo* including T cell cloning of in vivo expanded T cells with specific Vβ.

D Hafler: How long have you monitored the injected patients for?

Gregori: The range time follow-up is between 0.5–2.5 years. Sometimes these patients have infection that can trigger graft versus host disease that is moderate and can be controlled by immunosuppression.

Insel: Can you comment on the side effects of IL10 when used *in vivo*?

Gregori: IL10 has an effect on B cells. It can prime B cells to produce Ig switching. But we plan to treat patients with IL10 and rapamycin in order to control these possible side effects.

Bonifacio: The safety profile of IL10 is good in trials.

Kay: But IL10 died in trials, didn't it?

Bonifacio: The reason it was stopped is because Schering had another drug that would work better.

D Hafler: Have you ever thought of making a bifunctional anti-CD3/anti-CD46 monoclonal antibody? It really whacks IL10 secretion.

Gregori: No. We were not able to generate Tr1 cells using anti-CD46 mAb.

D Hafler: It depends which one you use. There is only one that works.

Gregori: We tried to reproduce the data of the *Nature* paper (Kemper et al 2003) however we did not generate Tr1 cells.

D Hafler: We were able to reproduce these data.

Flavell: Going back to your point, Matthias von Herrath, incremental IL10 might give much more, so it's not that crazy an idea. Naïve T cells plus IL10 give Tr1s.

von Herrath: Do we know from the previous experiments that you can map Tr1 cell efficacy to IL10 only? Do Tr1 cells operate just by IL10 or also by other factors?

Gregori: Tr1 cells suppress T cell responses via both IL10 and TGFβ.

von Herrath: In terms of transferring IL10-positive Tr1s compared to their IL10 negative counterparts, and similar sorting experiments, what are the results? You could also put them in an IL10-deficient host to see whether host IL10 is needed.

Gregori: Transfer in SCID mice of OVA-specific Tr1 prevents inflammatory bowel disease induced by pathogenic $CD4^{+}CD45RB^{hi}$ splenic T cells. Importantly, Tr1 cells are effective only upon stimulation *in vivo* by feeding the mice with OVA. In these experiments the role of IL10 was not investigated. However, in other systems it was demonstrated that disease protection mediated by Tr1 cells is abrogated by administration of anti-IL10R mAbs.

Bonifacio: There is also the transplant work by Manuela (Battaglia et al 2006) where she has tolerance and then adds anti-IL10 receptor she loses it.

von Herrath: That's convincing.

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Translating mucosal antigen based prevention of autoimmune diabetes to human

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Abstract. Mucosal administration of autoantigen (insulin) to animal models has been demonstrated to be effective in preventing autoimmune diabetes. Efficacy is dependent upon the dose and the age at which it is delivered. Because of its low toxicity, mucosal administration of insulin represents an attractive preventive therapy in human. Translation of what is efficacious in animal models is, however, challenging. We have proposed mucosal insulin vaccination as a primary prevention strategy in children on the basis that children with extreme type 1 diabetes risk (>50%) can be identified and that insulin has been shown to be the first target of autoimmunity in children. Novel, and similar to what is efficacious in mice, is that insulin will be administered when the children are still autoantibody negative in order to induce protective immunity prior to initiation of autoimmunity. The efficacy of increasing doses of mucosal insulin to induce protective immunity will be assessed as the primary end point of the trial. The rationale for primary vaccination and the trial strategy are discussed.

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The medical problem

Type 1 diabetes (T1D) is an autoimmune disease with worldwide increasing incidence rate. T1D is one of the most common chronic diseases of childhood, is the cause of serious late-stage health problems including kidney disease, and is associated with large social and economic burden. Preventing/delaying T1D and/or its long-term complications is therefore of major benefit to society.

Current therapy does not prevent complications

The Diabetes Control and Complications Trial (DCCT) showed that complications in patients with T1D can be reduced if glycaemia is kept under control by

intensive insulin therapy (EDIC 2003). Unfortunately, such metabolic control is (1) difficult to achieve throughout life, and (2) associated with a substantially increased number of life-threatening episodes of hypoglycaemia. It is generally agreed, therefore, that experimental interventions to improve metabolic control in patients with T1D or prevent T1D in individuals at risk for T1D are a high priority.

Rationale for primary intervention

Experimental interventions applied late in the disease process have had limited success

Interventions at or after T1D onset are typically immunomodulatory or immunosuppressive in nature. Some have shown a beneficial effect in reducing insulin requirement to achieve reasonable metabolic control, but the side effects were either too great (The Canadian-European Randomized Control Trial Group 1988) or as yet uncertain in the case of monoclonal antibody therapy (Herold et al 2002, Keymeulen et al 2005). Preventative therapies can be applied prior to diabetes onset in individuals who are genetically at risk and who have evidence of autoimmunity indicating a high risk of later development of diabetes. Three large multicentre trials have been performed in this group; two showed no β cell protection by treatment with nicotinamide or parenteral insulin (Gale et al 2004, Diabetes Prevention Trial–Type 1 Diabetes Study Group 2002), and the third showed no benefit from treatment with oral insulin in the study group as a whole, but some encouraging effect after post-hoc stratification of subjects (Skyler et al 2005). It is clear that although not impossible, preventing diabetes onset in subjects with an immune system primed to destroy insulin-producing β cells is also likely to require potentially toxic therapies. An alternative is to intervene prior to the appearance of autoimmunity.

The infrastructure for performing primary intervention trials in T1D is established

Identification of neonates with high T1D risk using genetic typing in affected families can be achieved (Bonifacio et al 2004, Barker et al 2004), and families of these children are willing to participate in primary intervention trials that are relatively demanding with respect to lifestyle change, time and blood collection (Schmid et al 2004). Early specific markers that predict who will develop T1D in childhood are available (Hummel et al 2004, Achenbach et al 2004), thereby allowing primary intervention trials to be carried out within a relatively short time frame. Thus, provided suitable treatments are identified, the infrastructure to perform primary intervention trials for childhood T1D is available.

Identification of appropriate subjects for primary intervention

Neonates with very high T1D risk can be identified and monitored for the appearance of diabetes relevant autoimmunity

It has been demonstrated that neonates who have a first degree family history of T1D and the HLA risk genotypes DR3/4-DQ8 or DR4-DQ8/DR4-DQ8 have a 20% or higher risk for developing islet autoantibodies during childhood (Barker et al 2004, Walter et al 2003). Risk can be further stratified by selection of neonates with susceptible genotypes at other diabetes genes (Walter et al 2003, Laine et al 2004, Steck et al 2005), by selection of neonates with multiple family history of diabetes (Bonifacio et al 2004) and by selection of relatives that are HLA identical to the proband (Aly et al 2006). In some cases, the risk for developing islet autoantibodies can exceed 50% (Fig. 1). In the BABYDIAB cohort, 55% (95% CI, 30–80%) of neonates who had a multiplex first degree family history of T1D and who had either the DR3/4-DQ8 or DR4-DQ8/DR4-DQ8 genotypes developed diabetes relevant autoantibodies (multiple islet autoantibodies—IAA, followed by GAD and IA-2 antibodies) in the first years of life, and the majority of those who developed these antibodies progressed to disease in childhood (Bonifacio et al 2004). In the DAISY cohort, siblings of children with T1D who have the HLA DR3/DR4-DQ8 genotype and are identical by descent for both HLA haplotypes with their diabetic proband sibling had a 65% risk for developing islet autoantibodies by age 7 years and a 50% risk of developing diabetes by age 10 years (Aly et al 2006). The overall risk for developing T1D in these two high risk groups was 50% by 10 years of age. Thus, the development of multiple islet autoantibodies is an early and specific marker that represents a valid end point for primary prevention trials in high risk children, and it is possible to identify children with a 50% or higher risk of developing multiple antibodies in childhood.

Rationale for antigen-specific primary intervention with oral/intranasal insulin

(Pro)insulin is a primary autoantigen in children developing T1D

(Pro)insulin is a β cell-specific antigen, and has been suggested to be important in driving autoimmune β cell destruction in the NOD mouse (French et al 1997, Thebault-Baumont et al 2003, Moriyama et al 2003, Jaekel et al 2004). Most recently, it was demonstrated that expression of a non-mutated proinsulin gene in the NOD mouse was essential for disease implying that (pro)insulin is a primary autoantigen in this model (Nakayama et al 2005). Autoimmunity against insulin is also a characteristic feature in the pathogenesis of human childhood T1D. Insulin autoantibodies (IAA) are present in sera of almost all children at diabetes

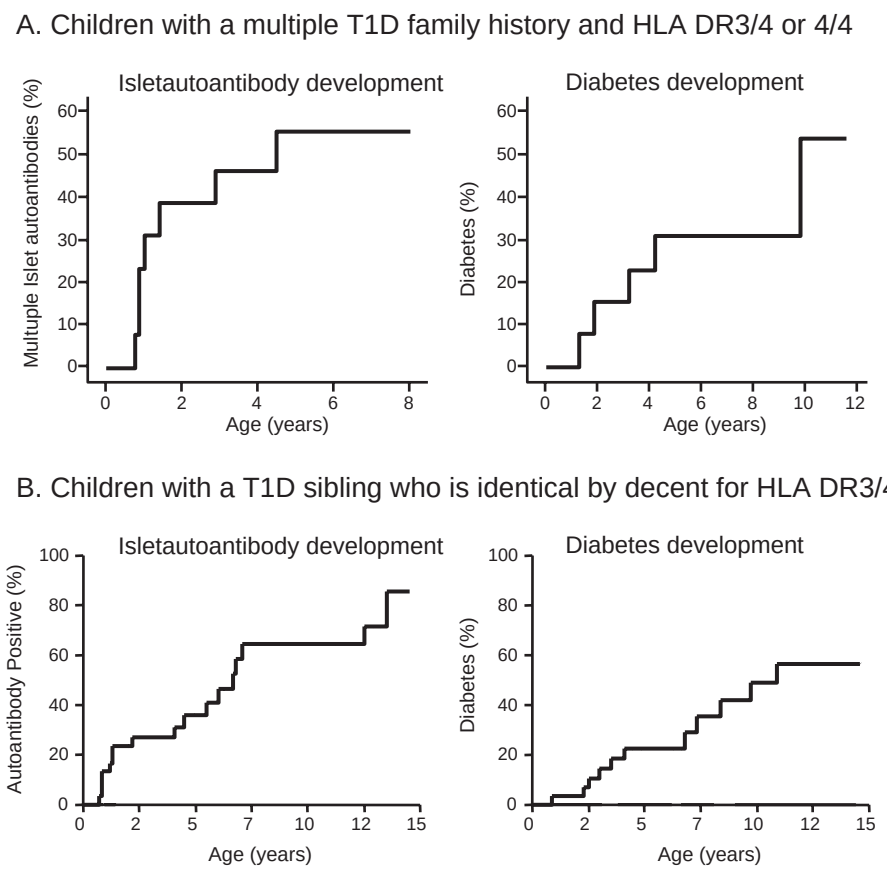

FIG. 1. Cumulative development of islet autoantibodies and diabetes in children with very high type 1 diabetes genetic risk. Adapted from Hummel et al (2004) and from Aly et al (2006).

onset (Vardi et al 1988). Prospective studies investigating the natural history of T1D in children from birth such as the German BabyDiab study (Ziegler et al 1999), the Finnish DIPP study (Kimpimaki et al 2001), the US DAISY study (Barker et al 2004), and the Australian BabyDiab study have shown that IAA are the first or among the first islet autoantibodies detected early in infancy (Fig. 2). The early appearance of IAA is often followed by autoantibodies to other β cell antigens such as GAD or IA-2, and children who progress to multiple islet autoantibodies are more likely to develop T1D (Barker et al 2004, Hummel et al 2004, Ziegler et al 1999, Kimpimaki et al 2001, 2002, Colman et al 2000). Only IAA of high affinity are predictive of progression to multiple autoantibodies and T1D, and

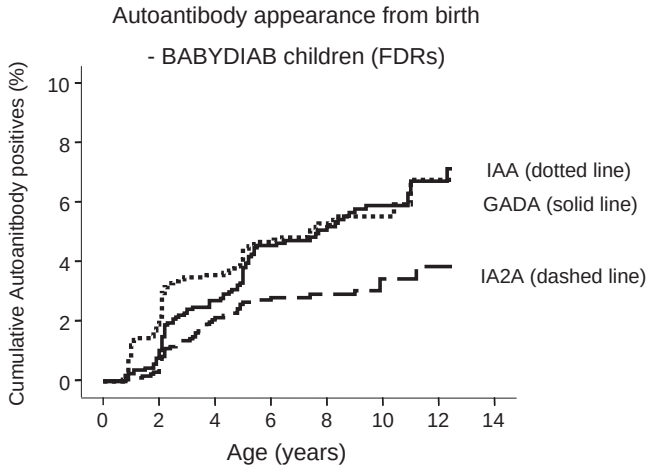

FIG. 2. Cumulative development of insulin autoantibodies (IAA, dotted line) GAD antibodies (GADA, solid line) and IA-2 antibodies (IA-2A, dashed line) from birth in children of parents with type 1 diabetes participating in the BABYDIAB study. Adapted from Hummel et al (2004).

these are a specific marker of future disease in genetically at-risk children (Achenbach et al 2004). In the BabyDiab cohort, the majority (25 of 27 cases) of children developing T1D before 10 years of age have detectable high affinity IAA early in life (Ziegler et al 1999). Moreover, children who have these IAA have a 50% risk to develop T1D before puberty (Hummel et al 2004). Finally, studies on lymph nodes from patients with T1D showed that the predominant T cell reactivity was against a peptide of the insulin A chain, suggesting that insulin remains a major autoimmune target years after diabetes onset (Kent et al 2005). Altogether, the findings point to insulin as a primary autoantigen in the disease process, and suggest that a deleterious immune response to insulin is an early event that if altered could change the course of disease.

Early antigen (insulin) specific intervention is an attractive and available candidate therapy

Modification of environment, although likely to be effective for some cases, is unlikely to be a universal method of preventing T1D. The use of non-specific agents is likely to also affect responses to vaccines and infection. In contrast, specifically targeting the immune response to autoantigens may provide protective immunity that does not affect immune responses to other vaccines or pathogens. Insulin is almost always amongst the first autoantigens recognized by antibodies in cases of childhood T1D (Hummel et al 2004, Yu et al 2000),

thereby representing an obvious target for antigen-specific intervention. Moreover, there is a strong relationship between diabetes-relevant insulin autoimmunity and the presence of HLA DR4-DQ8 allele (Achenbach et al 2004), thereby establishing a molecular basis for antigen-specific intervention. Human insulin has been used for decades to treat patients by injection, and more recently it has been given via oral, intranasal and inhaled routes in both patients and healthy subjects (Skyler at al 2005, Chaillous et al 2000, Pozzilli et al 2000, Kupila et al 2003, Harrison et al 2004). Mucosal administration of insulin is effective in inducing regulatory immune responses that can prevent autoimmune diabetes in animal models (Bergerot et al 1994, Harrison et al 1996, Zhang et al 1991, Maron et al 1999, Daniel & Wegmann 1996).

Preclinical studies using mucosal insulin

Oral/intranasal insulin prevents diabetes in animal models

In experimental animal models, administration of self-antigens to mucosa-associated lymphoid tissues can induce immune tolerance and prevent autoimmune disease (Weiner 1997, Harrison & Haffler 2000). Mucosal immunity generates a protective immune response manifested by Th2-type cytokines such as IL4, IL10 and TGFβ (Gottlieb & Eisenbarth 2002). In the NOD mouse, oral or intranasal administration of insulin or insulin peptides induces regulatory T cells that prevent autoimmune diabetes (Bergerot et al 1994, Harrison et al 1996, Zhang et al 1991, Maron et al 1999, Daniel & Wegmann 1996). Oral insulin therapy in NOD mice is most successful when given to neonates (Maron et al 2001).

Clinical studies using mucosal insulin

Mucosal insulin trials in human

Studies using oral or intranasal antigen therapy in human, although reassuring from a safety perspective (Kupila et al 2003, Harrison et al 2004), have so far not proven to have clinical benefit in human autoimmune diseases (Chaillous et al 2000, Pozzilli et al 2000, Weiner et al 1993, Trentham et al 1993). Oral insulin has been given to both diabetic and non-diabetic subjects without side effects (Skyler et al 2005, Kupila et al 2003, Harrison et al 2004). Children as young as 3 years of age have received oral insulin without side effects. Two studies have been performed administering insulin orally at doses ranging between 2.5 and 7.5 mg per day (together with standard insulin replacement therapy) in patients with new onset T1D (Chaillous et al 2000, Pozzilli et al 2000). Neither demonstrated obvious benefit with respect to preservation of residual β cell function. One of these studies demonstrated increases in the regulatory cytokine TGFβ (Monetini et al 2004).

Human recombinant insulin (7.5 mg per day) has been administered orally to *pre-diabetic* ICA and IAA positive first degree relatives of T1D patients without significant beneficial effects (Skyler et al 2005). A sub-analysis of the data, however, showed significant benefit in those relatives with higher titre IAA, raising hopes that antigen therapy may be effective if we optimize the conditions and timing of administration. In this regard, a *post-hoc* analysis of the oral insulin DPT1 data showed that after stratification of subjects by age, a potential benefit was best observed in the children aged less than 5 years of age (Dr J. Barker, unpublished personal communication). Immunoregulatory effects of antigen vaccination may not be able to counteract the pathogenic immunity in end-stage autoimmune disease, and asymptomatic individuals prior to preclinical autoimmune disease may be preferred candidates for such immunoregulatory therapy. No study in human has attempted to use autoantigen therapy for primary prevention of autoimmune disease. The advantage of primary prevention is that protection would be present before and at the time of initiation of the autoimmune process. This implies that autoimmunity *per se* could be prevented, rather than having to reverse a determined disease process. On this basis, a placebo-controlled, double-blind/double-masked, primary intervention pilot study of mucosal (oral/intranasal) insulin treatment in high risk children (Pre-POINT study) is proposed.

The Pre-POINT study

The Pre-POINT study is designed as a randomized, placebo-controlled, double blind/double masked, multicentre, dose-escalation, primary intervention pilot study in which oral or intranasal insulin will be administered daily to islet autoantibody negative children at high genetic risk for developing T1D. The study will be monitored by an external Data Monitoring and Safety Committee. Recruitment will be carried out in both Europe and the USA to identify children who have an estimated genetic risk of >50% to develop islet autoantibodies and T1D by adolescence. Forty children will be randomized to treatment or placebo groups (3:1 randomization). Pre-POINT will assess safety of treatment with oral/intranasal insulin at increasing doses and determine the bioavailability of mucosal insulin to the immune system. A dose-finding committee will evaluate data pertaining to safety and immune bioavailability, and determine the dose and route of mucosal insulin to be used in a phase II/III primary intervention study (POINT study).

Rationale for choosing the dose and route of mucosal insulin

In human, oral insulin has been administered at 2.5, 5, and 7.5 mg/day (corresponding to around 0.03–0.8 mg/kg/day for 9 kg to 85 kg participants) with no adverse effects. The potential beneficial effect on diabetes described in the DPT1

cohort (Skyler et al 2005) was achieved at a dose of 7.5 mg/day. Based upon FDA conversion charts (*http://www.fda.gov/cder/cancer/animalframe.htm*), the proposed dose of 7.5 mg in a child aged 6 months to 6 years corresponds to approximately 9 to 18 mg/m2/dose. Previous studies in mice used doses ranging from 1 to 100 mg/kg per day in various time schedules (Zhang et al 1991, Hancock et al 1995, von Herrath et al 1996, Bergerot et al 1996). In the study of Zhang, the optimal dose for efficacy in the mouse model was around 100 mg/kg given twice a week (Zhang et al 1991) corresponding to 300 mg/m2/dose or 600 mg/m2/week in human (Table 1A). A partial effect was observed at 10–20 mg/kg per day, but not at 1–2 mg/kg per day. Translation of doses from mouse to human is based on a number of assumptions, but one can see that on a dose per surface area basis,

TABLE 1 Oral insulin doses used in mice in relation to those used in Pre-POINT

(A) Mouse—fed twice weekly from age 5 weeks, once weekly from age 10 weeks[a]

	Diabetes incidence (%)			*Mouse mg/kg dose*		*Human equivalent mg/m²*	
Dose (mg)	*6 mo*	*12 mo*	*Effect*	*Per dose*	*Per Week*	*Per dose*	*Per Week*
PBS	20.5	49.2					
0.01	16.7	37.3	None	1	2	3	6
0.1	11.1	43.8	Slight, NS	10	20	30	60
1.0	0	8.0	$P < 0.02$	100	200	**300**[b]	**600**[b]

[a] data from Zhang et al PNAS 1991; [b] Efficacious dose equivalent in human

(B) Proposed dose of oral insulin for children in Pre-POINT—given daily

	Age of child			
Dose	*6 mo*	*2 yr*	*4 yr*	*6 yr*
2.5 mg				
— mg/m²/day	5.9	4.3	3.6	3.1
— mg/m²/week	41	30	25	22
7.5 mg				
— mg/m²/day	17.7	12.8	10.7	9.3
— mg/m²/week	124	90	75	65
22.5 mg				
— mg/m²/day	53.1	38.4	32.1	27.9
— mg/m²/week	372	270	225	195
67.5 mg				
— mg/m²/day	159.3	115.2	96.3	83.7
— mg/m²/week	1116	810	675	585

we need to approach 50 mg per day to achieve a dose equivalent that was efficacious in the mouse (Table 1B). Pre-POINT will test four doses of oral insulin: 2.5 mg, 7.5 mg, 22.5 mg and 67.5 mg per day (threefold increases between doses). The lowest dose (2.5 mg) is equivalent to the average dose used in the DPT1 study (corrected for weight of subject). The highest dose (67.5 mg) is in the range of an efficacious dose in the mouse.

Intranasal administration of insulin offers an alternative mucosal route to oral insulin. Experiments in mice have shown greater bioavailability of antigen to the immune system when it is administered intranasally than orally. Studies have been performed with intranasal insulin in islet autoantibody positive subjects as young as 2 years of age (Kupila et al 2003). Pre-POINT will include a parallel dose-finding study with intranasal administration of insulin, which will test four doses of intranasal insulin: 0.28 mg, 0.83 mg, 2.5 mg and 7.5 mg per day (threefold increases between doses). Doses were selected on the basis of prior experience in the INIT I study (1.6 mg per day) (Harrison et al 2004), and planned doses in the INIT II study (1.6 mg and 16 mg per day).

Measuring bioavailability of insulin to immune system

The proposed mechanism of action of protection is that through mucosal exposure to the study drug a protective immune response to the insulin autoantigen will be achieved, and that this response will subsequently be favoured if events that normally lead to a β cell destructive insulin autoimmunity arise. Although other mechanisms of action could be operative, the major focus of the mechanistic studies in Pre-POINT will be to determine whether administration of oral insulin leads to an immune response to insulin that has characteristics consistent with protection. The mechanistic studies will examine both B and T cell responses to insulin.

B cell responses

We have demonstrated that the IAA preceding T1D are of IgG isotype, typically initiating with IgG1 followed by expansion to include IgG4 clones (Bonifacio et al 1999), are reactive against proinsulin and rapidly become high affinity (Achenbach et al 2004). Antibody responses to insulin administered to the mucosa have not been convincing. No significant response was observed in IAA-positive subjects in the oral insulin DPT1 trial (Barker et al 2007). Some responses were noted in the INIT intranasal insulin trial (Harrison et al 2004). However, detection in these trials is hampered by the fact that subjects already have circulating antibodies to insulin. In contrast, Pre-POINT will look or responses in the absence of previous priming. We postulate that mucosal

exposure to insulin may result in antibody responses that differ in characteristics to IAA, and include IgA-insulin antibodies (that may be restricted to administration site), lower affinity insulin antibodies and insulin antibodies that do not react with proinsulin.

T cell responses

While antibody responses may be a sensitive means to detect vaccination against insulin, it is the T cell response that is likely to be informative about whether responses are likely to be protective. T cell responses to autoantigen are notoriously troublesome (Roep 1999). Nevertheless, qualitative responses with respect to cytokine profile are possible and have been reported to distinguish aggressive responses from benign or protective responses (Arif et al 2004). Recent focus on measuring the memory cell responses to antigen (Danke et al 2005) is also likely to improve our ability to detect true T cell response to study drug.

Using both B and T cells response measurements and a dose amplification strategy, Pre-POINT will base its decision to proceed to a phase II/III study on safety and proven immune bioavailability of the study drug. Success will lead to a phase II/III primary intervention trial (POINT study) to determine whether administration of mucosal insulin before the appearance of islet autoimmunity can delay or prevent the development of islet autoantibodies or diabetes in children who have both familial and HLA genetic risk for T1D.

Concluding remark

Primary intervention therapy would offer a number of advantages over intervention at a later stage of the disease process. A major benefit is that public health measures for screening and prevention could be applied to a disease that is currently increasing in prevalence and considered a worldwide burden.

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DISCUSSION

D Hafler: Getting back to MS, it is interesting that one of the dominant responses to myelin basic protein (MBP) by autoantibodies is to the same dominant epitope as seen by T cells. There aren't that many peptides there, so the chance of having them overlap is maybe random, which means we have to be careful of extrapolations. But this does raise the interesting question of whether it is related or not. It also raises the question as to whether the response we are seeing in the islet is because of the B cells and their antibodies and antigen receptors.

Peakman: Were you tempted to model this effect in animals at all, to look and see whether there is a dose effect in animals?

Bonifacio: We looked in Balb/c mice, giving increasing oral doses of insulin to high levels. We did not generate an insulin antibody response. It was hard for us to choose the mouse. If you choose a non-diabetic mouse with the right MHC, many of them have insulin antibodies to start off with. We should probably find

an insulin antibody negative mouse with the right MHC to look at this. Len Harrison always says that there is antibody production in the intranasally treated mice, but I haven't seen these data.

Herold: It looked like the insulin antibodies come up and then go down again when you stop treatment in the trial. Is that correct?

Bonifacio: They looked like they were coming down in those few that went up. These were our measurements that were different to Len's. If I look at Len's data, which he shows as an average using his measurement, it went up and then sort of went down a little bit. The ones that I showed that went up looked as if they had come down in titre when they went off the antigen.

Herold: For how long were they exposed?

Bonifacio: Six months. In the dose finding trial they will be on for at least three months and up to 15 months.

Kay: What age will they be?

Bonifacio: The pre-point will include two to seven year olds. One of the reasons for this is so that we can get sufficient blood to do the immune response.

Kay: So you will have missed the first peak?

Bonifacio: Yes. George Eisenbarth and I have discussed this a lot. George still believes that it is linear. Our data say that we will miss this first peak.

Foulis: Will breast feeding confound this trial? There is insulin in breast milk.

Bonifacio: That's right. Some will have had oral exposure to insulin and some won't.

Roep: There is insulin in cow's milk, too.

D Hafler: What is the concentration of insulin in breast milk?

Bonifacio: It's not a huge amount. It's a bit homeopathic. The doses of insulin we are giving are enormously higher, and they are also high compared with what patients inject.

Roep: Do you see any hypoglycaemia?

Bonifacio: We check for this. To get something as simple as this going from a regulatory perspective, in two continents, has not been easy.

Lew: Len definitely didn't see any hypoglycaemia in his mice.

D Hafler: We have looked at this extensively early on.

Roep: It is quite a different dosage in the mice.

Bonifacio: There is a lot of safety stuff in there, and there is a lot of insistence that we don't move to another dose until this has been cleared up.

Bresson: You have shown data on the use of insulin as mucosal antigen vaccine. We have seen data from Bart Roep using GAD65 to induce tolerance. So which antigen should we use to induce tolerance in humans? Is there a possibility of defining the antigen that will be the most beneficial before the patient is treated? You have shown the data with the DPT1 trial, where patients with the highest insulin antibody levels seem to show the best efficacy. In Bart's trial, patients with

the highest GAD65 antibody levels were the ones responding. At entry, can we therefore look at antibody levels and then define which antigen will be the best to use to induce tolerance in clinical trials?

Bonifacio: It might be that the antibodies are picking up antigen and doing some presentation. I have no idea. Currently, the trials are selecting insulin on the basis that you are insulin antibody positive, and to give GAD on the basis that you are GAD antibody positive.

Eisenbarth: There is an alternative hypothesis for IAA discrimination. There is a higher risk in those individuals. In the diabetes prevention trial they changed the entry criteria and lowered the insulin autoantibody. It turns out the highest risk children were the ones where an effect was seen. You might have diluted out the effect you see by putting in children with no risk. It could be that the insulin antibody levels are telling us something biologically, but the jury is out.

Roep: What about the low risk patients with multiple antibodies?

Eisenbarth: It turned out there were very few with multiple antibodies.

Bonifacio: You can get the same effect if you look at multiple antibody positive patients only.

D Hafler: If you believe the mechanism of bystander suppression, both antigens may work.

Bresson: You still need to induce a good antigenic response to induce regulatory cells and observe bystander suppression. In humans, even though the genetic background is restricted from patient to patient, there is a difference between patients; it is not like in NOD mice. You want to induce a T cell response against the antigen in all the treated patients, so what is the best antigen for inducing these antigen-specific Tregs in a particular patient?

D Hafler: It is an experimental question.

Eisenbarth: I have one caveat. It is difficult to study GAD in NOD mice. Mice have almost no GAD in their islets. With GAD in mice we are looking at something that is not an antigen-specific therapy. Is there enough GAD67 in the islet, or is it more like Freund's adjuvant. It is hard to make a T cell clone that causes diabetes to GAD. There are data that if you remove the immune response to GAD in the mice they develop diabetes happily. I am somewhat sceptical. In human I think it is a great antigen.

Roep: It may be an advantage to have some sort of systemic exposure, if you know that you get the effect in all the places you want it.

Closing remarks

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Several people said they wanted to see a synopsis of this meeting. So Mark Atkinson, Mark Peakman and I took a few brief moments to develop one. In these closing remarks, I shall briefly summarize the issues we found most striking that arose from the discussions at this meeting. While each talk was informative and interesting, and we thank all for their participation, we thought five concepts were particularly noteworthy.

It is interesting to see that, compared to animal models, human insulitis is infrequent and mild; especially in those patients who get the disease slower when they are older. The fact that only a small fraction of islets is affected by infiltration illustrates the slower kinetics of human type 1 diabetes (T1D). This will most likely translate in finding even lower precursor frequencies of autoreactive T cells in the blood of human prediabetic patients in comparison to the NOD mouse, where detection of such cells is already difficult. Furthermore, the cellular composition of human insulitis is probably important: there are many more $CD8^+$ cells than $CD4^+$ cells in human insulitis.

Related to this, many of us found it striking that there can be a uniform class I MHC up-regulation and interferon (IFN)α up-regulation in human islets within the type 1 pancreas that otherwise look relatively normal and do not appear to have any cellular infiltrate. This might tell us that it is still worth searching for a persistent driver, for example an islet-specific viral infection. It would explain how such a slow process could sustain itself.

Next, immunologically, from what we have learned of self-propagating immune responses in animal models, the human response in T1D is just below the threshold of any known self-sustaining immune response. If you destroy one islet every few months in humans, except in young children where you would almost have to postulate that something is in the β cells that would instigate the immune response to start this process again. Perhaps some studies of the molecular details of the IFN response systems might be warranted. Overall, there is no doubt that we need more data from prediabetic human pancreata (for example those obtained with the JDRF nPOD initiative) and very good virological analyses. I don't know how much prediabetic pancreata we will need to generate solid conclusions. Perhaps we can

also define new pathways from pancreata of diabetic patients, which are also present after diagnosis.

It is also interesting that lower avidity T cells apoptose less easily and might cause disease, or even regulate it. The lower avidity or degenerate T cell receptors might be prevalent in the autoreactive repertoire as opposed to the host defence; in other words, a foreign reactive repertoire. Some T1D genes could influence this. What we need to do to answer this question is to develop a precise mapping of human autoreactive T cell receptors, and we need to assess the affinity of these in the human repertoire.

Next, we turn to potential therapies for T1D. It seems likely that an effective therapeutic approach should consist of a combination of immunosuppression, immuneregulation and islet regeneration. All three are likely to be needed for a therapy to provide sustained efficacy. Unfortunately, we do not understand the kinetics of islet destruction very well, nor the chronic drivers of the immune response. There is a need for prolonged immune modulation. Also, islets under attack seem to regenerate or replicate β cells.

Fourth, there are some encouraging data on biomarkers of destruction and immune regulation. Interleukin (IL)10 appears to be major regulator in many systems and a very good predictor of glycaemic control.

Finally, what do we need to do? First, we should look at the genotype/phenotype of the disease in each patient as a prelude to selecting particular therapies. The kinetics of single islet destruction *in vivo* need to be mapped. In addition, IL10-based therapies need to be pursued, as well as the engineering/induction of regulatory T cells, tolerogenic APCs and antigen-specific immunotherapies. I thank you all for your participation.

Contributor index

Non-participating co-authors are indicated by asterisks. Entries in bold indicate papers; other entries refer to discussion contributions.

This index was prepared by Neil Manley

Subject index

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