

Role of β -Cells in Type 1 Diabetes Pathogenesis

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Whether autoimmunity results primarily from a defect of the immune system, target organ dysfunction, or both remains an open issue in most human autoimmune diseases. The highly multigenic background on which diabetes develops in the NOD mouse and in the human suggests that numerous gene variants associate in contributing to activation of autoimmunity to β -cells. Both immune genes and islet-related genes are involved. The presence of β -cells is required for initiation of diabetes autoimmunity to proceed. Available experiments in the NOD mouse and epidemiological evidence in the human point to proinsulin as a key autoantigen in diabetes. The functional importance of insulin, the high number of autoantigens characterized at different stages of diabetes, and their clustering within β -cell subparticles point to the islet as a starting point in the initiation phase of the disease. Genes that direct the autoimmune reaction toward the β -cell target, autoantigens that are recognized by autoreactive B- and T-cells along the autoimmune process, the importance of β -cells in the activation of autoreactive lymphocytes, and the expression level of key β -cell molecules along diabetes development are successively considered in this review. *Diabetes* 54 (Suppl. 2):S87–S96, 2005

Type 1 diabetes is the result of an autoimmune reaction that develops against pancreatic β -cells. Indirect evidence for autoimmunity in human type 1 diabetes relies on the detection of insulinitis, islet cell antibodies, T-cell responses to β -cell antigens, and the association of diabetes with a restricted set of class II major histocompatibility complex (MHC) alleles. However, mechanisms that initiate the failure of immune tolerance remain elusive in common forms of type 1 diabetes, a multifactorial disease in which environmental factors concur with a highly multigenic susceptibility background to allow failure of immune tolerance to β -cells to develop.

Since the experimental demonstration in the mid-1950s that autoimmune diseases are elicited by immunization against self-tissues or self-antigens in complete Freund's adjuvant, the rationale pursued for years assimilated autoimmune reactions to immune responses to foreign antigens. A leading scheme postulated that an environmental factor mimics the presentation of autoantigens in Freund's adjuvant and fouls the immune system in activating and expanding autoreactive lymphocytes. In these experimen-

tal conditions, autoimmunity results from presentation of autoantigens by professional antigen-presenting cells that deliver costimulatory signals to autoreactive T-cells. The presentation of autoantigens occurs at sites that are distant from target tissues. Autoimmunity in these models does not require prior functional or anatomical modifications of target tissues. In the human, uncommon situations may proceed along this scheme, as in autoimmunity observed in paraneoplastic syndromes, during pregnancy, or after infections in which tumoral cells, the placenta, and infectious agents, respectively, express autoantigens shared with peripheral tissues. However, whether autoimmunity results primarily from a defect of the immune system, target organ dysfunction, or both remains an open issue in most human autoimmune diseases.

Animal models of spontaneous diabetes and epidemiological data in the human challenge the obligatory role of environmental factors in triggering diabetes development. Animal models also indicate that pathological modifications of the islets are required for full-blown diabetes to develop. Furthermore, no experimental model has been obtained in which diabetes is induced by immunization against β -cells or β -cell autoantigens in adjuvant. In the human, the role of a genetic region that controls the expression of insulin also points to the islet as an important parameter in the development of autoimmune diabetes. The role of local factors in the disease process is further underscored by the striking heterogeneity of the islet infiltrates within individual pancreases.

Genetic and experimental evidence that make the islet and β -cells key players in the development of autoimmune diabetes will be detailed in this review.

TYPE 1 DIABETES: GENES VERSUS ENVIRONMENT

Physiological tolerance of self-antigens relies on negative selection or silencing of lymphocyte precursors in central lymphoid organs and deletion, silencing, or deviation of self-reactive clones that escape central deletion in peripheral tissues. The pathological conditions that induce abnormal presentation of autoantigens by professional antigen-presenting cells in human autoimmune diseases have been the matter of relentless research. Epidemiological observations that have accumulated over the years have failed to identify undisputable environmental factors that trigger autoimmune diabetes (1,2).

Environmental factors in human type 1 diabetes. There is evidence in experimental models that infections can trigger autoimmune reactions to β -cells. Molecular mimicry between viral proteins and β -cell transgenes (3), bystander activation of autoreactive T-cells in transgenic mice expressing a biased T-cell repertoire (4), and disruption of Th1/Th2 T balance, as following infection by the Kilham virus in the rat (1), have been reported to induce diabetes. There is no such strong evidence to support the role of infections in triggering autoimmunity in human type 1 diabetes (1,2). There are anecdotal reports of coincidence of viral infections and onset of type 1 diabetes

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LCMV, lymphochoriomeningitis virus; MHC, major histocompatibility complex; VNTR, variable number of tandem repeats.

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and evidence for an increased prevalence of virus-specific IgM antibodies or detection of viral RNA or DNA in recent-onset type 1 diabetic patients. Viruses that have been most frequently involved are rubella virus, cytomegalovirus, mumps virus, and Coxsackie's virus B4. This does not provide direct evidence, however, that viruses initiate the failure of tolerance to β -cell antigens in human diabetes. If one excepts the case of congenital rubella, no unique virus has been convincingly reported to trigger diabetes development. Bystander activation of autoreactive T-cells by superantigens has also been suggested to trigger diabetes in the human (5), but direct evidence favoring this mechanism remains elusive. The role of infectious agents in triggering autoimmunity through molecular mimicry or broad activation of T-cells by superantigens implies that autoimmunity arises against an autoantigen, the expression of which is restricted to cells that will be destroyed by the autoimmune reaction, unless a low sensitivity threshold to destruction predisposes a given tissue to destruction by the autoimmune reaction. The evidence that this is the case in type 1 diabetes is disputed. Many autoantigens are targets of autoantibodies and T-cells in type 1 diabetes, most of which are expressed by β -cells but also by other islet endocrine cells and by neurons that are not damaged by the autoimmune process in common forms of the disease (6).

Environmental factors in animal models of autoimmune diabetes. The role of environmental factors in triggering diabetes autoimmunity is further challenged by animal models in which autoimmune diabetes develops spontaneously, especially the nonobese diabetic (NOD) mouse and the BioBreeding (BB) rat (7,8). The NOD mouse is an inbred strain that shows striking similarities with human diabetes. NOD mice develop diabetes from 12 weeks on, with a higher prevalence in females than in males. Early histological alterations at 3 weeks of age consist of swelling of endothelial cells within islet vessels and infiltration by dendritic cells and macrophages around some islets. A peri-insular infiltration by T-cells, mostly CD4⁺ T-cells, is seen secondarily. T-cells control activation of the autoimmune reaction and mediate β -cell destruction, as evidenced by transfer of diabetes in naive recipients by T-cells from diabetic NOD mice. Diabetes is prevented by injection of monoclonal antibodies that target membrane molecules involved in T-cell interaction with antigen-presenting cells or T-cell signaling that follows antigen recognition. Diabetes prevalence varies from one colony to another, depending on the environment and possibly on genetic drift from the original stock. However, most infectious agents have shown neutral or protective effects rather than induction of diabetes in the NOD mouse. NOD mice develop insulinitis and diabetes with the highest prevalence in pathogen-free colonies. All animals do not develop overt diabetes in specific pathogen-free colonies, independently of environmental factors. The NOD mouse makes a major case against the absolute need for environmental factors in triggering autoimmunity, raising the possibility that stochastic events influence diabetes development (9).

Type 1 diabetes: a highly multigenic disease. Extensive genome scanning studies have identified a long list of regions possibly involved in genetic susceptibility to diabetes in both the human and the NOD mouse (10,11). With the exception of MHC class II genes, the 5' flanking region of the insulin gene, and the *CTLA4* gene, most susceptibility genes within these regions remain unknown. The

likelihood that multiple genes contribute to susceptibility and that most of them have, individually, a weak effect has hampered characterization of further contributing genes. This is even complicated by genetic heterogeneity of diabetes. Susceptibility regions that are associated with diabetes partly differ in crosses of NOD mice with different strains. Resistance genes (12) are mixed with susceptibility genes on diabetes-prone backgrounds and may explain late development of diabetes in conventional NOD mice. Finally, distinct genes contribute to susceptibility as clusters in several genetic regions. Linkages have been evidenced between diabetes and markers located on numerous chromosomes, allowing an estimate of over 20 genetic loci possibly carrying diabetes susceptibility genes. In animal models, several localizations are shared by different crosses, but other resistance and susceptibility genes are only detected in specific crosses. Further genetic analysis suggests that strong interactions between different diabetes susceptibility (*idd*) genes modulate diabetes development. Moreover, congenic mice harboring non-NOD alleles at some of the *idd* loci indicate that several genes in a given location contribute to disease susceptibility. In the human, markers located in several chromosomal regions associate with other autoimmune diseases, indicating that genetic susceptibility is mediated by genes favoring general failure of immune tolerance, whereas others control targeting to β -cells. Overall, the MHC locus contributes 40% of genetic susceptibility to type 1 diabetes.

The aforementioned data need to be considered together to draw a possible scheme of the respective role of environmental factors and a multigenic susceptibility background in diabetes development. Diabetes occurs independently of environmental triggering events in the NOD model. Environmental factors even exert a protective effect on diabetes. Multiple genes also associate to favor disease development in this model. Human diabetes shares this multigenic background with the NOD mouse. A possible hypothesis is that numerous genes assemble to contribute to stochastic activation of the autoimmune reaction to β -cells, independently of environmental factors in the NOD mouse and, possibly, in common forms of diabetes in the human. Mutations of key genes controlling immune reactions to self, such as in the case of the *AIRE* gene in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) or the *FoxP3* gene in the immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (13,14), and environmental factors, as in the case of congenital rubella in the human or the Kilham virus in the rat, only precipitate diabetes in uncommon forms of type 1 diabetes. Susceptibility regions are thus likely to harbor gene variants that are possibly involved in functional features that are characteristic of the NOD mouse model, some of which are shared by comparable functional traits in the human. A major challenge in the future will be to tag each functional trait with identified genes or gene clusters (Fig. 1).

THE ROLE OF β -CELL ANTIGENS

The anatomical and physiological organization of target tissues shape many factors that are involved in the clinical expression of autoimmune diseases (15). These include the local homing of inflammatory cells and lymphocytes, antigen presentation, the level of autoantigen expression or expression of neoantigens, and spreading of the auto-

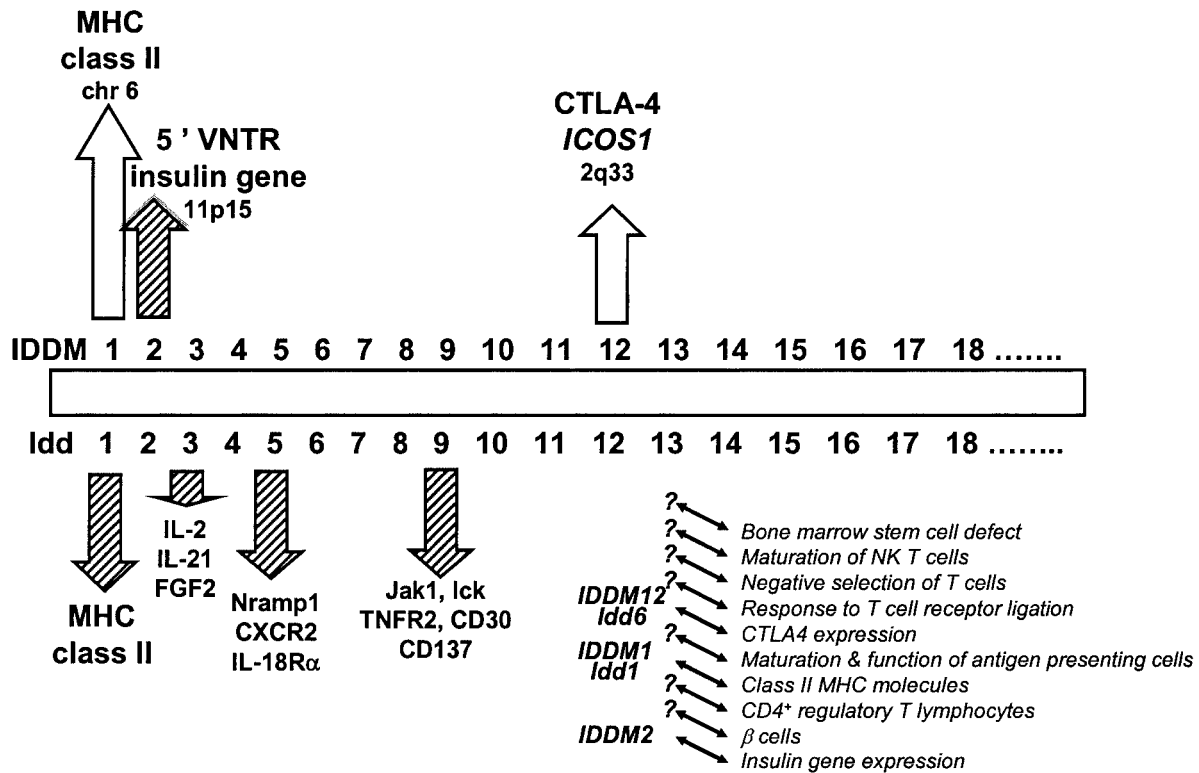


FIG. 1. Schematic representation of susceptibility regions (Idd/IDDM) that are referred to in this review and functional features found to be associated with diabetes in the mouse and in the human (hatched arrows, loci implicated in the targeting of the autoimmune reaction to β -cells). A major challenge in the future will be to tag each functional variant with identified genes or gene clusters. IDDM and Idd numbers refer to loci identified as associated to type 1 diabetes in the human and in the NOD mouse, respectively. Candidate genes within loci referred to in this review are indicated above (*upper panel*) and beneath (*lower panel*) open and hatched arrows. Double-headed arrows in the lower right panel point to functional features that have been associated with type 1 diabetes and corresponding diabetes-associated loci, when identified. The question marks point to traits that have not been associated with identified loci.

immune reaction from a single to multiple antigenic epitopes or from an "initial" autoantigen to other autoantigens. Several lines of evidence point to a direct role of the islet of Langerhans in the general process that drives diabetes autoimmunity. Genes that direct the autoimmune reaction toward the β -cell target, autoantigens that are recognized by autoreactive B- and T-cells along the autoimmune process, the importance of β -cells in the activation of autoreactive lymphocytes, and the expression level of key β -cell molecules along diabetes development will be successively considered.

Why do β -cells rather than other target tissues participate in an autoimmune setting? The clustering of organ-specific autoimmune diseases observed in human type 1 diabetes is shared by the NOD mouse and the BB rat. Beyond inheritance of genes that predispose to general failure of self-tolerance on both backgrounds, some susceptibility regions identified by analysis of crosses between the NOD and conventional mouse strains harbor genes that control the targeting of autoimmunity toward β -cells. Among these is the major diabetes susceptibility region that maps to the MHC. In the human, a strong association between type 1 diabetes and a restricted set of class II MHC alleles (*IDDM1*) has been evidenced. Diabetes susceptibility alleles carry a serine, an alanine, or a valine residue in position 57 of the class II DQ β chain in the human. Class II alleles carrying an aspartic acid in DQ β 57 are neutral or confer dominant protection (HLA DQB1*0602). The presence of an aspartic acid at residue 57 alters the peptide binding cleft of the class II molecule

and modifies the sequence profile of peptides that are presented to T-cell clones. Class II knockout mice carrying the human DQ8 and DQ6 alleles along with a B7 transgene under the control of the rat insulin promoter provide definitive evidence for a direct role of class II genes in diabetes susceptibility (16). In the mouse, the class II I-A^{G7} allele is associated with diabetes. I-A^{G7} carries a unique A β chain that shows structural homology with human DQ β susceptibility alleles.

Diabetes class II susceptibility alleles are disease specific. They differ from class II alleles that predispose to nonpancreatic autoimmune diseases. Congenic NOD mice expressing class II molecules that are not associated with diabetes in the mouse, such as I-A^{H4} or I-A^B, fail to develop diabetes. They instead develop extensive thyroiditis, especially in the presence of increased dietary iodine, and destructive sialitis, respectively (17). However, non-class II genes and environmental factors also contribute to directing the autoimmune response toward a target tissue on autoimmune-prone backgrounds. This has been shown in the case of B7 costimulatory molecules. B7.2 knockout NOD mice, which fail to develop diabetes, develop autoimmune peripheral neuropathy (18). Infection by mycobacteria favors the development of a lupus phenotype in conventional NOD mice (19).

Beyond the role of class II MHC alleles and B.7.2, other regions have been implicated in directing the autoimmune reaction against nonislet tissues. A low frequency of diabetes is observed in congenic NOD mice carrying B6 or B10 gene fragments defined within diabetes susceptibility

loci. This is the case for *Idd3* (chromosome 3), *Idd9* (chromosome 4), or *Idd5* (chromosome 1) congenics. In the case of *Idd9*, resistance to diabetes is contrasted by persistence of extensive insulinitis in NOD.B10 *Idd9* congenics (20). Introgression of two resistance regions from the B6 (*Idd3*) and B10 (*Idd9*) backgrounds onto the NOD background in NOD.B6/B10 *Idd3/Idd9* congenics results in a more complete protection from diabetes than introgression of single B6 (*Idd3*) or B10 (*Idd9*) regions. Interestingly, NOD.B6/B10 *Idd3/Idd9* congenics develop liver cysts and infiltration, along with anti-DNA and anti-Sm autoantibodies that are characteristic of lupus syndromes (21). NOD.B6 *Idd3/Idd5* congenics develop extensive autoimmune exocrinopathy that leads to functional defects reminiscent of Sjogren's syndrome (22). Like NOD.H2^b, NOD.B10 *Idd9.1/9.2/9.3* congenics show a low incidence of diabetes but still develop significant sialitis and salivary dysfunction (20). If one excepts the role of class II molecules, non-MHC genes involved in directing the autoimmune reaction toward β-cells and underlying mechanisms remain to be characterized.

Type 1 diabetes: multiple autoantigens and multiple T-cell epitopes. The destruction of β-cells in type 1 diabetes is highly selective. These cells represent 60–70% of the endocrine compartment of the islet of Langerhans, in which they are surrounded by α-cells, δ-cells, and PP cells. The prediction in the 1980s that autoimmunity arises against a single autoantigen that is specific to β-cells has not met the experimental challenge. B- and T-cells recognize several autoantigens along the diabetes process, and most autoantigens are expressed by β-cells but also by cells that are not damaged by the autoimmune reaction. Furthermore, the striking clustering of autoantigens into intracellular subparticles, i.e., secretory granules and synaptic-like vesicles, hints at a relationship between key β-cell functions and autoimmune development (6). This brings indirect evidence that diabetes develops as a β-cell disease rather than an isolated failure of immune tolerance to a single autoantigen. A major challenge remains in deciphering the role of individual autoantigens and characterizing epitopes that are recognized by T-cells along the disease process. Most autoantigens, especially GAD and IA-2, two major autoantigens identified in human diabetes, and glima 38, an autoantigen to which autoantibodies are detected in 15–20% of the patients, are expressed by all the endocrine cells composing the islet and by neurons. Insulin and GAD are expressed in the thymus. A β-cell-specific autoantigen, the glucose-6-phosphatase catalytic subunit-related protein, has recently been identified in the NOD mouse (23), but the evidence that it is involved in human diabetes is lacking.

Evidence that an autoantigen is critical has been investigated by attempts to prevent diabetes in NOD mice upon tolerogenic delivery of autoantigens, by inducing diabetes by transfer of autoantigen-specific T-cell clones, or by inducing an immune response to the islets after immunization of normal mice against candidate antigens (24–30). Other manipulations have been explored to prevent diabetes, as in transgenic NOD mice expressing proinsulin or heat shock protein 60 in antigen-presenting cells, proinsulin in pituitary cells, or GAD or GAD antisense in β-cells (31–35) or in mice injected with plasmid DNA encoding for candidate autoantigens (36–39). Epitopes recognized by T-cells (insulin B9-23, heat shock protein 60 p277, and GAD 524–543) have further been defined and used to prevent diabetes in susceptible mice (24–27). The screen-

ing of IA^{g7}-restricted hybridomas obtained after immunization against GAD65 has allowed mapping of eight epitopes on GAD65 (40) and eight epitopes on proinsulin 1 and proinsulin 2 (41) in the NOD mouse model. Similar studies in class II^{-/-} mice expressing human class II alleles have defined GAD and insulin epitopes (42,43). However, many experiments reported face major caveats. Transfer of diabetes by T-cell clones does not allow defining of autoantigens recognized at initiation of the disease process. Immunization against autoantigens may activate T-cell clones independently of their role in the spontaneous disease. Tolerization procedures may bypass tolerance through secretion of inhibitory cytokines. The failure to transfer diabetes in GAD antisense transgenic mice (35) remains unexplained.

We focused in recent studies on the role of proinsulin as an autoantigen in type 1 diabetes (41). This choice is justified by the fact that proinsulin is a predominant β-cell protein and the only candidate autoantigen the expression of which is largely restricted to β-cells. Rodents express two insulin isoforms encoded by distinct genes located on chromosome 7 and 19, respectively, in the mouse. The two isoforms differ by two amino acids in the B-chain, three amino acids in C-peptide, and several differences in the leader peptide sequence. Proinsulin 1 and 2 are controlled by distinct promoter regions and show distinct tissue distributions. Both isoforms are expressed in β-cells. Expression of proinsulin 2 has been evidenced in the thymus. To obtain an unbiased image of the repertoire of T-cells that invade the islets of Langerhans along diabetes development, we fused islet-infiltrating T-cells (Fig. 2) with the BW 5147 thymoma line *ex vivo* and screened hybridomas using a peptide library spanning the entire sequence of preproinsulin 1 and 2. We identified CD4⁺ T-cells specific for four different proinsulin epitopes within the islet cell infiltrate of pre-diabetic female NOD mice. These were among the immunogenic epitopes to which a T-cell response was detected after immunization of 8-week-old NOD mice against individual peptides in complete Freund's adjuvant. One epitope (II 33-47) was identical to a previously defined proinsulin 2 peptide that was recognized by NOD islet T-cell lines that were expanded in the presence of exogenous insulin *in vitro* (i.e., B9-23) (28). This epitope is located in the middle fragment of the insulin B-chain. It overlaps a nonamer (B9-15) that is also recognized by NOD CD8⁺ T-cells (44). CD4⁺ immunogenic epitopes were found on both isoforms of proinsulin. The percentage of hybridomas that were responsive to islet cells was in the order of 15–25% of the panel of hybridoma obtained both at 8 and 14 weeks of age in pre-diabetic female NOD mice. The percentage of proinsulin-reactive hybridomas was 3.6%. Presumably, this illustrates the diversity of islet autoantigens to which CD4⁺ T-cells respond in type 1 diabetes. Only two out of the 192 T hybridomas obtained responded to the proinsulin 2 peptide II 33-47/B9-23 epitope, indicating that the corresponding T-cells were not predominant in the repertoire of proinsulin-specific T-cells involved in the autoimmune response to the islets. Other infiltrating T-cells were specific for proinsulin I 33-47 and I 71-86, and proinsulin II 26-41, indicating that a wide array of proinsulin epitopes were recognized along the autoimmune process. Because protection from diabetes has been observed in NOD mice injected with insulin B-chain peptide B9-23, we evaluated interleukin-4 and interferon-γ production by NOD T-cells after immunization against peptide II 33-47 in complete

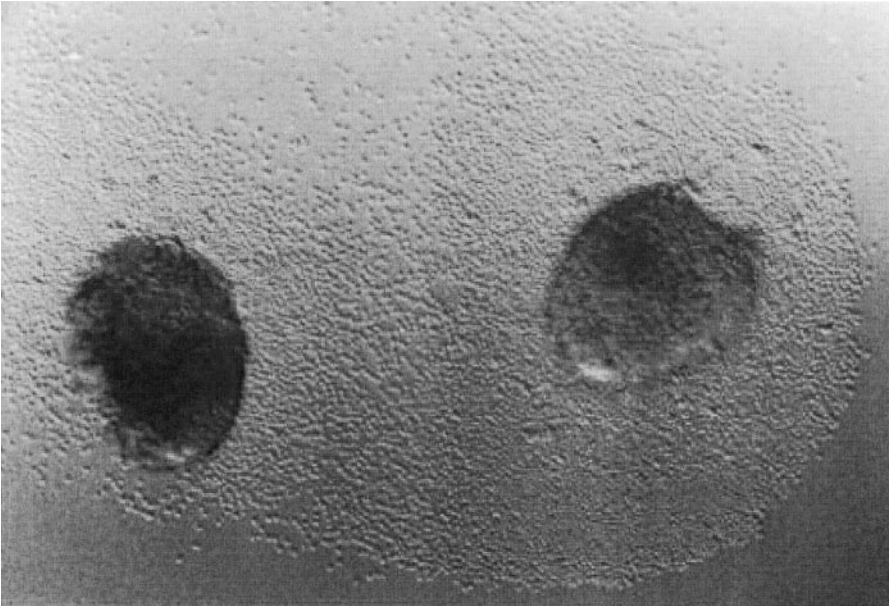


FIG. 2. Infiltrated islets obtained by collagenase digestion of a 14-week-old female NOD mice. Lymphocytes are seen extruding from the islets by waves within the first 24 h of culture.

Freund's adjuvant using an ELISPOT assay. No interferon- γ response was observed to peptide II 33-47 as opposed to a significant response to control peptide I 20-35. Several proinsulin peptides recognized by islet-infiltrating T-cells or to which an immune response was observed after immunization in complete Freund's adjuvant were fitted with anchoring positions defined by I-A^{S7} crystallographic studies. Peptides I 7-23, I 33-47, I 71-86, II 33-47, and II 71-88 could be fitted using the optimal alignment for the P4, P6, P7, and P9 positions. Peptide II 14-30 shared optimal residues for P4, P6, and P9 pockets. Peptides I 20-35 and I 77-92 shared optimal residues for P7 and P9 pockets with the aforementioned peptides.

The development of diabetes in mice lacking the expression of β -cell autoantigens. Transgenic NOD mice expressing GAD antisense in β -cells have been shown to be protected from diabetes (35). However, other models in which the expression of most of the GAD sequence was disrupted have failed to show any significant difference in the incidence of spontaneous diabetes or insulinitis compared with wild-type mice. This does not favor a key role of GAD in diabetes on the NOD background (45). Seemingly, disruption of the IA-2 gene in 129 mice and backcrosses of IA-2 knockout onto the NOD background allowed the role of IA-2 in the NOD mouse to be addressed. IA-2-deficient mice failed to show any difference in diabetes susceptibility when compared with wild-type mice, especially after injection of cyclophosphamide, making a key role of IA-2 in the development of the autoimmune process against β -cells unlikely (46).

We undertook a similar approach to evaluate the role of proinsulin isoforms in diabetes in the NOD mouse. We introduced a null mutation for the proinsulin 1 or proinsulin 2 gene by crossing proinsulin 2^{-/-} 129 mice with NOD mice. Breeders were selected for carrying genetic markers (including the I-A^{S7} MHC molecule) associated with diabetes in the NOD model. An increased incidence of diabetes was observed in proinsulin 2^{+/-} heterozygotes compared with wild-type mice at each of the three backcross (BC) generations that were analyzed (i.e., BC2, BC7, BC8) (47). Furthermore, proinsulin 2^{-/-} mice generated by intercrossing BC4 mice developed highly accelerated insulinitis and diabetes. The high prevalence of anti-insulin

autoantibodies in proinsulin 2^{-/-} mice indicates that diabetes acceleration relates to altered recognition of proinsulin. The prevalence of anti-glutamate decarboxylase autoantibodies and of sialitis was not increased in proinsulin 2^{-/-} mice. Using this model, we provide evidence that proinsulin 2 expression leads to silencing of T-cells specific for an epitope overlapping the C-peptide and the A-chain sequence and shared by proinsulin 1 and 2 (II 88-103/I 86-101) (48). Acceleration of diabetes has now been confirmed in intercrosses obtained from backcross 12 mice. A lesser but significant increase in diabetes prevalence has been observed in heterozygote proinsulin 2^{+/-} mice in these intercrosses. In contrast with data obtained in proinsulin 2^{-/-} mice, the follow-up study of proinsulin 1^{-/-} NOD mice showed strong protection against the development of diabetes. It has been reported that proinsulin 1^{-/-} islets that were grafted under the kidney capsule of diabetic NOD mice were resistant to recurrence of diabetes. This has been taken as the evidence that proinsulin 1 is a primary autoantigen in the NOD model (49). However, we have evidence that proinsulin 1^{-/-} mice keep full susceptibility to diabetes induction by cyclophosphamide at 8 weeks of age, indicating that protection in this model does not relate with an intrinsic resistance of proinsulin 1^{-/-} islets to destruction by T-cells.

Our data provide evidence that expression of proinsulin 2 is not a prerequisite for development of autoimmunity to β -cells in the NOD mouse and that proinsulin 1 is an autoantigen in this model. The expression of proinsulin 1 is only not a prerequisite for development of diabetes on the NOD background. Increased immune recognition of proinsulin and acceleration of diabetes in proinsulin 2^{-/-} mice possibly relate to several mechanisms. The first possibility is an increased frequency of effector T-cells in proinsulin 2^{-/-} mice. We have excluded this possibility by transfer experiments in which different numbers of T-cells from diabetic proinsulin 2^{-/-} mice or wild-type mice were transferred into NODscid recipients. The frequency of effector T-cells was comparable in both diabetic congenic mice. A second possibility is that proinsulin 2 epitopes specifically activate regulatory T-cells. Seemingly, the frequency of effector T-cells is not decreased in proinsulin

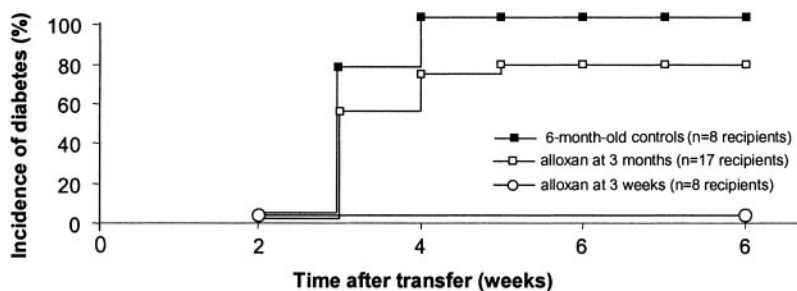


FIG. 3. Transfer of diabetes by spleen cells from β -cell-deprived NOD mice. The 10×10^6 spleen cells from β -cell-deprived or control female NOD mice that received a single injection of alloxan (200 mg/kg) at 3 weeks or 3 months of age were injected into pre-irradiated 8-week-old male recipients. Recipients were followed for development of diabetes (glycemia >250 mg/dl twice at 24-h intervals).

1^{-/-} mice. This hypothesis fits with previous reports pointing to the protection induced by administration of insulin B-chain peptide B9-23 to nondiabetic NOD mice. However, the demonstration that regulatory T-cells specifically recognize peptide B9-23 has not been obtained, and antigen specificity of regulatory T-cells remains elusive in most models. By performing co-transfer experiments in which purified effector T-cells were injected into naive NODscid recipients along with T-cells from diabetes-resistant proinsulin 1^{-/-} mice or wild-type mice, we were unable to evidence the role of regulatory T-cells in the protection observed.

An alternative hypothesis is that proinsulin 2 operates by controlling the peripheral T-cell repertoire. Proinsulin 2 is the predominant isoform expressed within the thymus. The importance of proinsulin expression in the thymus is strengthened by data showing that allelic variation of proinsulin expression influences the risk for type 1 diabetes in the human and that insulin expression in the thymus modulates T-cell tolerance to insulin in the mouse. The role of thymic expression of viral antigens in the mouse has further been shown to control both the frequency of specific T-cell precursors and the severity of diabetes in transgenic mice expressing lymphochoriomeningitis proteins in β -cells. Our data demonstrating that proinsulin 2^{-/-} mice showed a response to a peptide that was not recognized in wild-type mice provide evidence that T-cell precursors are eliminated or silenced in mice expressing proinsulin 2. This favors the role of thymic expression of proinsulin 2 in modulating the peripheral proinsulin-specific immune repertoire and diabetes development. There is strong evidence in the human that variable number of tandem repeats (VNTR) alleles flanking the insulin gene are key determinants of diabetes susceptibility. They may control the β -cell response to glucose within the islets of Langerhans and the expression of proinsulin in the thymus. In the mouse, there is no allelic variability in the 5' VNTR flanking the insulin gene. Proinsulin 2^{-/-} NOD mice may thus represent a relevant model to study the role of thymic expression of insulin on susceptibility to type 1 diabetes (50–52).

To further evaluate the role of insulin expression in immune tolerance to proinsulin in the absence of diabetes susceptibility genes, we studied nonautoimmune-prone proinsulin 2^{-/-} and proinsulin 1^{-/-} 129 mice (53). The repertoire of immunogenic proinsulin peptides was identical in wild-type and proinsulin 1^{-/-} 129 mice. A T-cell response was observed against six preproinsulin 1 and five preproinsulin 2 peptides in both mouse lines. But an additional response was observed in proinsulin 2^{-/-} mice against proinsulin peptide II56-71. Expression of preproinsulin 2 was thus associated with a loss of response to proinsulin 2. T-cell hybridomas were generated against both peptides to further study epitope presentation by

pancreatic islet cells. Whereas proinsulin 2-specific hybridomas generated in proinsulin 2^{-/-} 129 mice were responsive to islets in vitro, T-cell hybridomas that were raised against proinsulin 2 peptides in wild-type mice were unresponsive to islets, indicating that corresponding T-cells are functionally eliminated in mice expressing proinsulin 2. We used a transplantation model to further evaluate the functional relevance of responses to proinsulin 2 in vivo. When wild-type islets were grafted under the kidney capsule of 129 proinsulin 2^{-/-} mice, a B- and T-cell response was observed to proinsulin. Spontaneous insulinitis was observed in grafted proinsulin 2^{+/+} islets and was further enhanced by immunization with proinsulin II56-71. Anti-insulin antibodies were detected in grafted mice. Proinsulin 2 expression thus shapes the proinsulin-specific T-cell repertoire and prevents self-reactivity to the islet. Moreover, islet proinsulin 2 primes an immune response to proinsulin in proinsulin 2^{-/-} mice. Thymus and bone marrow chimeras allowed identification of the thymic epithelium as the relevant site of proinsulin 2 expression that controls T-cell responses to proinsulin 2, providing evidence that thymic expression of proinsulin 2 is directly responsible for repertoire selection bias seen in proinsulin 2^{-/-} 129 mice.

The presence of β -cells: a requirement for the activation of autoreactive T-cells. Whether the activation of autoimmunity proceeds from intrinsic immune dysregulation or requires the presence of β -cells has been addressed in β -cell-deprived NOD mice, obtained after a single injection of a toxic dose of alloxan given at 3 weeks of age (54). Whereas spleen cells from 6-month-old nondiabetic NOD mice efficiently transfer diabetes into irradiated 8-week-old NOD male recipients, spleen cells from 6-month-old female NOD mice that have been deprived of β -cells since the age of 3 weeks fail to transfer diabetes. Importantly, the development of sialitis, as well as control humoral and T-cell-mediated immune responses, are not affected in β -cell-deprived NOD mice. By contrast, spleen cells from NOD mice that have been deprived of β -cells at a later stage of the disease process (i.e., 3 months of age) transfer diabetes into pre-irradiated recipients with full efficiency (Fig. 3). The expression of autoantigens in the target organ is thus required for initial activation of autoreactive lymphocytes. But the presence of β -cells is not required for activation and expansion of autoreactive T-cells once the autoimmune reaction is triggered.

The importance of β -cells in the development of autoimmunity is further strengthened by the demonstration, in the NOD mouse, both of intramolecular and intermolecular spreading of the autoimmune reaction to distinct epitopes within a given autoantigen and to different autoantigens (55). However, in the NOD mouse and in most cases of human type 1 diabetes, autoimmunity does not extend to cells that share antigens with β -cells, particu-

larly non-insulin-secreting endocrine islet cells and neuronal cells that express GAD and IA-2. It is thus likely that both the expression by target cells of autoantigens and their presentation under conditions that allow recognition by T-cells are prerequisites to the development of autoimmune lesions. It remains unresolved whether spontaneous diabetes in the NOD mouse corresponds quantitatively or qualitatively to pathological expression of autoantigens or simply requires their physiological expression. In a distinct model, the development of diabetes was reported in mice showing late expression of an SV40 T antigen transgene on β -cells, whereas early transgene expression led to tolerance (56). Along the same line, evidence has been obtained that the priming of T-cells against β -cell autoantigens occurs in draining pancreatic lymph nodes (57). The protection from diabetes observed after preventive excision of pancreatic lymph nodes at 3 weeks of age provides direct evidence for the importance of the local presentation of autoantigens in the diabetes process (whereas excision of mesenteric lymph node or pancreatic lymph nodes at 10 weeks of age has no effect). The direct visualization of the activation of autoreactive lymphocyte in draining pancreatic lymph nodes (58) and the role of early physiological β -cell apoptosis in this activation (59) have similar implications.

Islet and β -cell modifications: a requirement for development of full-blown diabetes. Human genetic studies indicate that an important contributing region to susceptibility to type 1 diabetes is the polymorphic VNTR minisatellite that lies in the promoter region of the insulin gene 365 bp upstream of the transcription initiation site on chromosome 11p15 (60–62). Polymorphism in this region is generated by variations of the number of repetitive units and by sequence heterogeneity of individual repeats. Alleles are usually categorized in two main classes: short (26–63 repeats) class I alleles and long (140–200 repeats) class III alleles. Homozygotes for class I alleles carry a risk for type 1 diabetes that is increased by 2–5, whereas class III alleles confer dominant protection that reduces the risk by 3–5. In Caucasians, allele distribution is bimodal, with a frequency of ~70% for class I alleles and 30% for class III alleles. The insulin gene VNTR contributes 10% of genetic susceptibility to type 1 diabetes. This region has been reported to bind the transcription factor Pur 1. Variations in this region modulate transcription of the insulin gene in the islet in response to glucose and in the thymus. Whether susceptibility or resistance to type 1 diabetes conferred by variants in the 11p15 VNTR, coined as *IDDM2*, relates to variations in expression of the insulin gene in β -cells or in the thymus remains unknown in the human. Aforementioned experiments in proinsulin-deficient mice, in which variation of proinsulin expression in the thymus controls the T-cell repertoire, support the role of the thymic expression of insulin in predisposition to diabetes.

Other pieces of evidence have been obtained that associate defined insulin secretion patterns with diabetes development, both in the human and in rodent models (15). Particularly, an increased insulin response to glucose correlates with the appearance of inflamed islets in the BB rat. In the NOD model, female mice show increased insulin responses to glucose at 6 weeks of age, correlating with development of insulinitis. Further evidence that β -cell function and products can modulate the development of type 1 diabetes comes from therapeutic approaches. In humans, intensive intravenous insulin therapy during the 15 days after the clinical onset of type 1 diabetes improves

endogenous insulin secretion evaluated 1 year after the onset of the disease. In rodent models, the development of diabetes is modulated by insulin, which modulates macrophage and T-cell functions. The incidence of diabetes is lower after prophylactic insulin therapy at the pre-diabetic stage in the BB rat and the NOD mouse, and the transfer of diabetes by diabetogenic T-cells into treated recipients is slightly delayed. Prophylactic insulin therapy in the NOD mouse reduces the local increase in antigen-presenting cells and islet hypertrophy and decreases the expression of β -cell autoantigens. Diazoxide, an inhibitor of endogenous insulin secretion, prevents diabetes in the BB rat. Neonatal injections of glucose modulate the incidence of diabetes in the NOD mouse, possibly through the modification of maturing β -cells exposed to high glucose levels.

The importance of islet modifications in the triggering of β -cell destruction has been recently underscored in a transgenic model of autoimmune diabetes in which interferon- α and the subsequent increase in expression of class I MHC molecules by islet cells preclude diabetes development (63). In this model, transgenic mice expressing the glycoprotein of the lymphochoriomeningitis virus (LCMV) under the control of the rat insulin promoter develop diabetes upon infection by LCMV, transfer of dendritic cells pulsed with a LCMV glycoprotein-derived peptide, or co-expression of B7, interferon- γ , or interleukin-6 by β -cell under the control of the rat insulin promoter. However, diabetes is not induced by immunization against the LCMV glycoprotein-derived peptide in complete Freund's adjuvant despite the presence of a large number of peptide-specific CD8⁺ T-cells and efficient homing and activation of these cells. Local expression of interferon- α and increased expression of class I MHC molecules correlate with diabetes in this model. Class I upregulation in this model depends on signaling through Toll-like receptors TLR3 and -7. Diabetes is induced in transgenic mice immunized against the LCMV glycoprotein-derived peptide by injection of interferon- α or TLR3 or TLR7 ligands. Expression of TLR3 has previously been reported within the pancreas.

Another approach undertaken to evaluate the role of islet cells in the development of the autoimmune process has been to study the consequence on diabetes development of experimental β -cell mass reduction. While a decreased delay to diabetes onset was expected in NOD mice submitted to surgical pancreatectomy, according to the classic scheme of a progressive β -cell destruction by immune cells, this procedure prevented the destruction of remaining β -cells. Indeed, with 90% pancreatectomy performed at the age of 7 weeks, diabetes incidence was reduced by >80% with reduction of insulinitis. The same procedure was ineffective when performed at the age of 13 weeks. Islet transplantations were used to test the hypothesis that protection was indeed due to loss of antigenic load to the immune system. When a mass of 500 or 125 islets was transplanted in pancreatectomized mice, diabetes was induced at around 100–200 days and the natural history of the disease was reconstituted. In contrast, when a number of islets (30) (representing roughly one-tenth of a normal mouse islet content) was transplanted, the effect of pancreatectomy was not abrogated, and most of the animals remained diabetes free. Interestingly, transplantation of allogeneic islets also promoted diabetes in this model (breakage of the protective effect of pancreatectomy), indicating that the antigen(s) involved are not specific to the NOD mouse and that the pathway of antigen

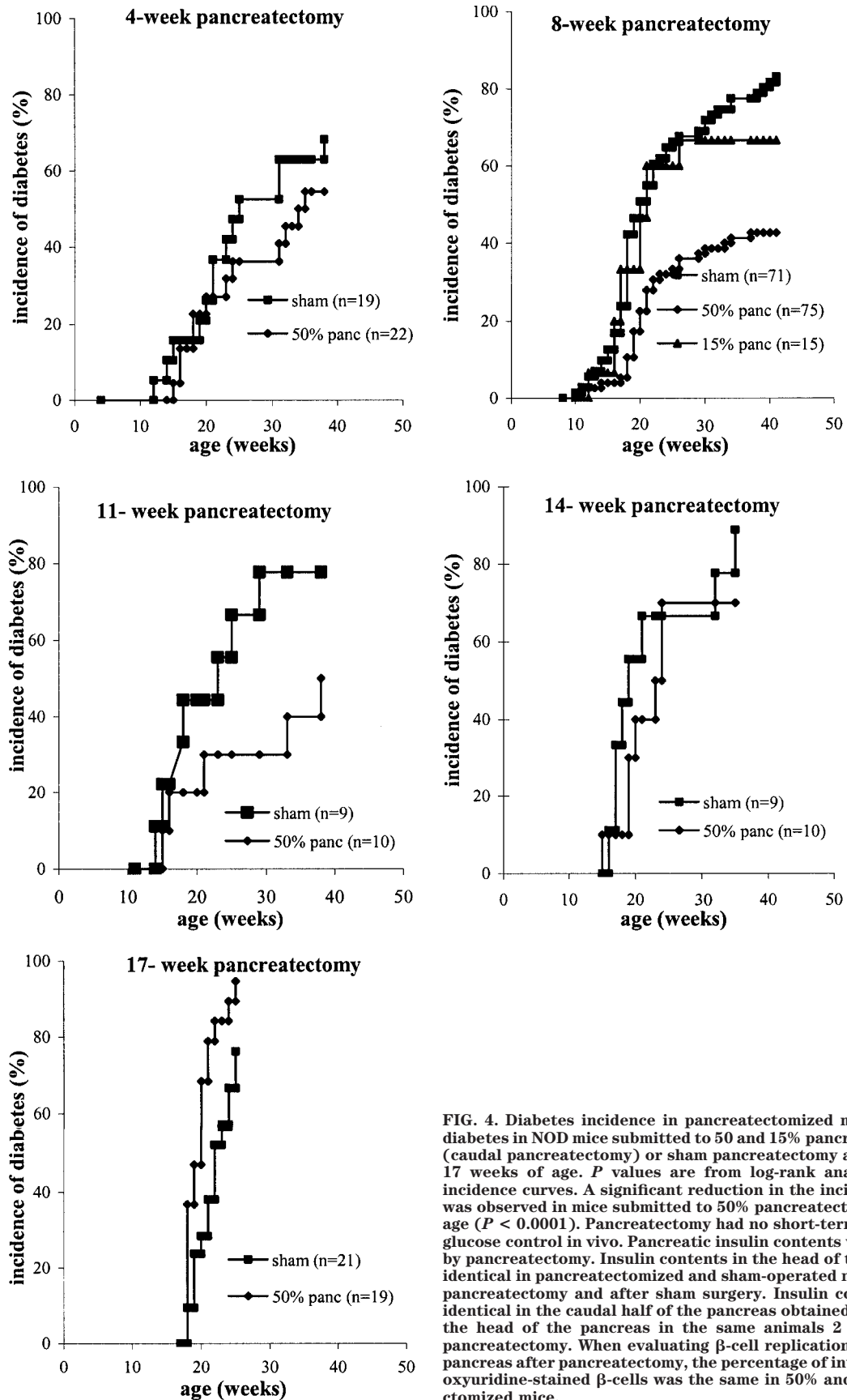


FIG. 4. Diabetes incidence in pancreatectomized mice. Incidence of diabetes in NOD mice submitted to 50 and 15% pancreatectomy (panc) (caudal pancreatectomy) or sham pancreatectomy at 4, 8, 11, 14, and 17 weeks of age. *P* values are from log-rank analysis of diabetes incidence curves. A significant reduction in the incidence of diabetes was observed in mice submitted to 50% pancreatectomy at 8 weeks of age ($P < 0.0001$). Pancreatectomy had no short-term effects on blood glucose control in vivo. Pancreatic insulin contents were not modified by pancreatectomy. Insulin contents in the head of the pancreas were identical in pancreatectomized and sham-operated mice 2 weeks after pancreatectomy and after sham surgery. Insulin contents were also identical in the caudal half of the pancreas obtained at surgery and in the head of the pancreas in the same animals 2 weeks after 50% pancreatectomy. When evaluating β -cell replication in the remaining pancreas after pancreatectomy, the percentage of intra-islet bromodeoxyuridine-stained β -cells was the same in 50% and sham-pancreatectomized mice.

presentation involved does not use specific MHC alleles expressed on the β -cell itself. In additional experiments, grafted NOD islets (500) were removed secondarily to elucidate if a continuous stimulation of the immune system was necessary to break tolerance and activate effector cells toward the islets that remained in situ. When the islets were removed 7 or 14 days after grafting, diabetes developed, whereas the removal of the islets 3 days after grafting did not alter the protective effect of the pancreatectomy. In addition, implantation of islets at 35 weeks of age was devoid of the diabetes-inducing effect. Altogether, these results confirm the role of islet antigens in the drive of the immune response. They also point to specific time windows for immune activation to proceed (64).

In similar experiments, we evaluated the effect of milder pancreatectomy on diabetes development. Fifty percent or 15% pancreatectomy was performed in NOD mice at different ages. Fifty percent pancreatectomy performed at the age of 8 weeks reduced diabetes incidence by 50%, as opposed to sham or 15% pancreatectomy (Fig. 4). When pancreatectomy was performed at 11 weeks of age, a less drastic effect was observed. Pancreatectomy at the age of 17 weeks even accelerated diabetes. Of interest, when pancreatectomy was performed earlier (4 weeks), the protective effect was partially lost. One interpretation of this last finding could be that regeneration of the islet cell mass had taken place before reaching the age of initiation of the disease process. Transfer experiments with splenocytes from diabetic donors confirmed that the remaining islets after pancreatectomy could still be the target of autoimmune effector cells. Altogether, our results confirm those of Itoh and Maki (64) but pose a theoretical challenge to elucidate how a 50% reduction of islet mass can prevent diabetes while the re-implantation of a relatively limited islet mass can re-induce diabetes in a 90% pancreatectomized animal. Nevertheless, altogether they confirm the extreme importance of the presence and timeliness of islet cells in the development of the diabetic process in the NOD mouse model.

CONCLUSION

Accumulating evidence suggests that immune tolerance to β -cells reflects the physiological interaction between the immune system and β -cells rather than intrinsic properties of tolerant lymphocytes. The presence of β -cells is required for initiation of diabetes autoimmunity to proceed. Available experiments in the NOD mouse and epidemiological evidence in the human (48,65) point to proinsulin as a key autoantigen in diabetes. The functional importance of insulin, the high number of autoantigens characterized in the different phases of diabetes, and their clustering within β -cell subparticles point to the islet as a starting point in the initiation phase of the disease. All the experimental evidence also indicates that diabetes development is irreversible once the initiation of autoimmunity is triggered. However, the evidence that islet dysfunction participates in eliciting the autoimmune reaction to β -cell remains indirect.

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