Pancreatic Expression of Interleukin-4 Abrogates Insulitis and Autoimmune Diabetes in Nonobese Diabetic (NOD) Mice

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Summary

Diabetes in nonobese diabetic (NOD) mice is a T cell–dependent autoimmune disease. The destructive activities of autoreactive T cells have been shown to be tightly regulated by effector molecules. In particular, T helper (Th) 1 cytokines have been linked to diabetes pathogenesis, whereas Th2 cytokines and the cells that release them have been postulated to be protective from disease. To test this hypothesis, we generated transgenic NOD mice that express interleukin (IL) 4 in their pancreatic β cells under the control of the human insulin promoter. We found that transgenic NOD–IL-4 mice, both females and males, were completely protected from insulitis and diabetes. Induction of functional tolerance to islet antigens in these mice was indicated by their inability to reject syngeneic pancreatic islets and the failure of diabetogenic spleen cells to induce diabetes in transgenic NOD–IL-4 recipients. Interestingly, however, islet expression of IL-4 was incapable of preventing islet rejection in overtly diabetic NOD recipient mice. These results demonstrate that the Th2 cytokine IL-4 can prevent the development of autoimmunity and destructive autoreactivity in the NOD mouse. Its ability to regulate the disease process in the periphery also indicates that autoimmune diabetes in NOD mice is not a systemic disease, and it can be modulated from the islet compartment.

The nonobese diabetic (NOD)[1] mouse develops autoimmune diabetes with immunopathological features resembling those of the human disease (1). In these mice, a pancreatic inflammation consisting of mononuclear leukocytes first surrounds and then infiltrates the islets and destroys the insulin-producing β cells (2). T cells play a pivotal role in the development of this disease, as demonstrated by antibody treatment (3–6) and splenocyte transfer experiments (7, 8).

T cells are important positive and negative regulators of (auto)immune responses. In humans and mice, at least two functionally distinct subsets of T cells exist, distinguishable by the cytokines they release upon activation (9). The $CD4^+$ compartment includes Th1 and Th2 subsets. Th1 cells release predominantly IFN-γ and are associated with pathogenic autoimmunity, whereas Th2 cells release IL-4 and IL-10 and are associated with allergic responses and, presumably, inhibition of spontaneous autoimmune disease. Apparently, the $CD8^+$ compartment also contains two cell categories, TC1 and TC2, with cytokine profiles similar to those of the CD4 compartment (10–12). The generation of both Th2 cells (13) and TC2 cells (11, 12) in vitro is critically dependent on the presence of IL-4 during T cell priming.

The immune system can be viewed as a dynamic balance between different types of T cells; any disturbance that enables one T cell subcategory to become dominant may prove deleterious. Indeed, substantial evidence has linked the pathogenesis of diabetes in NOD mice to the preferential activation of the Th1 subset (for review see reference 14). Direct evidence for the pathogenic effects of Th1 versus Th2 cells was recently provided by the injection of Th1- and Th2-like effector cells into neonatal NOD mice: Th1 cells rapidly promoted diabetes, whereas Th2 cells did not initiate the disease (15, 16). Interestingly, in co-transfer experiments using these in vitro–differentiated Th1 and Th2 cells, the Th2 cells did not confer protection from diabetes (16). Yet, such transfer experiments with in vitro– "predifferentiated" T cells of an individual clonotype do not reflect the situation in vivo in which antigen exposure and cytokine stimulation occur simultaneously within the lymphoid organs or in tissues, and multiple clonotypes of varying affinity coexist. Since naive islet-specific T cells are likely to encounter islet antigens in islets or adjacent lymphoid aggregates, we hypothesized that in situ expression of IL-4 within the islets could affect T cells that migrate within the pancreas, encouraging them to differentiate toward a nondestructive phenotype.

[1] *Abbreviations used in this paper:* GAD, glutamic acid decarboxylase; NOD, nonobese diabetic.

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Materials and Methods

Generation of Transgenic Mice. A plasmid containing the human insulin promoter and the terminator sequence from the hepatitis B virus gene (17) was cut at its unique BamHI site and blunt-ended by the Klenow fragment of DNA polymerase I. The 451-bp HindIII/EcoRI fragment encoding murine IL-4 was excised from a cDNA clone (British Biotechnology, Oxon, UK), blunt-ended, and inserted into the insulin promoter–containing plasmid. The hybrid DNA molecule (ins–IL-4) was cleaved with EcoRI and SphI, and the 3,097-bp fragment was isolated, purified, and microinjected into fertilized zygotes from NOD/Shi mice from the Scripps rodent colony. The presence of the transgene was confirmed by PCR typing of tail DNA. All mice were housed together under specific pathogen–free housing conditions at the Scripps rodent colony.

Islet and Thymocyte Culture and IL-4 Assay. Islets were isolated from pancreata of 7–12-wk-old NOD–IL-4 mice and their nontransgenic littermates as described (18). Briefly, pancreata were digested with collagenase P (Boehringer Mannheim Corp., Indianapolis, IN) and handpicked under an inverted microscope. 65 individual islets were cultured for 48 h in 500 μl RPMI 1640 medium supplemented with 20 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Thymocytes from three NOD–IL-4 mice and three nontransgenic littermates were cultured (5×10^6/ml) in RPMI 1640 supplemented with 1 mM NaPyruvate, 2 mM L-glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS for 24 h (19). Supernatants from islet cells and thymocytes were removed, and IL-4 levels were determined with an ELISA assay using mAb BVD4-1D11 and biotinylated Ab 11B11 as capture and detection Abs, respectively (PharMingen, San Diego, CA). The concentration of IL-4 in samples was interpolated from a standard curve using recombinant murine IL-4 (PharMingen). This standard curve was linear in the range of 19–1,250 pg/ml.

Measuring of Blood Glucose Levels. Twice a month, blood was withdrawn from the retroorbital sinus of anesthetized mice, and blood glucose levels were determined using blood glucose test strips (Glucofilm; Miles Diagnostics Division, Inc., Elkhart, IN).

Histological Evaluation of Pancreata. Multiple hematoxylin and eosin–stained pancreatic sections from three to five mice in each age group were scored for periinsulitis and insulitis. Approximately 20 islets were scored for each pancreas.

Grafting of Islets. For grafting into NOD–IL-4 females, islets were isolated from 4–5-wk-old NOD females as described above. 120–150 islets were transplanted under the right kidney capsule of 10–11-mo-old NOD–IL-4 females. For grafting into diabetic NOD females, islets were isolated from 5–6-wk-old NOD–IL-4 females and their nontransgenic female littermates. 100–200 individual islets were grafted under the right kidney capsule of diabetic NOD females. All grafted kidneys were removed 4 wk after surgery, and the grafts were examined histologically.

Splenocyte Transfer. Donor cell suspensions were prepared from spleens of diabetic NOD females. Red cells were lysed with 0.84% ammonium chloride. Remaining cells were washed and resuspended at 10^8 cells/ml in sterile PBS. 8–9-wk-old recipient NOD females were irradiated (700 rad) and injected intravenously with 200 μl of the donor splenocyte suspension. Blood glucose values were determined weekly starting at 2 wk after transfer.

Immunohistochemical Staining. Paraffin-embedded pancreata and graft tissue were stained with an immunoperoxidase method using polyclonal Abs to porcine insulin (Dako Corp., Carpinteria, CA), synthetic glucagon (Chemicon International, Inc., Temecula, CA, USA), and human somatostatin (Dako Corp.), followed by a biotinylated secondary Ab and an avidin–biotin complex (Vector Laboratories, Inc., Burlingame, CA). Frozen sections were stained with primary Abs to murine L3T4, Ly2, B 220 (all PharMingen), and F4/80 (Serotec Ltd., Oxford, UK) and the same immunoperoxidase technique.

Cytokine Release to Glutamic Acid Decarboxylase (GAD) Stimulation. Spleen cells were prepared from individual 7–8-wk-old NOD–IL-4 females and their nontransgenic female littermates and incubated at 12×10^6/ml in 0.5 ml culture medium (RPMI 1640, 1% Nutridoma, 5×10^{-5} M 2-ME) in the presence or absence of 20 μg/ml human GAD65 purified from recombinant bacteria (a kind gift of Dr. D. Kaufman, UCLA, Los Angeles, CA). After 72 h, supernatants were collected and analyzed for the presence of IFN-γ and IL-4 using an ELISA assay. IFN-γ levels were determined using mAb R4-6A2 and biotinylated Ab XMG1.2 as capture and detection Abs, respectively (PharMingen). The concentration of IFN-γ in samples was interpolated from a standard curve using recombinant murine IFN-γ (PharMingen). This standard curve was linear in the range of 39–2,500 pg/ml. IL-4 levels were determined as described as above.

Results and Discussion

To generate transgenic NOD mice, murine IL-4 was targeted to the pancreatic islets using the human insulin promoter (17, 20, 21). The Ins–IL-4 transgene construct was injected into fertilized NOD zygotes, and progeny positive for the Ins–IL-4 transgene were subsequently bred with NOD mice. Using this approach, four independent lines were obtained; one of these lines (designated NOD–IL-4 mice) was propagated for the current study. Appropriate expression of the transgene in NOD–IL-4 mice was verified at the mRNA and protein level. In situ hybridization with probes that detect the IL-4 coding sequence confirmed the exclusive localization of IL-4 to pancreatic islets in NOD–IL-4 mice (data not shown). To ensure that this IL-4 mRNA led to the production and secretion of IL-4 protein, islets were isolated from the pancreas of NOD–IL-4 mice and their nontransgenic littermates, cultured in vitro, and the amount of IL-4 released by individual islets was determined with an ELISA assay. We found that 65 islets isolated from NOD–IL-4 mice secreted from 50 to 95 pg IL-4/48 h. These amounts are in the range of that released by comparable numbers of stimulated T cells (22, 23) and allow the differentiation of Th2 effectors in vitro (16). IL-4 was not detectable in supernatants from islets of nontransgenic littermates. Constructs using the insulin promoter may direct expression of the transgene not only to pancreatic β cells but also to the thymus (24, 25). Furthermore, it has been shown that IL-4, when selectively overexpressed in the thymus of transgenic mice, significantly alters thymocyte development and thymus architecture (19). To address possible transgene expression in the thymus of NOD–IL-4 mice, IL-4 levels in supernatants from thymocytes after short-term in vitro culture were determined using an ELISA assay. Thymocyte cultures from neither NOD–IL-4 mice nor from their nontransgenic littermates

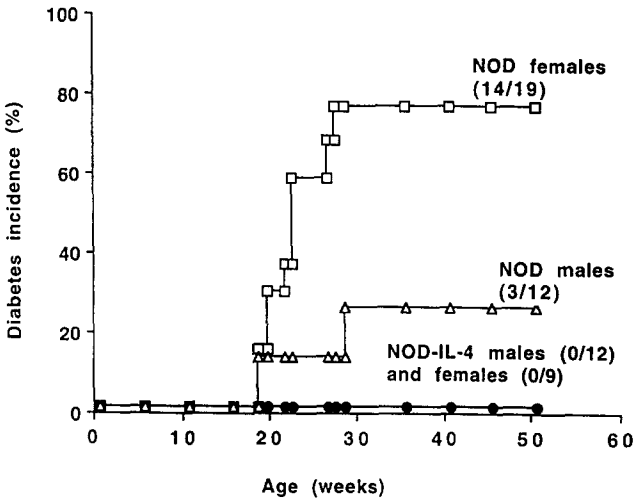

Figure 1. Cumulative diabetes incidence of NOD–IL-4 mice and their nontransgenic littermates. Blood glucose levels were determined bimonthly using Glucofilm blood glucose test strips. Mice were considered diabetic after two consecutive measurements >250 mg/dl. Number of diabetic mice/total number of mice is given in parentheses.

had detectable levels of IL-4, demonstrating that there was no significant amount of IL-4 released from their thymi. Hematoxylin and eosin–stained paraffin sections from thymi of NOD–IL-4 mice were further examined for the histological changes described previously (19). Thymi from NOD–IL-4 mice showed normal architecture and were indistinguishable from thymi of their nontransgenic littermates, rendering thymic expression of the transgene highly unlikely.

To determine whether in situ expression of IL-4 affects the development of diabetes, NOD–IL-4 mice and their nontransgenic littermates were measured for nonfasting blood glucose levels (Fig. 1). Nontransgenic NOD mice spontaneously developed diabetes starting at 18 wk of age, the cumulative incidence of diabetes reaching 75% in females and 25% in males by 28 wk of age, respectively. This disease course duplicated the one of our conventional SPF–NOD colony. In sharp contrast, none of the NOD–IL-4 mice, female or male, became diabetic during the 1-yr observation period. During the whole observation period, blood glucose values of these mice stayed below 200 mg/dl. Histological analysis of pancreata from nontransgenic NOD mice at the onset of diabetes revealed severe insulitis in a majority of islets (Fig. 2 *A*). However, pancreata of NOD–IL-4 mice at 9–11 mo of age contained intact islets occasionally surrounded by inflammatory cells (periinsulitis), but destructive insulitis was never detected (Fig. 2 *B*). Normal islet architecture and intact islet mass in NOD–IL-4 mice were furthermore confirmed using antibodies to the islet hormones insulin (Fig. 2 *C*), glucagon, and somatostatin (data not shown). The time course of insulitis development was analyzed in more detail by evaluating the rate and magnitude of the inflammatory infiltrate in pancreata from NOD–IL-4 mice and their nontransgenic littermates at varying ages (Fig. 3). In both groups, periinsulitis was apparent at the earliest age examined (<2 mo). However, whereas NOD mice steadily progressed to insulitis and islet destruction, NOD–IL-4 mice remained at the periinsulitis stage during their whole lifetime. The mononuclear cell in-

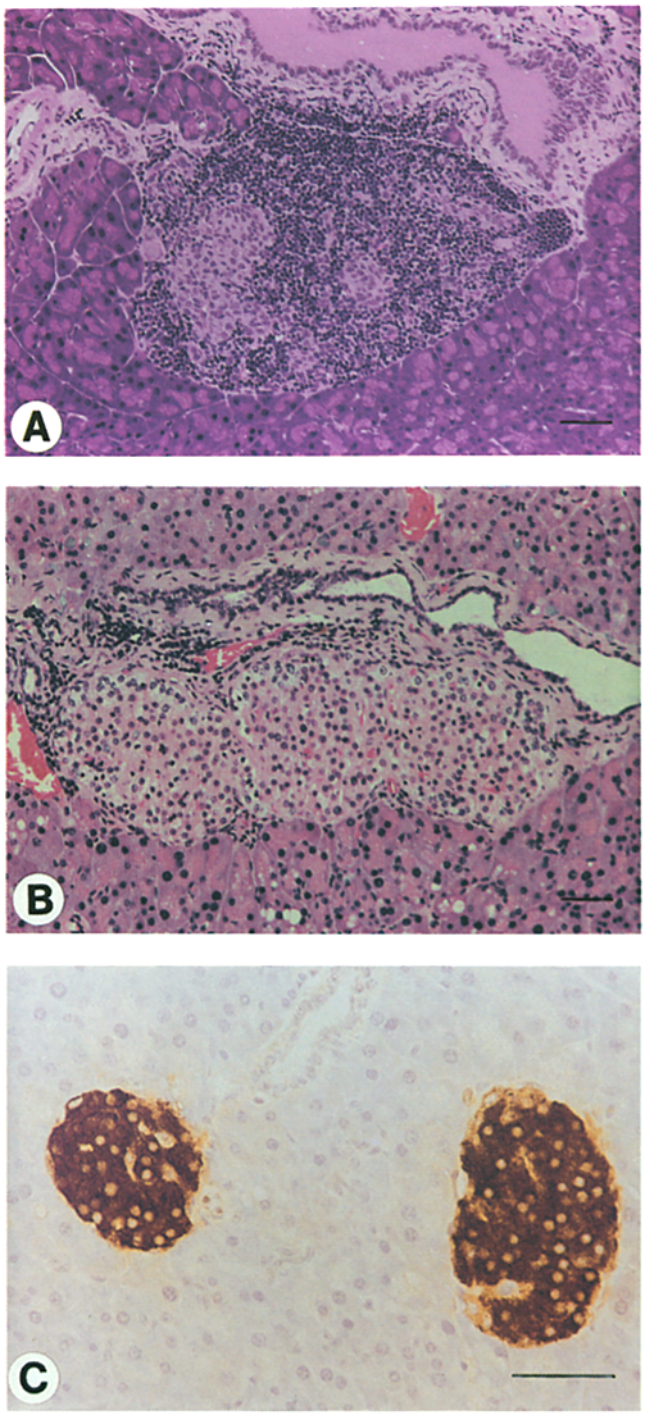

Figure 2. Histological analysis of pancreata of NOD–IL-4 mice and their nontransgenic littermates. Representative hematoxylin and eosin–stained sections of the pancreas reveal characteristic insulitis in the pancreas of a diabetic NOD female at 5 mo of age (*A*), but only weak periinsulitis in a 10-mo-old NOD–IL-4 female (*B*). The islet integrity in NOD–IL-4 mice was confirmed by immunocytochemistry using an antibody to insulin (*C*). Bar, 50 μm.

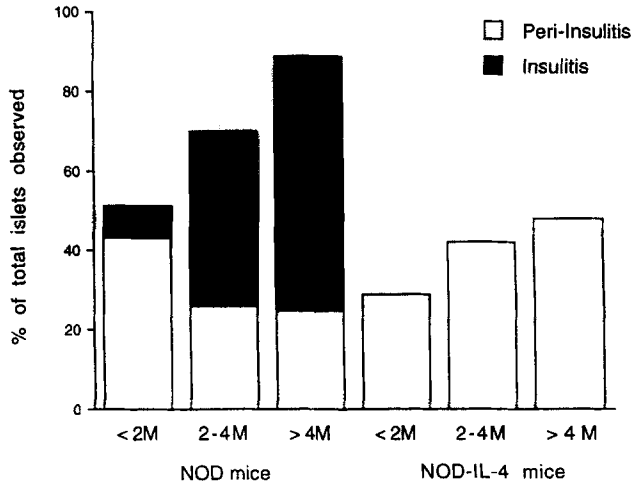

Figure 3. Summary of histological analysis of pancreatic sections from NOD–IL-4 mice and their nontransgenic NOD littermates. Mice (three to five per group) were killed at various ages (<2, 2–4, >4 mo), and their pancreata were examined for periinsulitis and insulitis. Approximately 20 islets were scored for each pancreas.

filtrate around the islets in NOD–IL-4 mice was comparable in cellular composition to the infiltrates observed in nontransgenic littermates: $CD4^+$ and $CD8^+$ T cells and macrophages were the main cell types identified by immunocytochemistry (data not shown). Apart from the pancreas, the salivary gland is the main organ for lymphocyte extravasation in NOD mice. Interestingly, all NOD–IL-4 mice still developed heavy sialitis in their submandibular glands, indicating that IL-4 induced a local immunosuppressive effect in the pancreas but not a generalized immune suppression.

To establish whether "functional tolerance" governed this profound protection from insulitis and diabetes in NOD–IL-4 mice, islet isografting experiments were performed by grafting islets from young NOD mice under the kidney capsule of 10–11-mo-old NOD–IL-4 mice. 4 wk after surgery, histological analysis of the kidneys where these islets were engrafted revealed that, in NOD–IL-4 mice, the grafts were completely free from infiltrating inflammatory cells and that they contained intact islets stain-

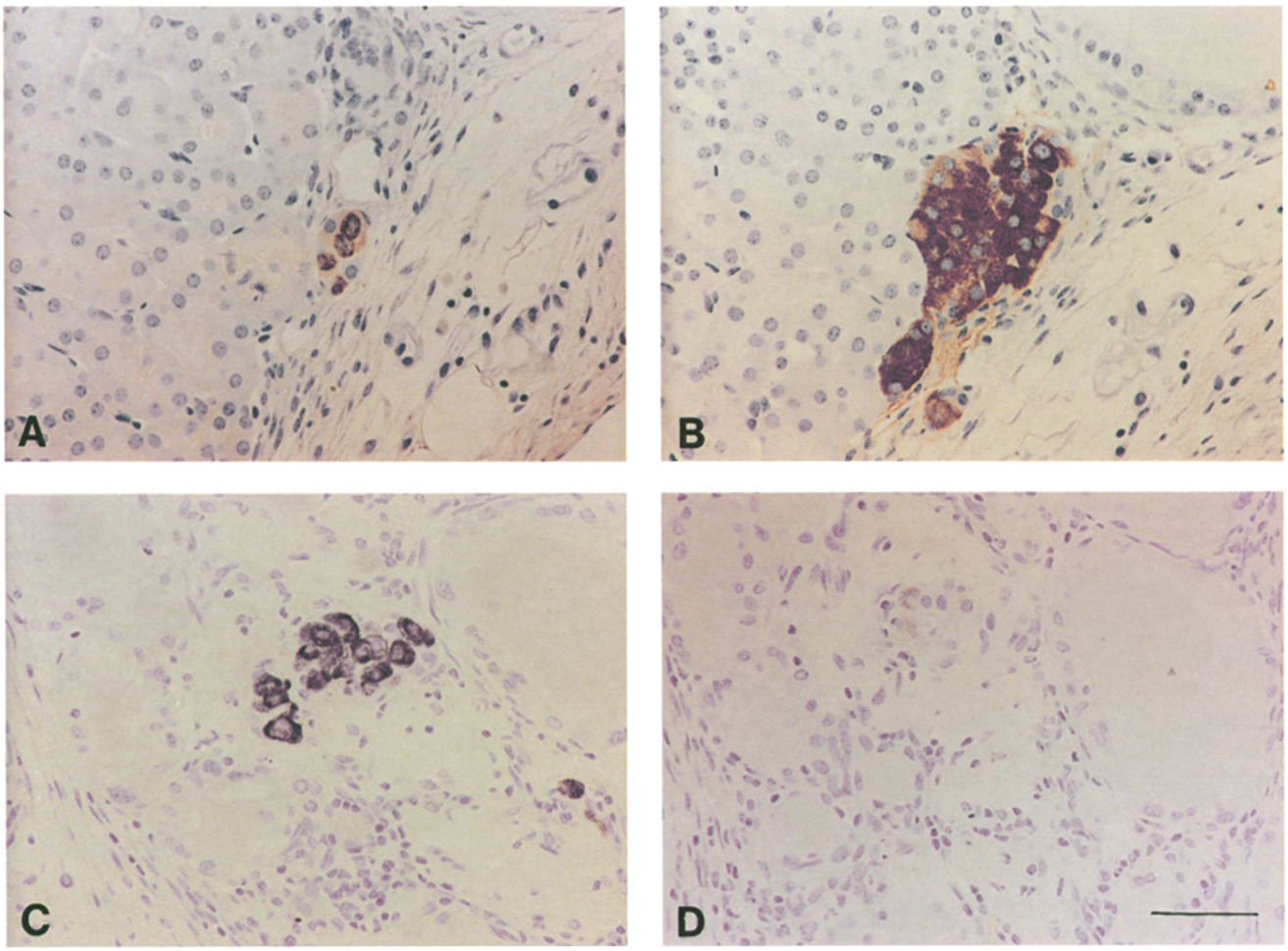

Figure 4. Islet isografting experiments with NOD–IL-4 mice and their nontransgenic littermates. (*A* and *B*) Transgenic expression of IL-4 induces tolerance in NOD–IL-4 mice. Islet isografts from NOD mice were tolerated by NOD–IL-4 mice as demonstrated by the presence of both α (*A*) and β cells (*B*). (*C* and *D*) Transgenic expression of IL-4 does not protect islets from destruction by primed T cells. Islet isografts from NOD–IL-4 mice were rejected in diabetic NOD recipients by an inflammatory infiltrate that left α cells unaffected (*C*) but selectively destroyed β cells (*D*). Bar, 50 μm.

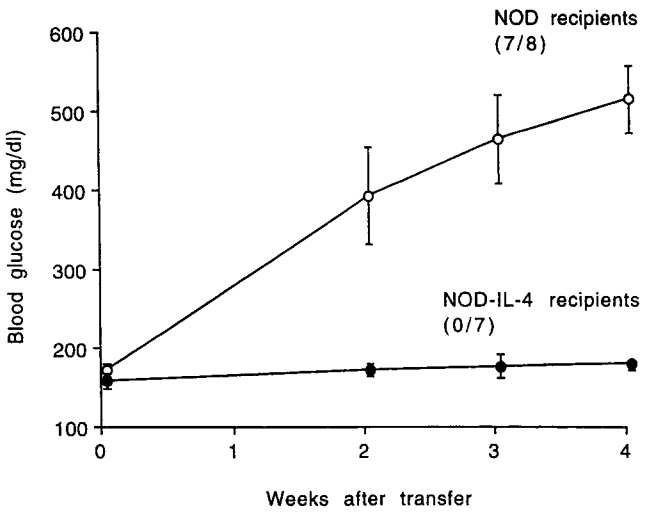

Figure 5. Transgenic expression of IL-4 in the pancreas prevents diabetes transfer. Irradiated NOD–IL-4 females and their nontransgenic female littermates (NOD) were injected with splenocytes from overtly diabetic NOD females. Blood glucose values of recipient mice were determined at 2, 3, and 4 wk after transfer. Number of diabetic mice/total number of mice is given in parentheses.

ing strongly for the islet hormones insulin and glucagon (Fig. 4, *A* and *B*). Consequently, IL-4 had induced a state of functional tolerance to islet antigens in NOD mice.

The inhibition of both diabetes and destruction of engrafted NOD islet cells could reflect a lack of autoreactive T cells in the transgenic animal. To create an experimental situation in which regulation of autoreactive cells could be observed, a transfer model (7) was used in which the ability of in situ IL-4 to block the diabetogenic actions of splenocytes isolated from diabetic NOD mice was assessed. To this end, 8–9-wk-old, sublethaly irradiated NOD–IL-4 mice and their nontransgenic littermates were injected with splenocytes from acutely diabetic NOD mice. Of eight NOD recipients, seven mice became overtly diabetic within 2–4 wk after transfer, with blood glucose values >500 mg/dl (Fig. 5). In contrast, blood glucose values of all seven NOD–IL-4 recipient mice remained <200 mg/dl within the 4-wk observation period after the transfer. Histological analysis revealed massive inflammatory infiltrates in islets of nontransgenic recipients, but NOD–IL-4 recipient mice had only periinsulitis (date not shown), indicating that diabetogenic splenocytes could home to the pancreatic islets but were not able to initiate a destructive autoimmune attack. In the converse experiment, islets were isolated from NOD–IL-4 mice and their nontransgenic littermates and grafted into diabetic NOD recipients, in which islet antigen–specific T cell memory has been demonstrated (26). 4 wk after surgery, the grafts were removed and examined histologically. In control experiments, diabetic NOD mice rejected islet isografts from NOD mice, as demonstrated by a mononuclear cell infiltrate and the selective loss of β cells (data not shown). The same result was observed with islet isografts from NOD–IL-4 mice; glucagon-producing α cells were clearly visible, but no insulin producing β cells could be observed (Fig. 4, *C* and *D*). Thus, whereas autoreactive T cells were inhibited in the splenocyte transfer model, they were unaffected in our grafting experiments.

GAD has been implicated as being the key target antigen in humans (27) and by extrapolation in the NOD mice based on the finding that a T cell response to GAD coincides with the onset of insulitis (28, 29). It has furthermore been demonstrated that this GAD-specific response is mediated by the Th1 subset. To determine whether pancreatic production of IL-4 had an influence on the cytokine pattern of GAD-reactive T cells in NOD–IL-4 mice, splenocytes were isolated from 7–8-wk-old mice stimulated in vitro with GAD65 as described previously (29), and the supernatants were tested for the presence of IFN-γ and IL-4. Although T cells from NOD–IL-4 mice released lower amounts of IFN-γ into the medium compared with T cells from their nontransgenic littermates (0.7 ± 0.24 vs. 1.03 ± 0.32 ng/ml), the difference did not reach statistical significance. Furthermore, supernatants from neither NOD nor NOD–IL-4 mice showed any detectable levels of IL-4. These results suggest that a switch from the production of Th1 to Th2 cytokines in the GAD-reactive T cell compartment present in the spleen is not the major mechanism by which NOD–IL-4 mice are protected from diabetes. Because of the low numbers of infiltrating cells within the pancreas itself, it has so far been very difficult to determine whether such a switch might be occurring at a local level.

The data presented in this study demonstrate that in situ islet expression of IL-4 is sufficient to protect NOD mice from insulitis and diabetes by rendering them functionally tolerant to islet antigens, and significantly extend previous findings based on the in vivo administration of rIL-4 to prediabetic NOD mice (30). The mechanism by which this profound effect is mediated is still under investigation. Although islets from NOD–IL-4 mice produce IL-4 in amounts that are sufficient to induce Th2 cells in vitro (16), no immune deviation toward a Th2 cytokine–producing phenotype could be observed among the GAD-reactive splenocytes in NOD–IL-4 mice. However, one has to consider the possibility that a phenotype switch among autoreactive T cells within the pancreas might not be reflected in the spleen cell compartment or that these cells are very short-lived (31).

One of the puzzling features of diabetes development in NOD mice is the crucial step from periinsulitis to insulitis and concomitant β cell destruction. Although all NOD mice start to exhibit insulitis around 3–5 wk of age, diabetes will ultimately develop in only in a portion of these mice. How this immune response cascade is started in some animals but not in others is still unclear. Strikingly, NOD–IL-4 mice, females and males, remained at the initial periinsulitis stage for all their lives and never progressed to insulitis and diabetes. Several lines of evidence suggest that $CD8^+$ T cells play an essential role in the initiation of the autoimmune response to β cells, possibly by recruiting the final effector cells (32–35). The absence of $CD8^+$ T cells in β2-microglobulin–deficient NOD mice, for instance, leads

to a phenotype that is in many ways very similar to NOD–IL-4 mice, as both strains develop only mild periinsulitis but no insulitis or diabetes. Downregulation or deregulation of the $CD8^+$ T cell compartment by IL-4 in NOD–IL-4 mice could therefore be a likely explanation for our findings. Indeed, Sad and Mosmann (36) recently reported that differentiated $CD8^+$ TC1 cells, when exposed to IL-4 in vitro, lost their ability to synthesize IL-2 and were thus unable to proliferate, suggesting that IL-4 in vivo might downregulate a TC1 response by preventing the clonal expansion of these effector cells. It is conceivable that in the case of NOD–IL-4 mice, this mechanism would result in the abrogation of the immune response cascade and thus stop the disease development at the periinsulitis stage. Committed TC1 cells present in splenocytes from diabetic NOD mice could be regulated by IL-4 in similar ways, thus inhibiting the transfer of disease into NOD–IL-4 mice. In addition to affecting TC1 cells, IL-4 might also act on macrophages in the pancreas by inhibiting their nitric oxide (37, 38) and TNF-α production (39). Interestingly, islets from NOD–IL-4 mice grafted into diabetic NOD mice were not protected from destruction, a seeming contradiction to the splenocyte transfer experiments. However, the immune response to an ectopically placed isograft is likely to be aggravated by an inflammatory response due to the operation procedure itself and can thus not readily be compared with the immune response observed to endogenous islets.

This is also the first report of a localized peripheral approach to the diversion of autoreactivity, demonstrating that the targeted immune intervention aiming to restore an immune balance is feasible. Although the etiology of diabetes in the NOD mouse is not yet understood, the presence of autoreactive cells has implied a defective negative selection in the thymus. However, that islet immunogenicity could now be corrected at this peripheral site suggests that a systemic defect is not the primary cause of diabetes. On the contrary, it seems that before disease onset, islet antigen–naive T cells emerge from the thymus and migrate through blood and lymph until antigen is encountered. The site of this priming event is debated, however, our experiments clearly demonstrate that regulation can occur from the remote islet site. Thus immune therapy by localized expression of IL-4 is a worthwhile consideration in approaching insulin-dependent diabetes mellitus, having the advantage of not inducing global immunosuppression.

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