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Production of interleukin 10 by islet cells accelerates immune-mediated destruction of beta cells in nonobese diabetic mice.

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Brief Definitive Report

Production of Interleukin 10 by Islet Cells Accelerates Immune-mediated Destruction of β Cells in Nonobese Diabetic Mice

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Summary

The T helper type 2 (Th2) cell product interleukin 10 (IL-10) inhibits the proliferation and function of Th1 lymphocytes and macrophages (Mø). The nonobese diabetic mouse strain (NOD/Shi) develops a Mø and T cell–dependent autoimmune diabetes that closely resembles human insulin-dependent diabetes mellitus (IDDM). The objective of the present study was to explore the consequences of localized production of IL-10 on diabetes development in NOD/Shi mice. Surprisingly, local production of IL-10 accelerated the onset and increased the prevalence of diabetes, since diabetes developed at 5–10 wk of age in 92% of IL-10 positive I-A$\beta^{g7/g7}$, I-E$^-$ mice in first (N2) and second (N3) generation backcrosses between IL-10 transgenic BALB/c mice and (NOD/Shi) mice. None of the IL-10 negative major histocompatibility complex-identical littermates were diabetic at this age. Furthermore, diabetes developed in 33% of I-A$\beta^{g7/d}$, I-E$^+$ N3 mice in the presence of IL-10 before the mice were 10 wk old. Our findings support the notion that IL-10 should not simply be regarded as an immunoinhibitory cytokine, since it possesses powerful, immunostimulatory properties as well. Furthermore, our observations suggest that β cell destruction in NOD mice may be a Th2-mediated event.

In mice, IL-10 is produced by the Th2 subset of CD4$^+$ T helper lymphocytes, Ly1$^+$ B lymphocytes and macrophages (Mø) (1, 2). It inhibits proliferation of Th1 lymphocytes and production of cytokines by blocking costimulatory functions of accessory cells (3–6). IL-10 is also a potent inhibitor of monocyte/Mø function and cytotoxicity (7–9). However, IL-10 is not a general inhibitor of immune responses. It potentiates IL-2–induced proliferation and differentiation of CD8$^+$ T cells (10), and stimulates expression of the high-affinity IgG Fc receptor type 1 (FcγR1) on monocytes, thereby stimulating monocyte-mediated antibody-dependent cellular toxicity (11).

Transgenic expression of IL-10 in pancreatic β cells of BALB/c mice (Ins-IL-10) leads to peri-islet inflammation (12). Characteristically, this lesion does not progress to insulitis and the mice never become diabetic. However, aside from attracting leukocytes, IL-10 may affect the functional specialization of lymphocytes within the inflammatory foci, promoting development of a Th2 cytokine response. Because a shift in the balance between Th1 and Th2 lymphocytes may change the outcome of immunological reactions (13, 14), the objective of this study was to explore the consequences of localized production of IL-10 on the development of insulin-dependent diabetes mellitus (IDDM) in nonobese diabetic (NOD/Shi) mice. To obtain islet-specific production of IL-10 on a diabetes susceptible genetic background, we backcrossed mice from two well-characterized, independent, Ins-IL-10 transgenic lines to NOD/Shi mice and examined the N2 and N3 progeny. Mice were examined for diabetes, and typed by the PCR for the MHC I-Aβ^d (BALB/c) and the presence of a functional I-E (I-E$^+$).

Materials and Methods

Animals. The two Ins-IL-10 transgenic BALB/c lines used for the breeding are described elsewhere (12). The NOD/Shi mice were part of a colony at The Scripps Research Institute; the original mice were donated by E. Leiter (The Jackson Laboratory, Bar Harbor, ME). Diabetes in the parental NOD/Shi mice develops when the mice are 13 wk old. In females, the prevalence of diabetes is ~50% at 16 wk and 75% at 36 wk of age. In males the prevalence is ~20% at 36 wk of age. NOD/Shi mice were outcrossed to the Ins-IL-10 strain and the progeny (F1) was backcrossed twice to obtain a N2 and N3 generation, which carry 75 and 87.5% of the NOD/Shi genes, respectively. Mice were maintained in microisolator cages under pathogen-free conditions. The animal care was in accordance with the institutional guidelines and recommendations from The National Institutes of Health.

The blood glucose concentration (BG) was determined in unanesthetized mice in a drop of tail blood using Chemstrip bG and an AccuChek II monitor (Boehringer Mannheim Diagnostics, Indianapolis, IN). As preliminary experiments strongly indicated that the presence of IL-10 accelerated the onset of diabetes in mice of both gender, BG levels were determined once a week beginning

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when the mice were 4 wk old. Mice were killed at the onset of diabetes, defined as a BG level >300 mg/dl, or at 10 wk of age (some IL-10 negative mice were killed on the same day that a transgenic littermate developed diabetes). A normal BG concentration was defined as a BG level less than 120 mg/dl.

Histology. Upon death all the pancreata were divided in two parts. The part of the pancreas used for evaluation of peri-islet inflammation and insulitis was fixed in Bouin's fixative overnight, followed by paraffin embedding and hematoxylin and eosin staining (H&E). Islets were counted in three discontinuous layers of pancreatic tissue, and scored for morphological abnormalities according to the following system: 0, normal islets; 1, peri-islet inflammation, only few cells are seen in the islets; 2, severe insulitis with evidence of β cell necrosis. The part for immunohistochemistry was snap-frozen in Tissue-Tek (Miles Laboratories Inc., Elkhart, IN), sectioned and stained with Mac-1 (Boehringer Mannheim Biochemicals), Ly5 (CDR45/B220), L3T4, and Ly2 for detection of macrophages, B lymphocytes, $CD4^+$ lymphocytes, and $CD8^+$ lymphocytes, respectively (PharMingen, San Diego, CA) (all antibodies were used at a concentration of 5 μg/ml). After incubation with biotin-labeled anti-rat IgG antibodies (Vector Laboratories, Inc., Burlingame, CA), sections were exposed to horseradish peroxidase (HRP)-labeled avidin-biotin complex (ABC-kit; Vector Laboratories, Inc.). HRP was visualized using 3,3′ diaminobenzidine as chromogene.

In Situ Hybridization. Deparaffinized, Bouin-fixed sections were prehybridized for 2–3 h at 42°C in a buffer composed of 50% formamide, 0.3 M NaCl, 20 mM tris, pH 8.0, 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, and 10 mM dithiothreitol. Hybridization with 750,000 cpm/section of ^{35}S-labeled sense or antisense RNA probe was done for 16 h at 42°C in a humidified chamber. This was followed by washing, dehydration, and drying. Sections were covered with Kodak NTB2 emulsion, and were developed after 3 wk. RNA probes were prepared by in vitro transcription of linearized plasmid containing IL-10 or IFN-γ cDNA.

Adoptive Transfer. Adoptive transfer was performed as described by Wicker et al. (15). Splenocytes were isolated from diabetic N2 (n = 7) and N3 donors (n = 1); all donors were I-A$\beta^{g7/g7}$, I-E$^-$. 7-wk-old NOD/Shi recipients were treated with 775 rad whole-body radiation from a cesium source. The mice were then given an intravenous injection containing 1–2 × 10^7 donor splenocytes. BG levels were measured weekly. Mice were killed at the onset of clinical diabetes or 6 wk after the transfer. Adoptive transfer from a diabetic NOD/Shi mouse to two 7-wk-old irradiated NOD/Shi mice was used as positive control. These mice became diabetic 2–3 wk after transfer. Untreated, age- and sex-matched NOD/Shi mice served as negative controls. We found no differences in pancreatic morphology between irradiated NOD/Shi mice left untreated for 6 wk (n = 2) and the untreated mice used as controls.

Genetic Analysis. The presence of the IL-10 transgene, I-Aβ^d and I-Eα^d were determined by PCR on tail DNA. I-Aβ^d–specific primers were: 5′ GATACATCTACAACCGGGAGGAG 3′ and 5′ CTGTTCCAGTACTCGGCGTCTG 3′ (16). The presence of I-Aβ^d was confirmed by immunohistochemical staining of thymic or splenic tissue from randomly selected mice; anti-mouse Ia monoclonal antibody M4/114, which does not react with I-Aβ^{g7}, was used. The presence of I-Eα^d was shown by using primers complementary to the signal peptide exon: 5′ ATGAGCTCCCAGAAGTCATGGG 3′ and 5′ GGAGAGACAGCAGCTCTCAGC 3′ (17). The 5′ end primer covers the SacI restriction site in the signal exon, which is deleted in mice not expressing I-E, such as the NOD strain (17). In each PCR reaction, we included DNA from NOD/Shi and BALB/c mice as controls.

Results and Discussion

Prevalence of Diabetes and Islet Inflammation in F1, N2, and N3 Backcross Animals. Diabetes did not develop in any of the F1 mice tested. At 8–12 wk of age, BG levels in IL-10 positive (n = 9) and IL-10 negative (n = 14) F1 mice were <120 mg/dl. Staining with H&E showed no evidence of leukocyte infiltration in pancreata from IL-10 negative F1 mice (n = 4). Pancreata from IL-10 positive F1 mice (n = 2) had peri-islet inflammation similar to that seen in the parental Ins-IL-10 lines (12).

The prevalence and time of onset of diabetes were determined in 47 mice of both genders from the first backcross (N2) (Table 1). Among mice that were I-A$\beta^{g7/g7}$, I-E$^-$, 16 of 17 IL-10 positive mice were diabetic by 4–10 wk of age; none of their MHC-identical, nontransgenic littermates (n = 6) were diabetic at this age (Table 1). The BG level in the IL-10 negative mice was normal at time of death. Diabetes did not develop in IL-10 positive mice that were

Table 1. *Prevalence of Spontaneous Diabetes in Male and Female Mice in First (N2) and Second (N3) Backcrosses in Relation to MHC Type*

N2 (n = 47)	MHC genotype: I-A$\beta^{g7/g7}$, I-E$^-$ (n = 23)	MHC genotype: I-A$\beta^{g7/d}$, I-E$^+$ (n = 24)	N3 (n = 33)	MHC genotype: I-A$\beta^{g7/g7}$, I-E$^-$ (n = 16)	MHC genotype: I-A$\beta^{g7/d}$, I-E$^+$ (n = 17)
	diabetes incidence (%)			*diabetes incidence (%)*	
IL-10 positive (n = 28)	16/17 (94)	0/11 (0)	IL-10 positive (n = 13)	6/7 (86)	2/6 (33)
IL-10 negative (n = 19)	0/6 (0)	0/13 (0)	IL-10 negative (n = 20)	0/9 (0)	0/11 (0)
p value	$p \leq 0.002$	ND	p value	$p \leq 0.01$	$p < 0.05$

Fisher's exact test was used for the statistical calculations.

Table 2. *Histopathological Analysis of Islets from Female and Male Mice from the First (N2) and Second (N3) Generation Backcrosses in Relation to MHC Type*

	MHC genotype: I-A$\beta^{g7/g7}$, I-E$^-$			MHC genotype: I-A$\beta^{g7/d}$, I-E$^+$		
	Normal islets	Islets showing peri-islet inflammation	Islets showing severe insulitis	Normal islets	Islets showing peri-islet inflammation	Islets showing severe insulitis
N2						
IL-10 positive	10%	38%	52%	5.5%	82%	12.5%
IL-10 negative	97.5%	2.5%	0%	99.6%	0.4%	0%
p values	p <0.001	p <0.001	p <0.001	p <0.001	p <0.001	p <0.001
N3						
IL-10 positive	4.8%	45.2%	50%	3.3%	59.3%	37.4%
IL-10 negative	75%	22.7%	2.3%	92%	7.1%	0.9%
p values	p <0.001	p <0.001	p <0.001	p <0.001	p <0.001	p <0.001

The following number of mice and islets (n) were tested for evaluation of histology. N2 generation: Mice expressing I-A$\beta^{g7/g7}$, I-E$^-$: 12 IL-10 positive (n = 145) and 5 IL-10 negative mice (n = 157); mice expressing I-A$\beta^{g7/d}$, I-E$^+$: 7 IL-10 positive (n = 168) and 10 IL-10 negative mice (n = 244). N3 generation: Mice expressing I-A$\beta^{g7/g7}$, I-E$^-$: 5 IL-10 positive (n = 42), 6 IL-10 negative mice (n = 172); mice expressing I-A$\beta^{g7/d}$, I-E$^+$: 4 IL-10 positive (n = 91), and 9 IL-10 negative mice (n = 222). Fisher's exact test was used for statistical calculations.

I-A$\beta^{g7/d}$, I-E$^+$ (n = 11) (Table 1). In addition, all of the IL-10 negative N2 mice obtained by backcrossing nontransgenic Ins-IL-10 littermates with NOD/Shi mice had normal BG levels at 10–13 wk of age. Diabetes in IL-10 positive I-A$\beta^{g7/g7}$, I-E$^-$, N2 mice was accompanied by pronounced insulitis (Table 2). This is in sharp contrast to the selective peri-insulitis in the parental Ins-IL-10 transgenic strain (12). Thus, localized production of IL-10 does not protect the islets of Langerhans against infiltration by inflammatory cells in the presence of diabetes susceptibility genes. Surprisingly, in I-A$\beta^{g7/d}$, I-E$^+$ mice, IL-10 induces infiltration of the islets of Langerhans, as no islets with insulitis were detected in MHC-identical IL-10 negative mice (Table 2).

The prevalence and time of onset of diabetes were also determined in 33 mice from the second backcross (N3). Among mice that were I-A$\beta^{g7/g7}$, I-E$^-$, six of seven IL-10 positive mice of both genders were diabetic by 4–10 wk of age; none of the MHC-identical, IL-10 negative littermates of these progeny were diabetic at this age (n = 9) (Table 1). As in the N2, the early onset of diabetes in I-A$\beta^{g7/g7}$, I-E$^-$, IL-

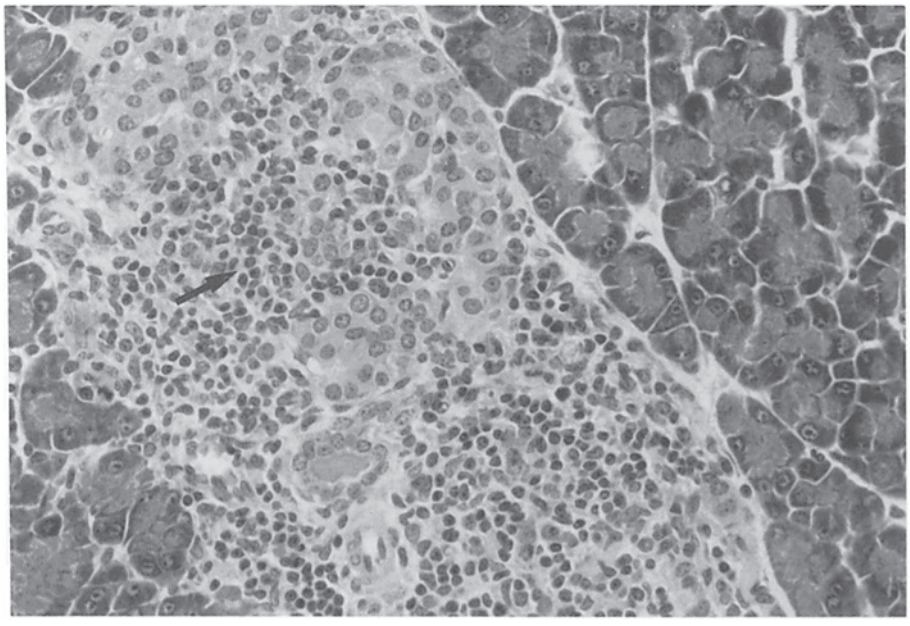

Figure 1. H&E staining of islet with insulitis (→). The pancreas was isolated from a diabetic, 5-wk-old, IL-10 transgenic N3 male expressing I-A$\beta^{g7/d}$, I-E$^+$.

10 positive mice was accompanied by an increase in the number of islets showing insulitis (Table 2). Among mice that were I-A$\beta^{g7/d}$, I-E$^+$ two of six mice that were IL-10 positive were diabetic at 5 and 6 wk of age (Table 1) (Fig. 1); none of their MHC-identical, IL-10 negative littermates were diabetic at 10 wk of age (n = 11). Parallel to the development of clinical diabetes in I-A$\beta^{g7/d}$ and I-E$^+$ mice that were IL-10 positive, the number of islets showing insulitis increased significantly from N2 (12.5%) to N3 (37.4%) (p <0.001) (Table 2). Introduction of more diabetes susceptibility genes from N2 to N3 is indicated by the increased presence of peri-islet inflammation in IL-10 negative mice that were I-A$\beta^{g7/g7}$, I-E$^-$ or I-A$\beta^{g7/d}$, I-E$^+$ (Table 2) (both p <0.001).

Because the accelerated onset and the high prevalence of diabetes in the N2 and N3 generations were accompanied by leukocytic infiltration of the islets of Langerhans, IL-10–induced diabetes in the NOD mice is most likely a consequence of an accelerated immune reaction.

Characterization of Leukocyte Subsets in Inflammatory Foci and IL-10 and IFN-γ Expression. Pancreatic tissue from IL-10 positive N2 mice with diabetes (n = 5, all I-A$\beta^{g7/g7}$, I-E$^-$), IL-10 positive N3 mice with or without diabetes (I-A$\beta^{g7/g7}$, I-E$^-$ [n = 3], and I-A$\beta^{g7/d}$, I-E$^+$ [n = 3]), and from NOD/Shi mice (n = 2) were selected for characterization of leukocyte subsets. The presence of IL-10 changed the subset of attracted leukocytes in both backcross progeny compared with the parental NOD/Shi mice. Characteristically, in 10 of 11 nondiabetic and diabetic IL-10 positive N2 and N3 mice,

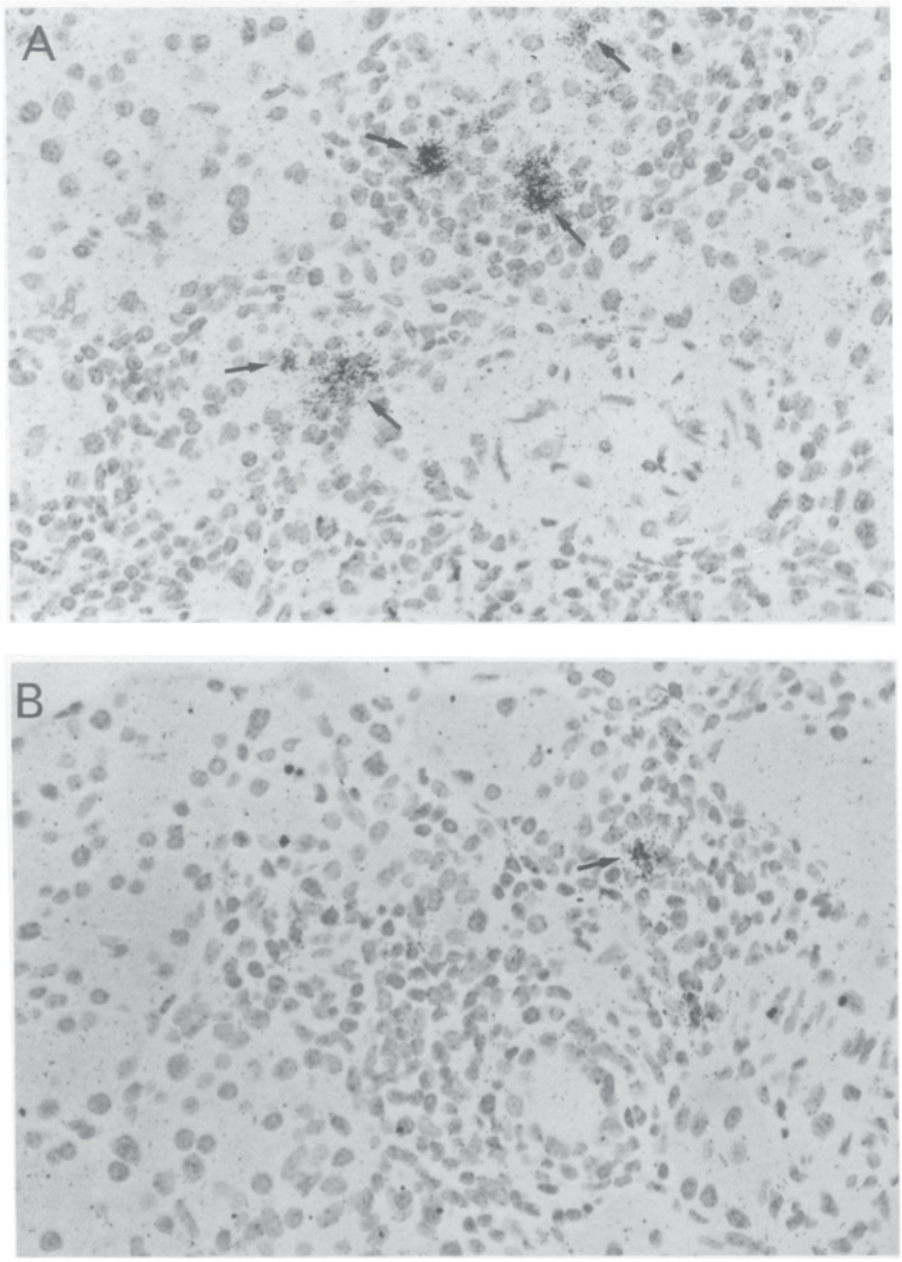

Figure 2. In situ hybridization demonstrating lymphocytic expression of IL-10 (*A*) (→) and IFN-γ (*B*) (→) in IL-10 transgenic BALB/c mice. No significant signals were seen using sense probes.

early as well as late lesions were dominated by Mø, $CD4^+$ T lymphocytes, and B lymphocytes, whereas only few $CD8^+$ cytotoxic T cells were present (data not shown). This subset distribution of attracted leukocytes is similar to that seen in the parental Ins-IL-10 lines (12). In contrast, in adult NOD mice, lesions were dominated by $CD4^+$ and $CD8^+$ T cells and B lymphocytes, whereas very few Mø were seen (n = 2) (data not shown). In the parental Ins-IL-10 strain, islet expression of IL-10 led to a Th2-like infiltration, as cells expressing IL-10 were frequently found in the inflammatory foci, as determined by in situ hybridization (Fig. 2 *A*), although occasionally cells expressing IFN-γ were also observed (Fig. 2 *B*). IL-10 containing cells were demonstrated in the inflammatory foci of N2 and N3 mice by immunohistochemistry (data not shown).

Splenocytes from Diabetic N2 and N3 Mice Infrequently Transfer Diabetes. The complete separation of peri-insulitis from insulitis and diabetes in the parental Ins-IL-10 mice suggests that immune sensitization to β cells does not occur in these mice (12). Sensitization to β cells after introduction of a limited number of diabetes susceptibility genes may explain the IL-10–induced insulitis and diabetes we observed. Therefore, we determined if splenocytes from diabetic N2 and N3 mice transferred disease to irradiated, 7-wk-old NOD/Shi recipients (n = 15). Only one recipient became diabetic after 3 wk ($p > 0.05$). At sacrifice, the BG levels in the recipients that did not become diabetic (n = 14) were similar to the BG levels in the negative control group (n = 8). In addition, the number of islets showing peri-islet inflammation or insulitis did not differ between the two groups ($p > 0.05$, Fisher's Exact test) (data not shown). The failure to transfer diabetes has several explanations. First, N2 and N3 mice still lack 25 and 12.5%, respectively, of the NOD genes and thus may lack some possible diabetes susceptibility genes important for development of antigen-specific autoimmunity, although one recipient did become diabetic. Second, IL-10–induced acceleration of insulitis and diabetes may be due to antigen-nonspecific immune reactions, which depend on the presence of IL-10 in the islets. Third, although donor splenocytes were I-A$\beta^{g7/g7}$, graft-vs.-host disease due to differences between minor antigens is possible, and this could prevent transfer of disease.

IL-10 Induces Diabetes in NOD Mice in the Presence of a Normally Protective MHC Haplotype. At least three to five genes (Idd-1–Idd-5) confer susceptibility to IDDM in NOD mice (18). Idd-1 is located in the MHC region on chromosome 17 and determines both development of insulitis and onset of diabetes. Two unusual features of the NOD MHC are the lack of I-E transcription, which is due to a deletion in the promoter region of the I-Eα chain gene (17, 19) and the presence of unique I-Aβ molecules (I-Aβ^{g7}) (16). Previous studies have suggested that the MHC contribution to IDDM is recessive, because coexpression of protective and diabetogenic I-A genes or expression of functionally active I-E inhibits development of diabetes and reduces insulitis (20–25). However, findings in breeding studies and in other studies with transgenic mice suggest that I-Aβ^{g7} is dominant with reduced penetrance in heterozygous form (26–29). Furthermore, $I\text{-}E^+$ MHC heterozygous NOD mice (I-A$\beta^{g7/nb1}$) have insulitis at 10 mo of age (30), and diabetes develops in very few I-A$\beta^{g7/b}$ or I-A$\beta^{g7/k}$ mice at 10–15 mo of age (31). Our results show that IL-10 can overcome the (partially) protective effect of $I\text{-}E^+$ and NOD MHC heterozygosity (I-A$\beta^{g7/d}$) on diabetes in N3 backcrosses before 10 wk of age.

In summary, we have shown that transgenic expression of IL-10 accelerates the prevalence and onset of diabetes in I-A$\beta^{g7/g7}$, $I\text{-}E^-$ N2 and N3 NOD mice and in some I-A$\beta^{g7/d}$, $I\text{-}E^+$ N3 mice. The effect of IL-10 is more than an acceleration since the cytokine can overcome genetic barriers imposed by less than complete set of genes from the NOD background. Furthermore, we have shown that transgenic expression of IL-10 promotes a Th2-like lymphokine pattern, at least as determined by the level of IL-10 expression of infiltrating cells. That IL-10 affects the balance between Th1 and Th2 lymphocytes in vivo is supported by recent observations obtained from IL-10–deficient mice (32).

Autoimmunity is usually attributed to Th1 cells, whereas Th2 responses are regarded as protective (13), although certain autoimmune diseases appear to be mediated by Th2 cells (33). However, recent observations indicate that the inflammatory foci in NOD mice are dominated by a Th2 cytokine response (34). Our observations support the notion that the autoimmune destruction of β cells in NOD mice is mediated by Th2 cells, and furthermore, that a Th2-mediated response is more harmful than generally believed.

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