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H-2D End Confers Dominant Protection from IL-10-Mediated Acceleration of Autoimmune Diabetes in the Nonobese Diabetic Mouse¹

Antonio La Cava,² Balaji Balasa,^{2,3} Augusta Good, Kurt van Gunst, Nadja Jung, and Nora Sarvetnick⁴

BALB/c mice that express IL-10 as a transgene in their pancreatic β cells (Ins-IL-10 mice) do not develop diabetes, even after crossing to nonobese diabetic (NOD) mice ((Ins-IL-10 \times NOD)F₁ mice). However, backcross of F₁ mice to NOD mice (NOD. Ins-IL-10 mice) results in N2 and N3 generations that develop accelerated diabetes. In this study, we found that NOD. Ins-IL-10 mice that expressed BALB/c-derived MHC molecules (NOD. Ins-IL-10(H-2^{g7/d}) mice) were protected from diabetes. This protection associated with peri-islet infiltration and preserved β cell function. Moreover, expression of I-A^d and I-E^d MHC class II molecules of BALB/c origin was not responsible for protection, but NOD. Ins-IL-10 mice that expressed BALB/c MHC class I D^d molecules (NOD. Ins-IL-10(H-2^{g7/d}) mice) did not develop diabetes. To directly test the possibility of a protective role of H-2D^d in the development of accelerated diabetes, we generated transgenic mice expressing D^d under the control of the MHC class I promoter. We found that double transgenic NOD. Ins-IL-10-D^d mice developed accelerated diabetes in a fashion similar to NOD. Ins-IL-10 mice that were D^d negative. Microsatellite analysis of H-2D^d-linked loci confirmed association between BALB/c-derived alleles and protection of NOD. Ins-IL-10(H-2^{g7/d}) mice. These results suggest a control of H-2D^d-linked gene(s) on IL-10-mediated acceleration of autoimmune diabetes and dominant protection of the D^d region in NOD. Ins-IL-10 mice. *The Journal of Immunology*, 2001, 167: 1066–1071.

Development of insulin-dependent diabetes mellitus (IDDM)⁵ in the nonobese diabetic (NOD) mouse closely recapitulates the clinical and genetic aspects of human type 1 diabetes (1–2). In both the NOD mouse and in humans, the autoimmune response that leads to loss of insulin-producing β cells and diabetes is under polygenic control, with stochastic and/or environmental factors influencing penetrance (3–4). At least 18 chromosome regions have been identified that control diabetes susceptibility or resistance in the NOD mouse (5–6). Among them, the most important region for susceptibility is the diabetes-susceptibility (*Idd*) *Idd-1* locus, corresponding to the unique NOD MHC H-2^{g7} (3). In addition to H-2^{g7}, other non-MHC genes exert diabetogenic influence (7–8).

We have previously reported that backcross of BALB/c mice transgenic for the expression of IL-10 in pancreatic β cells (Ins-IL-10 mice) (9) with NOD mice resulted in (Ins-IL-10 \times NOD)F₁

mice that did not develop autoimmune diabetes (10). Further backcross of F₁ mice to NOD mice resulted in progenies of NOD. Ins-IL-10 mice that developed accelerated diabetes in an MHC-dependent manner (10). The underlying mechanism(s) responsible for this outcome were not clear (11–12). However, some NOD. Ins-IL-10 mice did not develop diabetes (10, 13), suggesting that segregation of some BALB/c genes could protect from IL-10-mediated acceleration of IDDM (14).

In a previous analysis of MHC congenic stocks, we found that pancreatic IL-10 in NOD.B6PL-Thy1a-*Idd3-Idd10* congenic mice could specifically overcome the absence of susceptibility alleles at *Idd3* and *Idd10* loci (15). Furthermore, we showed that pancreatic IL-10 could overcome the absence of NOD homozygosity of all non-MHC *Idd* alleles (10, 15).

Here we performed genetic segregation analysis on high backcross generations of NOD. Ins-IL-10 mice to identify BALB/c-derived protective genetic region(s). We report that protection from IL-10-mediated acceleration of diabetes in the NOD mouse is influenced by H-2D-linked loci and that a single dose of D^d can protect from disease.

Materials and Methods

Mice

BALB/c, NOD/Shi, and NOD.SCID mice were purchased from The Scripps Research Institute's rodent breeding colony (La Jolla, CA). Transgenic Ins-IL-10 mice (9) (expressing IL-10 in the pancreatic islets under the control of the human insulin promoter) were bred with NOD mice to generate (Ins-IL-10 \times NOD)F₁ progeny, which was backcrossed to NOD mice (NOD. Ins-IL-10) up to the N11 generation. NOD. Ins-IL-10 mice were also crossed to NOD.SCID mice to obtain NOD.SCID. Ins-IL-10 mice. The SCID mutation was verified by flow cytometry. All mice were housed under specific pathogen-free conditions at The Scripps Research Institute's rodent breeding colony.

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⁵ Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; Ins-IL-10 mice, transgenic BALB/c mice expressing IL-10 in pancreatic β cells; NOD. Ins-IL-10, progeny from backcross of (Ins-IL-10 \times NOD)F₁ mice to NOD mice; H&E, hematoxylin and eosin.

Table I. Strain, transgene, MHC haplotype, and genetic background of the mice used in this study

Mice	Transgene	MHC Haplotype	Background (approximate %)	
			NOD	BALB/c
BALB/c	none	H-2 ^d	0	100
NOD	none	H-2 ^{g7}	100	0
NOD.scid	none	H-2 ^{g7}	100	0
NOD-D ^d	D ^d	H-2 ^{g7}	100	0
NOD.scid.D ^d	D ^d	H-2 ^{g7}	100	0
Ins.IL-10	IL-10	H-2 ^d	0	100
(Ins.IL-10 × NOD)F1	IL-10	H-2 ^{g7} , H-2 ^d	50	50
NOD.IL-10 N2 backcross	IL-10	H-2 ^{g7} , H-2 ^d	75	25
NOD.IL-10 N3 backcross	IL-10	H-2 ^{g7} , H-2 ^d	87.5	12.5
NOD.IL-10 N4 backcross	IL-10	H-2 ^{g7} , H-2 ^d	93.75	6.25
NOD.IL-10 N5 backcross	IL-10	H-2 ^{g7} , H-2 ^d	96.88	3.12
NOD.IL-10 N6 backcross	IL-10	H-2 ^{g7} , H-2 ^d	98.44	1.56
NOD.IL-10 N7 backcross	IL-10	H-2 ^{g7} , H-2 ^d	99.22	0.78
NOD.IL-10 N8 backcross	IL-10	H-2 ^{g7} , H-2 ^d	99.61	0.39
NOD.IL-10 N9 backcross	IL-10	H-2 ^{g7} , H-2 ^d	99.81	0.19
NOD.IL-10 N10 backcross	IL-10	H-2 ^{g7} , H-2 ^d	99.91	0.09
NOD.IL-10 N11 backcross	IL-10	H-2 ^{g7} , H-2 ^d	99.96	0.04

Production of D^d-transgenic mice

Transgenic NOD mice expressing the D^d MHC class I molecule under its own natural promoter were produced at the Transgenic and Embryonic Stem Cell Core Facility of The Scripps Research Institute. Genomic D^d DNA (Medline access no. 85140250; Ref. 16) in pSV2-Neo was a gift of D.B. Williams (University of Toronto, Toronto, Canada). After digestion with *EcoRI* from plasmid, purified D^d fragment was injected into NOD mouse zygotes that were implanted into pseudopregnant females. Three mice proved to be transgenic by Southern hybridization. Founder mice were crossed with NOD/Shi mice, NOD.IL-10, and NOD.SCID.IL-10 mice.

Genotyping analysis

The MHC haplotype and transgene expression of the mice used in this study are shown in Table I. DNA was extracted from tails according to standard protocols using proteinase K and phenol/chloroform. DNA samples were subject to genotyping for the IL-10 transgene and the H-2 haplotypes as previously described (10) or analyzed for polymorphism of microsatellite markers *D17Nds3*, *TNF*, and *D17 Mit13* (Whitehead Institute for Biomedical Research/Massachusetts Institute of Technology Center for Genome Research, Cambridge, MA). PCR conditions, primers, and polymorphism for microsatellite analysis were derived from The Jackson Laboratory (Bar Harbor, ME) database (www.informatics.jax.org).

Histology

Pancreatic tissue was fixed in 10% neutral buffered formalin for 24 h, dehydrated, cleared in toluene, and infiltrated with paraffin. Six-micrometer sections were cut at several levels throughout the organ and stained with either hematoxylin and eosin (H&E) or with an immunoperoxidase method (Vector Laboratories, Burlingame, CA) for the detection of insulin with polyclonal Abs to porcine insulin (Dako, Carpinteria, CA).

Flow cytometry

PBL of transgenic NOD-D^d mice were analyzed for the expression of MHC class I D^d molecules by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ) with specific FITC-conjugated mAb (clone 34-2-12; BD PharMingen, San Diego, CA). An isotype- and fluorochrome-matched control (BD PharMingen) was used to set background fluorescence.

Blood glucose measurement

Mice were tested for diabetes once a week by measuring blood glucose levels with a one-step Bayer Glucometer Elite (Bayer, Elkhart, IN). Animals were considered diabetic when blood glucose levels were ≥ 300 mg/dl in two consecutive measurements.

Statistical analysis

Significance of the data ($p < 0.05$) was evaluated by the χ^2 test using Statview software (Abacus Concepts, Berkeley, CA).

Results

NOD.IL-10(H-2^{g7/d}) but not NOD.IL-10(H-2^{g7/g7}) mice are protected from accelerated diabetes

Pancreatic expression of IL-10 in BALB/c mice (Ins-IL-10) did not cause diabetes (9), but most of the progeny from their backcrosses to NOD mice (NOD.IL-10) developed accelerated diabetes beginning at 4 wk of age (11). Nonetheless, some NOD.IL-10 mice were protected from diabetes. To study this protection, we typed mice for the expression of BALB/c-derived H-2 molecules and monitored the progeny for diabetes.

Table II and Fig. 1 summarize the incidence of diabetes in NOD.IL-10 mice of N6 to N11 backcross generations. Complete protection was observed at N6 and maintained thereafter in mice expressing BALB/c-derived H-2^d molecules (NOD.IL-10(H-2^{g7/d}) mice). On the contrary, NOD.IL-10(H-2^{g7/g7}) mice developed diabetes and rapidly succumbed to wasting disease. These data indicated that absence of diabetes in NOD.IL-10(H-2^{g7/d}) mice associated with inheritance of BALB/c-derived MHC molecules.

BALB/c-derived H-2 class II molecules do not protect NOD.IL-10 mice from IDDM

The H-2^{g7} haplotype of the NOD mouse is characterized by lack of expression of class II I-E molecules and by expression of the I-A^{g7} locus (17). The unique MHC class II of the NOD mouse is important for development of autoimmunity (18), because expression of I-A^d or I-E protects NOD mice from development of IDDM (19–20).

Table II. Diabetes incidence in Ins-IL-10 mice backcrossed to NOD mice

Backcross Generation	NOD.IL-10 Mice	
	NOD.IL-10 (H-2 ^{g7/g7})	NOD.IL-10 (H-2 ^{g7/d})
	No. diabetic/total (%)	
Fifth (N6)	11/12 (91)	0/10 (0)
Sixth (N7)	5/6 (83)	0/10 (0)
Seventh (N8)	6/6 (100)	0/6 (0)
Eighth (N9)	13/13 (100)	0/7 (0)
Ninth (N10)	6/6 (100)	0/9 (0)
Tenth (N11)	11/11 (100)	0/11 (0)
Total	52/54 (96)	0/53 (0)

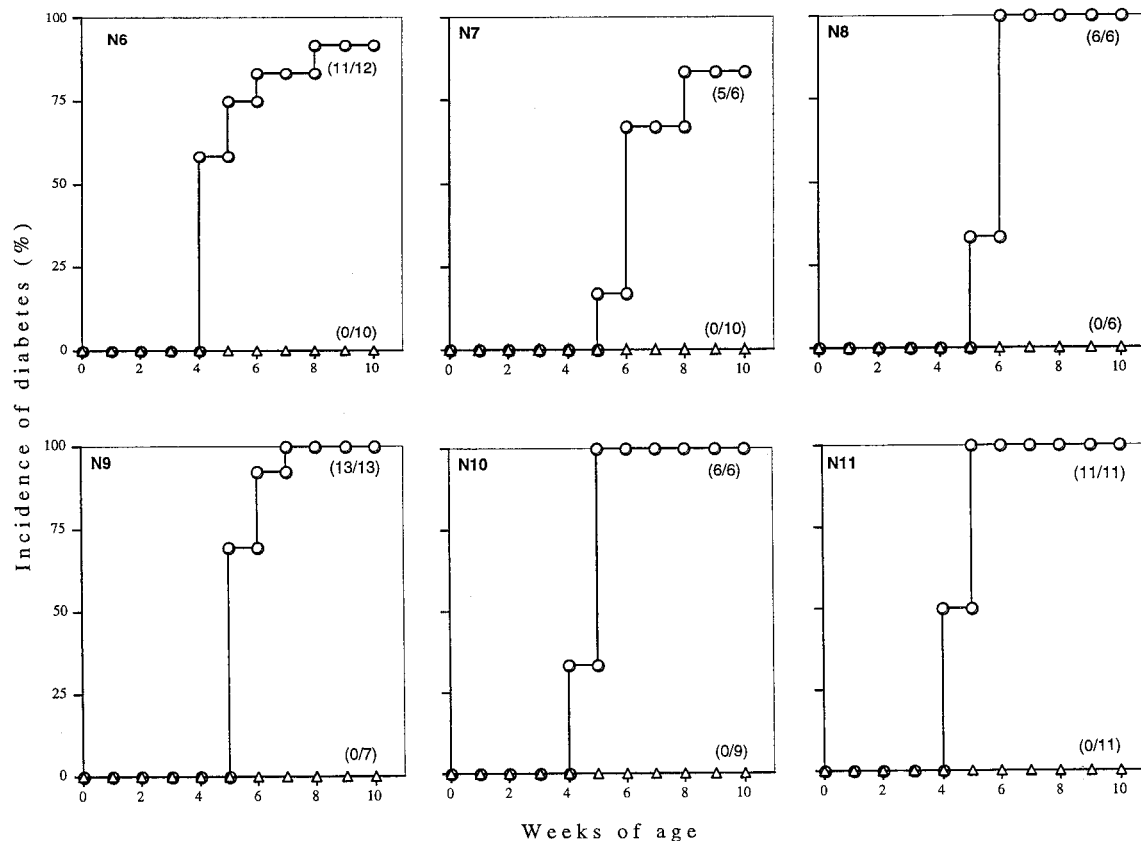


FIGURE 1. Incidence of IDDM in N6 to N11 backcrosses of NOD.Ins-IL-10 mice that were either H-2^{g7/g7} (Δ) homozygous or H-2^{g7/d} (○) heterozygous.

Therefore, we analyzed the progeny of high backcross generations for the segregation of BALB/c (H-2^d) MHC class II molecules. The results are shown in Table III. It was found that NOD.Ins-IL-10 mice expressing either I-A^d ($n = 3$) or I-E^d ($n = 3$) (BALB/c-derived) class II molecules readily developed diabetes. A NOD.Ins-IL-10 mouse expressing both I-A^d and I-E^d molecules also developed diabetes (the limited number of NOD.Ins-IL-10 mice expressing I-A^d or I-E^d class II molecules was dictated by the tightly linked cosegregation of MHC class II genes). These findings indicated that expression of BALB/c-derived class II MHC molecules did not influence development of IDDM in NOD.Ins-IL-10 mice.

Association of H-2D^d with protection of NOD.Ins-IL-10 mice from IDDM

Although expression of BALB/c-derived I-A^d and I-E^d MHC class II molecules did not protect NOD.Ins-IL-10 mice from accelerated

Table III. The incidence of diabetes in NOD.Ins-IL-10 mice is not regulated by BALB/c-derived MHC class II molecules

No. of Mice	H-2 Haplotype			Incidence of IDDM at 8 wk of Age ^a	% Protection
	I-A ^d	I-E ^d	D ^d		
12	-	-	-	12/12	0
3	+	-	-	3/3	0
4	+	-	+	0/4	100
3	-	+	-	3/3	0
5	-	+	+	0/5	100
1	+	+	-	1/1	0
9	+	+	+	0/9	100

^a Blood glucose level was measured at weekly intervals starting at 4 wk of age from N7 to N11 backcross generations of NOD.Ins-IL-10 mice. Mice with a blood glucose level ≥ 300 mg/dl were considered diabetic.

IDDM, protection occurred when D^d molecules were coexpressed (Table III). Therefore, we focused our attention on the possibility that BALB/c-derived H-2D^d class I molecules could confer protection from IDDM. As shown in Table IV, expression of BALB/c MHC class I D^d molecules associated with complete protection from IL-10-mediated acceleration of IDDM. One dose of D^d exerted protective effects on the development of diabetes, with phenotypic dominance when coexpressed with the NOD-derived D^b.

Pancreata from protected NOD.Ins-IL-10(H-2^{g7/d}) mice exhibit periinsulinitis and perivascular infiltrates

Insulinitis precedes the development of IDDM in NOD.Ins-IL-10 mice (11). Therefore, we asked whether protected NOD.Ins-IL-10(H-2^{g7/d}) mice were free from insulinitis. Paraffin-embedded sections from protected mice or from diabetes-prone NOD.Ins-IL-10(H-2^{g7/g7}) mice were compared for mononuclear infiltration by H&E staining at 8 wk of age. Pancreata of NOD.Ins-IL-10(H-2^{g7/d}) mice had periinsulinitis and/or perivascular infiltrate, but they

Table IV. Protection from diabetes in NOD.Ins-IL-10 mice correlates with the expression of BALB/c-derived MHC I D^d

No. of Mice	H-2D Haplotype		Incidence of IDDM at 8 wk of Age ^a	% Protection
	D ^b	D ^d		
30	+	-	28/30	6.7
14	-	+	0/14	100
10	+	+	0/10	100

^a Blood glucose level was measured at weekly intervals starting at 4 wk of age from N7 to N11 backcross generations of NOD.Ins-IL-10 mice. Mice with a blood glucose level ≥ 300 mg/dl were considered diabetic.

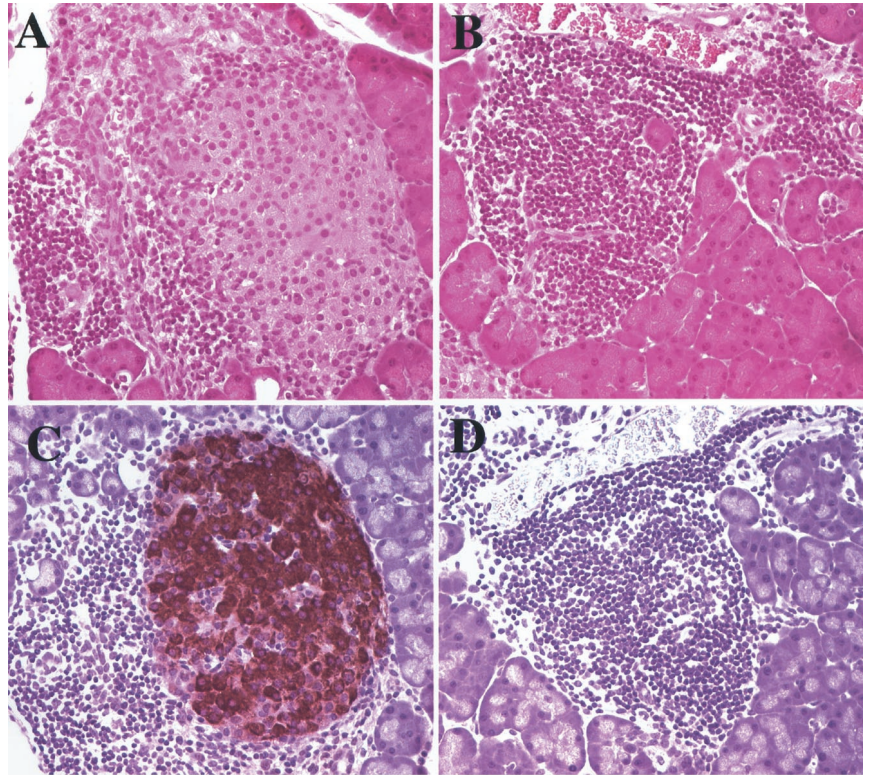


FIGURE 2. Paraffin-embedded pancreatic sections of NOD.Ins-IL-10(H-2^{g7/d}) mice protected from diabetes (A and C) and NOD.Ins-IL-10(H-2^{g7/g7}) mice that developed diabetes (B and D). H&E (A and B) and insulin (C and D) staining. Original magnification: $\times 250$. E, Insulinitis score on ~ 20 – 30 islets for each pancreas from NOD.Ins-IL-10(H-2^{g7/d}) ($n = 6$) or NOD.Ins-IL-10(H-2^{g7/g7}) ($n = 5$) mice indicates severe islet infiltration in diabetes-prone mice.

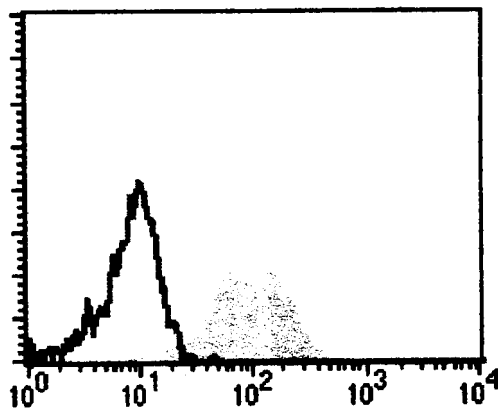
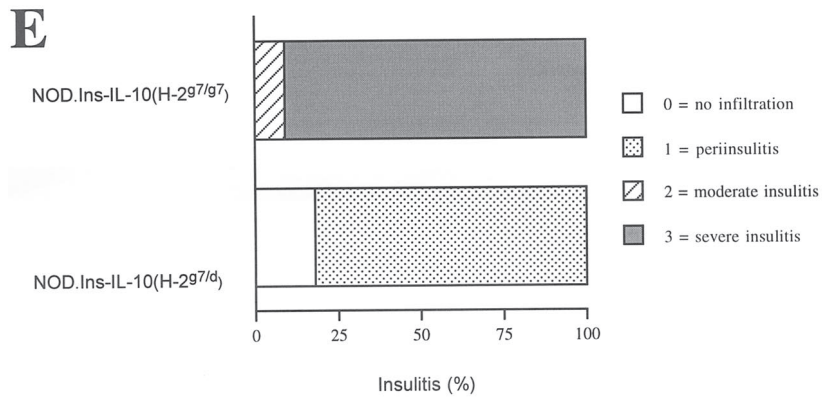


FIGURE 3. Flow cytometry surface expression of H-2D^d molecules on splenocytes from transgenic NOD-D^d mice (filled histogram). The negative control (open histogram) is set with an isotype-matched control.

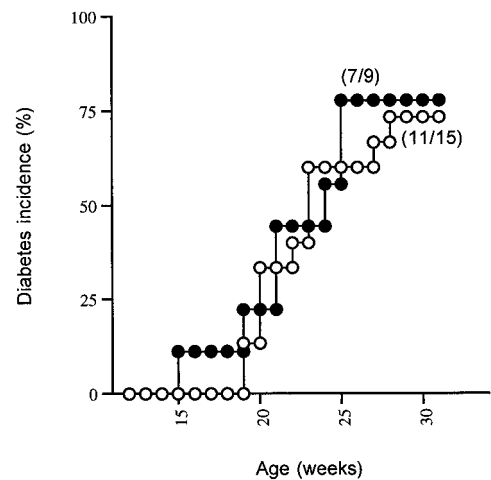


FIGURE 4. Incidence of diabetes in wild-type NOD (○) and transgenic NOD-D^d mice (●) indicates that transgenic expression of H-2D^d molecules does not protect from autoimmune diabetes.

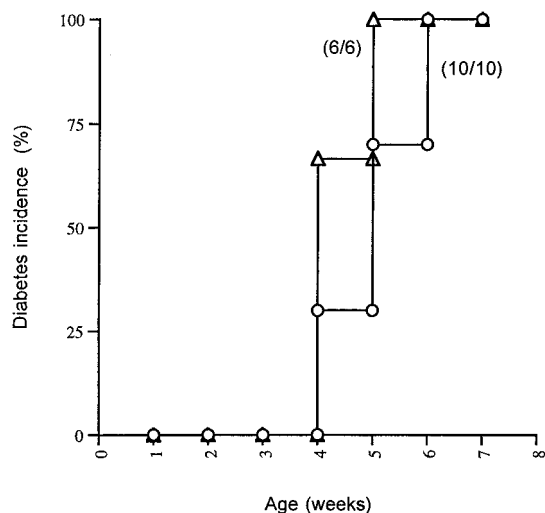


FIGURE 5. Heterologous expression of H-2D^d molecules does not prevent accelerated IDDM in NOD.SCID/wild-type.Ins-IL-10-D^d mice (Δ), as compared with NOD.Ins-IL-10 mice (\circ).

were free from insulinitis and β cells were intact, as indicated by insulin staining (Fig. 2, A and B). On the contrary, sections from age-matched NOD.Ins-IL-10(H-2^{g7/g7}) mice revealed severe insulinitis and β cell loss (Fig. 2, C and D). Furthermore, the incidence and severity of insulinitis were greater in NOD.Ins-IL-10(H-2^{g7/g7}) mice as compared with NOD.Ins-IL-10(H-2^{g7/d}) mice ($p < 0.001$), because $\sim 91\%$ of pancreatic islets from H-2^{g7/g7} homozygous mice were heavily infiltrated by lymphocytes, but none of the islets of heterozygous H-2^{g7/d} mice showed full-blown insulinitis (Fig. 2E).

Expression of MHC class I D^d molecules does not confer protection from IL-10-accelerated IDDM

The above data suggested that expression of H-2D^d molecules in NOD.Ins-IL-10 mice could favor protection from IDDM. Therefore, we decided to directly test the possibility that D^d molecules were responsible for protection. We generated transgenic NOD mice expressing D^d under the control of the MHC class I promoter. D^d transcription was properly regulated and found up-regulated on β cells in islets with periinsulinitis in NOD-D^d mice (data not shown). Fig. 3 shows the flow cytometry profile of the constitutive transgene expression in NOD-D^d mice.

We found that transgenic NOD-D^d mice developed IDDM with an incidence similar to nontransgenic NOD mice (Fig. 4). Next, to directly analyze the influence of H-2D^d on the incidence of diabetes, we bred transgenic NOD-D^d mice with immunodeficient NOD.scid/scid.IL-10 mice (which do not develop diabetes) to generate double transgenic NOD.SCID/wild-type.Ins-IL-10-D^d mice. Fig. 5 shows that expression of H-2D^d in NOD.SCID/wild-type.Ins-IL-10-D^d mice did not protect from accelerated IDDM.

Taken together, these findings indicated that protection from IDDM was not associated with H-2D^d genes, but rather due to genes in linkage with H-2D^d.

Microsatellite analysis of H-2D^d-linked polymorphism

Next, we asked whether allelic variation of genetic markers linked to H-2D^d (21–22) was associated with protection of heterozygous NOD.Ins-IL-10(H-2^{g7/d}) mice from insulinitis and diabetes. We compared *D17Nds3*, *TNF*, and *D17 Mit13* microsatellite marker polymorphism of N5 to N7 backcross generations of D^d-positive NOD.Ins-IL-10 mice protected from diabetes with backcross-matched D^d-negative NOD.Ins-IL-10 diabetic mice. We found that segregation of *D17Nds3*, *TNF*, or *D17 Mit13* alleles of the BALB/c background into NOD.Ins-IL-10 mice correlated with protection from diabetes (Table V). In fact, diabetic NOD.Ins-IL-10 mice only expressed NOD-derived alleles, whereas protected mice also expressed BALB/c-derived alleles. Thus, genetic polymorphism at the *D17Nds3*, *TNF*, and *D17 Mit13* loci confirmed association with the observed dominant protection.

Discussion

Transgenic NOD.Ins-IL-10 mice develop accelerated autoimmune diabetes and rapidly succumb to wasting disease. Here we show that H-2^{g7/d} heterozygosity completely protects NOD.Ins-IL-10 mice because of the presence of dominant H-2D-linked gene(s) but not because of the expression of MHC class II molecules. These data support the concept that, while the MHC clearly is important to IDDM development, other MHC-linked loci may also play a major contribution to the process(es) leading to accelerated onset and progression of the disease. Furthermore, we also show in genetic backcrosses that heterozygous expression of the diabetogenic H-2^{g7} haplotype is not sufficient per se for development of insulinitis when coexpressed with BALB/c-derived MHC class I D^d genes, thus indicating that BALB/c gene(s) can exert local protection in the pancreas. How the H-2D-linked gene(s) can favor protection from accelerated diabetes remains elusive. Until now, consideration of potential candidate genes contributing to differential IDDM susceptibility has primarily focused on loci controlling Ag presentation or immune responses. According to this view, the gene(s) involved in the protection of heterozygous segregants (H-2^{g7/d}) might control the immune homeostasis at least on two different levels. First, by influencing (auto)Ag presentation, because protected NOD.Ins-IL-10 mice had preserved β cells. Although protected NOD.Ins-IL-10(H-2^{g7/d}) mice had periinsulinitis, islet damage was limited and islet infiltration possibly related to ICAM-1 hyperexpression on the pancreatic vascular endothelium (9, 23). Second, the islet lymphomononuclear infiltrates did not progress to massive infiltration probably because of local immune regulation. In fact, T cell responses to glutamic acid decarboxylase were reduced in NOD.Ins-IL-10(H-2^{g7/d}) mice as compared with NOD.Ins-IL-10(H-2^{g7/g7}) mice (data not shown). Regulatory

Table V. Diabetes protection is associated with BALB/c alleles at the H-2D end region

NOD.Ins-IL-10 mouse ^a		1	2	3	4	5	6	7	8	9	10	11	12
IDDM		–	–	–	–	–	+	–	–	+	–	–	–
Marker	cM ^b												
<i>D17Mit13</i>	18.95	F	F	F	F	F	N	F	F	N	F	F	F
<i>D17Nds3</i>	19.06	F	F	F	F	F	N	F	F	N	F	F	F
<i>TNF</i>	19.06	F	F	F	F	F	N	F	F	N	F	F	F
H-2D ^d	19.09	+	+	+	+	+	–	+	+	–	+	+	+

^a Progeny of N5 to N7 backcrosses of NOD.Ins-IL-10 mice tested.

^b Position as recombinant distance from centromere markers (www.informatics.jax.org). N, Homozygous for the NOD marker allele; F, heterozygous NOD/BALB/c.

mechanisms should be particularly effective, because NOD.Ins-IL-10(H-2^{g7/d}) mice were also completely resistant to cyclophosphamide-induced diabetes (A.L.C., B.B., and N.S., unpublished observations).

Although the tight linkage association of the H-2D end with the protective phenotype of heterozygous H-2D^{g7/d} mice still requires the identification of the gene(s) involved, our finding of an H-2D^d-linked resistance to IDDM is of interest also in consideration of the recent observations by Hattori et al. of undefined MHC-linked gene(s) controlling diabetogenesis in the NOD mouse (24). These authors have suggested that this gene(s) might influence not only diabetes but other diseases as well. How this putative gene(s) could counterregulate pathogenetic autoimmunity needs to be elucidated (24). It would be interesting to know whether the region indicated by Hattori et al. is homologous to the human region encoding the MHC class I chain-related gene A (MICA) gene (and its homologue MICB). Indeed, polymorphism of stress-inducible MICA (25) has been shown to associate with incidence of IDDM (26–27), suggesting a pathogenetic role of this MHC-linked region. Unfortunately, whether a mouse homologue of MICA may exist is still not known.

Additional evidence for the influence of the H-2D end on susceptibility to IDDM also comes from the studies by Mathews et al. (28). The MHC of the NOD-related CTS/Shi mouse (H2^{ct}) shares MHC class II region identity with the H2^{g7} haplotype of the NOD mouse but differs at the H2-D end of the MHC complex. Mathews et al. found that congenic transfer of the MHC haplotype of the CTS/Shi strain (H2^{ct}) onto the NOD background resulted in significant reduction of diabetes frequency in congenic females associated with a protective MHC-linked gene at the H2-D end (28).

Our finding of an H-2D-linked resistance to accelerated diabetes in NOD.Ins-IL-10 mice and its inheritance as a dominant trait suggest that an understanding of this gene(s) could have direct applications. In fact, expression of such gene(s) (i.e., by transduction) or its regulated induction during severe IDDM could possibly have an influence on the clinical course of disease.

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