Resistance Alleles at Two Non-Major Histocompatibility Complex–linked Insulin-dependent Diabetes Loci on Chromosome 3, *Idd3* and *Idd10*, Protect Nonobese Diabetic Mice from Diabetes

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Summary

Development of diabetes in NOD mice is polygenic and dependent on both major histocompatibility complex (MHC)–linked and non-MHC–linked insulin-dependent diabetes (*Idd*) genes. In (F_1 × NOD) backcross analyses using the B10.*H-2*^{g7} or B6.PL-*Thy1*^{a} strains as the outcross partner, we previously identified several non-MHC *Idd* loci, including two located on chromosome 3 (*Idd3* and *Idd10*). In the current study, we report that protection from diabetes is observed in NOD congenic strains having B6.PL-*Thy1*^{a}- or B10-derived alleles at *Idd3* or *Idd10*. It is important to note that only partial protection is provided by two doses of the resistance allele at either *Idd3* or *Idd10*. However, nearly complete protection from diabetes is achieved when resistance alleles are expressed at both loci. Development of these congenic strains has allowed *Idd3* to be localized between *Glut2* and *D3Mit6*, close to the *Il2* locus.

The nonobese diabetic (NOD)[1] mouse develops autoimmune diabetes and is thought to be a relevant model of human insulin–dependent diabetes mellitus (IDDM). Many features of diabetes are shared when comparing human IDDM with the disease developed by NOD mice (1, 2). In both species, autoantibodies and T cells specific for the insulin-producing β cells in the pancreas are detected (3–9). It has been demonstrated that both CD4$^+$ and CD8$^+$ T cells are required for the destruction of β cells in the NOD mouse (10–12). In humans, T cell infiltrates are found within the islets of Langerhans and are thought to mediate the autoimmune destruction (13–15).

Disease development in the NOD mouse is complex and polygenic. A major genetic component of the disease is a gene (or genes) linked to the MHC, termed *Idd1* in the mouse (16–18). The primary candidate genes for the MHC-linked diabetogenic loci are those which encode the α and β chains of I-A^{g7} in NOD mice (19). Evidence for the role of class II molecules comes from the observation that the transgenic introduction of an I-E α gene into NOD mice, which results in the expression of I-E, can completely protect from diabetes as well as insulitis (20, 21). Similar protective effects have been observed with other I-A and I-E molecules introduced by transgenic technology and conventional breeding strategies (21–25). One mechanism for disease protection by non-NOD class II molecules may be that presentation of autoantigens by I-A^{g7} is less efficient or nonexistent (26), or alternatively, a non-I-A^{g7} cell–specific nonpathogenic immune response may actively suppress the I-A^{g7}–restricted response.

Although diabetes is controlled by a number of genes in the NOD mouse, the individual contribution of each *Idd* locus can be ascertained using a series of congenic NOD mouse strains, each possessing a resistance allele at a single *Idd* locus. Such *Idd* congenic mice are produced by introgressing *Idd*-containing chromosomal regions from normal nondiabetic mice into the genome of the NOD strain by repetitive backcrossing. We (24, 25, 27) and others (28, 29), developed MHC (*Idd1*) congenic NOD mice, such as NOD.*H-2*^{b}, NOD.*H-2*^{nb1}, and NOD.*H-2*^{i5}, and found that these mice are completely protected from diabetes and insulitis. By studying the F_1 progeny of the *Idd1* congenic strain and NOD, the inheritance of protection by one dose of an *Idd* allele can be established. In the case of (NOD.*H-2*^{b} × NOD)F_1 mice, insulitis is observed in ~50% of female mice, whereas diabetes develops in <5% of female mice (24, 27).

Our long-term goal is to define the inheritance and biologic activity of other *Idd* loci using the same congenic mouse

[1] *Abbreviations used in this paper: Idd,* a gene controlling insulin-dependent diabetes; NOD, nonobese diabetic.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/11/1705/09 $2.00
Volume 180 November 1994 1705–1713

strategy that proved to be informative in the case of *Idd1*. The chromosomal locations of some of the non-MHC–linked loci influencing the development of diabetes have been detected in humans and in the NOD mouse. In humans, one locus outside of the MHC has been shown to contribute to disease susceptibility, the insulin gene region on chromosome 11p15 (30, 31). In a backcross analysis of (NON × NOD)F_1 mice to the NOD parent, *Idd2* on chromosome 9 was the first non-MHC locus identified that influenced the incidence of diabetes (17). Additional diabetogenic loci, *Idd3, 4,5,6,7,8,9,* and *10*, have been localized to chromosomes 3 (centromeric), 11, 1, 6, 7, 14, 4, and 3 (distal), respectively, from a (B10.*H-2*^{g7} × NOD)F_1 × NOD backcross analysis (32–36). It is interesting to note that NOD homozygosity is not always associated with increase risk of diabetes for all of the *Idd* loci. In the case of *Idd7* and *Idd8*, the B10 alleles are more diabetogenic and homozygosity for the NOD allele at these loci is protective (33, 35).

To characterize further the non-MHC–linked *Idd* loci, we describe several new NOD congenic strains which express the B10- or B6.PL-*Thy1*^{a}-derived allele at *Idd3* and/or *Idd10*. Results from these strains demonstrate that the non-NOD alleles at *Idd3* and *Idd10* individually contribute to disease protection, although protection is only partial. The combination of resistance alleles at both *Idd3* and *Idd10* on the NOD background results in profound disease protection. This study demonstrates the usefulness of NOD congenic strains of mice in the dissection of autoimmune diabetes.

Materials and Methods

Animals. NOD/MrkTacfBR (NOD) mice were purchased from Taconic Farms Inc. (Germantown, NY) and C57BL/10SnJ (B10) and B6.PL-*Thy1*^{a}/Cy (B6.PL-*Thy1*^{a}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The B6.PL-*Thy1*^{a} congenic strain was derived at The Jackson Laboratory by backcrossing (B6 × PL)F_1 mice to the C57BL/6 (B6) strain with selection for the PL *Thy1* allele on chromosome 9. All mice were housed under sterile, specific pathogen-free conditions. NOD mice (K^d, I-A^{g7}, D^b, and no expression of I-E) were outcrossed to B10 or B6.PL-*Thy1*^{a} mice (both strains express K^b, I-A^b, and D^b, and lack I-E expression) and the resulting F_1 mice backcrossed to the NOD parental strain as reported earlier. First backcross mice were selected for homozygosity at the NOD MHC by testing for the expression of I-A^b and I-A^{g7} on the surface of PBMC as previously described (24). Further selection criteria based on the development of diabetes and insulitis are described in Results.

At the fifth or sixth backcross generation, DNA was isolated from peripheral blood leukocytes of mice from pedigrees relatively resistant to diabetes and tested for the presence of genetic markers on chromosome 3. Mice expressing B10 or B6.PL-*Thy1*^{a} alleles at various loci on chromosome 3 were selectively bred and used to develop the congenic lines described in this study. All microsatellite markers, with the exception of *D3Nds7* and *D3Nds28*, have been described previously (35–38). The oligonucleotide primer set for *D3Nds7* is 5′-TGCTCCTCACCGTCA-TGC-3′ and 5′-TAGTGT-TTGCCATGGCTCTC-3′ and for *D3Nds28* is 5′-GGTAGGAT-T-TGATGGAGGC-3′ and 5′-TTCTGCAGCAGATGGGAAC-3′ (Denny, P., and E. Hopes, personal communication).

Assessment of Diabetes and Insulitis. Mice were monitored for the development of diabetes by testing for elevated levels of urinary glucose with Tes-Tape (Eli Lilly, Indianapolis, IN). Animals were classified as diabetic after producing Tes-Tape values of 3+ or higher. We previously demonstrated that elevated urinary glucose measurements with Tes-Tape ≥1+ corresponded with blood glucose levels >320 mg/dl (39). Diabetic mice also displayed polydipsia, polyuria, and weight loss. The presence of insulitis was assessed after fixation of pancreata in buffered 10% formalin and paraffin sectioning. Tissue sections (5 μm) were stained with hematoxylin and eosin and examined for the presence of mononuclear cell infiltration. Two noncontiguous sections of each tissue were examined. Classification of each animal was made using the most severe inflammatory lesion observed.

Cyclophosphamide Treatment. Lyophilized cytoxan (cyclophosphamide for injection, Mead Johnson Oncology Products, Evansville, IN) was prepared immediately before injection by adding sterile distilled water for a final concentration of 20 mg/ml. Mice received 200 mg/kg by i.p. injection on days 0 and 14. All mice were nondiabetic before treatment and diabetes was monitored on a weekly basis up to day 29 after the first injection.

Results

Development of NOD Congenic Strains. The B10 and B6 strains of mice have previously been shown to possess four to five diabetes-resistant loci (18, 29). To begin defining these loci, we developed a series of NOD-related strains bred for diabetes-resistance after outcrosses to both the B10 and B6.PL-*Thy1*^{a} strains. We used the B6.PL-*Thy1*^{a} strain rather than B6 in order to follow segregation of *Idd2*, which is linked to the *Thy1* locus (17). At the first backcross generation, we selected nondiabetic mice that were homozygous for the NOD MHC, these mice were then continually backcrossed to the NOD strain with selection for diabetes resistance. Diabetes resistance was defined as a lack of spontaneous or cyclophosphamide-induced diabetes and little or no insulitis. Female mice at each backcross were assessed for resistance to cyclophosphamide-induced diabetes and insulitis after the production of at least two litters. Subsequently, in a backcross analysis using the NOD and B10.*H-2*^{nod} strains, we discovered that a region on chromosome 3 was a major determinant of diabetes susceptibility in the NOD mouse (33, 35). This linkage to diabetes on chromosome 3 was also observed in a backcross analysis performed with the NOD and B6.PL-*Thy1*^{a} strains (33, 36). Two diabetes-resistant strains were still segregating a large section of chromosome 3 derived from either B10 (at the sixth backcross generation) or B6.PL-*Thy1*^{a} (at the fifth backcross generation). These two congenic strains are described in this report as the NOD.B10$^{Il2\text{-}Tshb}$ and NOD.B6$^{Il2\text{-}Tshb}$ strains, respectively (Table 1). Using a battery of microsatellite markers which are polymorphic between NOD and B10 or B6.PL-*Thy1*^{a} (35), we continued to backcross these two congenic strains and to screen progeny for additional recombination events on chromosome 3. To facilitate the fine-mapping of the diabetes resistance gene on chromosome 3, such recombinant mice were used to initiate new congenic strains in which smaller portions of non-NOD chromosome 3 DNA were introgressed into the NOD genome (Table 1).

Table 1. *Genetic Characterization of Chromosome 3 Congenic Strains*

		Congenic Strain					
θ	Marker locus	NOD.B6$^{Il2\text{-}Tshb}$‡	NOD.B6^{Il2}§	NOD.B6^{Tshb}‖	NOD.B6^{D3Nds1}¶	NOD.B10$^{Il2\text{-}Tshb}$**	NOD.B10^{Tshb}‡‡
	D3Nds28	NOD	NOD	NOD	NOD	NOD	NOD
0.053	*D3Nds12 (Glut-2)*	B6	B6	NOD	NOD	NOD	NOD
0.053	*D3Nds6 (Il2)*	B6	B6	NOD	NOD	B10	NOD
0.046	*D3Mit6*	B6	B6	NOD	NOD	B10	NOD
0.077	*D3Nds1*	B6	B6	NOD	B6	B10	NOD
0.053	*D3Mit22*	B6	B6	NOD	B6	B10	NOD
0.038	*D3Mit51*	B6	B6	B6	B6	B10	NOD
0.063	*D3Mit40*	B6	NOD	B6	NOD	B10	B10
0.008	*D3Nds7 (Cacy)*	B6	NOD	B6	NOD	B10	B10
0.037	*D3Nds11 (Fcgr1)*	B6	NOD	B6	NOD	B10	B10
0.008	*D3Mit10*	B6	NOD	B6	NOD	B10	B10
0.022	*D3Nds8 (Tshb)*	B6	NOD	B6	NOD	B10	B10
0.217	*D3Nds9 (Adh-1)*	NOD	NOD	NOD	NOD	NOD	B10

* The recombinant fraction between loci using data obtained from 60–132 animals.
‡ The NOD.B6.PL-*Thy1^a D3Nds12 D3Nds8* (N6F3-5) strain.
§ The NOD.B6.PL-*Thy1^a D3Nds12 D3Mit51* (N7F2-3) strain.
‖ The NOD.B6.PL-*Thy1^a D3Mit51 D3Nds8* (N7F3-5) strain.
¶ The NOD.B6.PL-*Thy1^a D3Nds1 D3Mit51* (N10F2-4) strain.
** The NOD.B10-*D3Nds6 D3Nds8* (N7F4-5) strain.
‡‡ The NOD.B10-*D3Mit40 D3Nds9* (N7F4-5) strain.

During the selection process, congenic strains were analyzed for the presence of other chromosomal regions known to contribute to diabetes resistance in the NOD mouse, specifically *Idd2, 4, 5, 6, 7, 8,* and *9* (35). In addition, a panel of microsatellite markers sampling the entire genome were used to characterize the congenic strains (35). Regions outside of chromosome 3 that were detected as non-NOD were replaced with the NOD alleles by selective breeding. As detailed later, segregation analysis of the NOD.B6$^{Il2\text{-}Tshb}$ strain has been performed and results are consistent with the hypothesis that only regions on chromosome 3 are contributing to diabetes resistance. It is also important to note that the statistical probability of achieving homozygosity at loci unlinked to the selected locus increases with the number of backcrosses performed. All of the congenic strains in the current report were studied at the N6, N7, or N10 generations (see Table 1) in which there is a 96.9, 98.4, and 99.8% chance of homozygosity for the NOD allele at unlinked loci (40).

The NOD.B6$^{Il2\text{-}Tshb}$ Strain Shows Profound Protection from Diabetes. Development of spontaneous diabetes in male and female NOD.B6$^{Il2\text{-}Tshb}$ mice was monitored for 7 mo (Fig. 1, *A* and *B*, Table 2). Almost complete protection from disease was observed in both female (Fig. 1 *A*) and male (Fig. 1 *B*) NOD.B6$^{Il2\text{-}Tshb}$ mice; only 2 of 159 (1.2%) females and no males (0/145) developed spontaneous diabetes (Table 2). This near lack of diabetes is in marked contrast to the parental frequency of disease observed in our NOD colony during this time: 78% in females and 38% in males (Fig. 1, Table 2).

(NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F$_1$ mice, which possess only one copy of the protective chromosome 3–derived DNA, showed a reduced, but significant, level of protection from diabetes (Fig. 1, *A* and *B*). Whereas the disease incidence was reduced by half in F$_1$ females as compared with NOD females (41 vs. 78% incidence of diabetes at 7 mo; $p < 10^{-4}$), male (NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F$_1$ mice remained nearly disease free at 7 mo with only 4 of 132 male F$_1$ mice diabetic by this time. It is striking that with (NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F$_1$ mice, the usual twofold higher risk of disease development with NOD females compared with NOD males had increased to a 13-fold difference in disease incidence with 41% of females and 3% of males developing diabetes.

The NOD.B6^{Il2} and NOD.B6^{Tshb} Strains Have Higher Frequency of Diabetes than the NOD.B6$^{Il2\text{-}Tshb}$ Strain. Since the NOD.B6^{Il2} and NOD.B6^{Tshb} congenic strains essentially divide in half the region introgressed in the NOD.B6 $^{Il2\text{-}Tshb}$ congenic strain (Table 1), we expected that one or the other of the two strains would resemble the NOD.B6$^{Il2\text{-}Tshb}$ strain with respect to diabetes incidence. To our surprise, neither strain showed the same pattern of protection from diabetes seen in NOD.B6$^{Il2\text{-}Tshb}$ mice. In the case of the NOD.B6^{Il2} congenic strain (Fig. 1, *C* and *D*), male homozygotes were almost completely protected with only 1 of 84 mice developing diabetes. This value was not significantly different than the

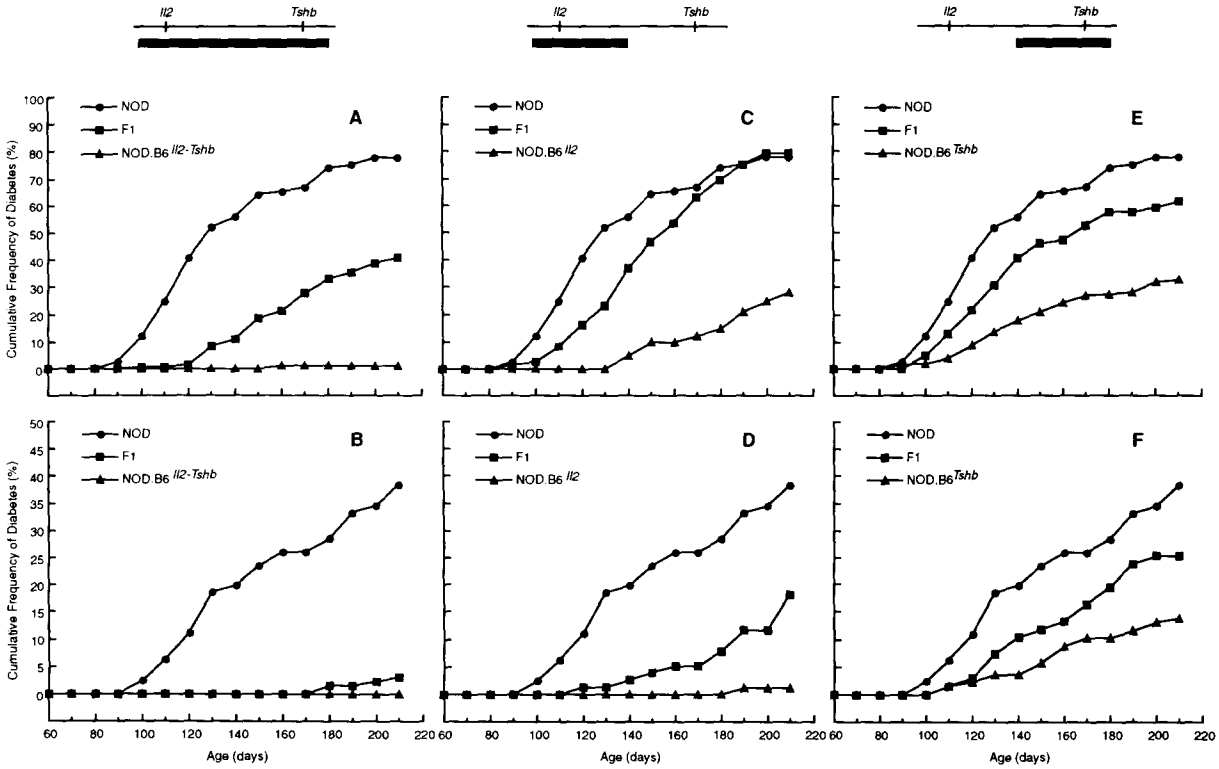

Figure 1. Development of diabetes in NOD congenic mice. (*A*) 159 NOD.B6$^{Il2\text{-}Tshb}$ (▲) and 118 (NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F_1 (■) female mice were observed for the development of spontaneous diabetes. (*B*) 145 NOD.B6$^{Il2\text{-}Tshb}$ (▲) and 132 (NOD.B6$^{Il2\text{-}Tshb}$ × NOD-F_1 (■) male mice were observed for the development of spontaneous diabetes. (*C*) 81 NOD.B6^{Il2} (▲) and 73 (NOD.B6^{Il2} × NOD)F_1 (■) female mice were observed for the development of spontaneous diabetes. (*D*) 84 NOD.B6^{Il2} (▲) and 77 (NOD.B6^{Il2} × NOD)F_1 (■) male mice were observed for the development of spontaneous diabetes. (*E*) 62 NOD.B6^{Tshb} (▲) and 78 (NOD.B6^{Tshb} × NOD)F_1 (■) female mice were observed for the development of spontaneous diabetes. (*F*) 63 NOD.B6^{Tshb} (▲) and 67 (NOD.B6^{Tshb} × NOD)F_1 (■) male mice were observed for the development of spontaneous diabetes. For comparison, the cumulative incidence of diabetes in 50 female (*A*, *C*, and *E*) and 49 male (*B*, *D*, and *F*) NOD mice is shown (●).

frequency observed in NOD.B6$^{Il2\text{-}Tshb}$ males. In contrast, the frequency of diabetes in NOD.B6^{Il2} females (28%) was significantly higher ($p < 10^{-4}$) than that observed in NOD.B6$^{Il2\text{-}Tshb}$ females. The NOD.B6^{Tshb} congenic strain (Fig. 1, *E* and *F*) showed a distinct pattern of protection from diabetes compared with the NOD.B6^{Il2} strain since both female and male NOD.B6^{Tshb} mice had a higher frequency of diabetes, 33 and 14%, respectively, as compared with the NOD.B6$^{Il2\text{-}Tshb}$ congenic strain ($p < 10^{-4}$ for both sexes).

The NOD.B6^{Il2} and NOD.B6^{Tshb} strains also produced different patterns of diabetes frequency when present in (NOD.B6^{Il2} × NOD)F_1 and (NOD.B6^{Tshb} × NOD)F_1

Table 2. *Incidence of Diabetes in NOD Congenic Strains*

Strain	Female incidence of diabetes*	*p* value vs. female NOD‡	Male incidence of diabetes*	*p* value vs. male NOD‡
NOD.B6$^{Il2\text{-}Tshb}$	2/159 (1.2%)	$<10^{-4}$	0/145	$<10^{-4}$
NOD.B6^{Il2}	23/81 (28.4%)	$<10^{-4}$	1/84 (1.2%)	$<10^{-4}$
NOD.B6^{Tshb}	50/152 (32.9%)	$<10^{-4}$	19/137 (13.9%)§	$<10^{-4}$
NOD.B6^{D3Nds1}	50/68 (73.5%)	>0.05	18/60 (30.0%)	>0.05
NOD.B10$^{Il2\text{-}Tshb}$	2/72 (2.8%)	$<10^{-4}$	0/87	$<10^{-4}$
NOD.B10^{Tshb}	23/59 (39.0%)	$<10^{-4}$	4/76 (5.3%)	$<10^{-4}$
NOD	63/81 (77.8%)		31/81 (38.3%)	

* The incidence of diabetes refers to the percentage of mice diabetic by 7 mo of age.

‡ Determined using Fisher's Exact Test.

§ The diabetes incidence for NOD.B6^{Tshb} males was not significantly different than the incidence for NOD.B10^{Tshb} males ($p > 0.05$).

mice, respectively (Fig. 1, *C–F*). Female (NOD.B6^{Il2} × NOD)F_1 mice were not protected from diabetes whereas male (NOD.B6^{Il2} × NOD)F_1 mice were significantly protected against disease at 7 mo of age (14/77 diabetic, p = 0.0078 vs NOD males). In contrast, for the (NOD.B6^{Tshb} × NOD)F_1 strain, female mice were marginally protected from disease (48/78, p = 0.0376 vs. NOD females) but males were not (17/67, p = 0.1136 vs. NOD males).

At Least Two Genes Account for the Resistance to Diabetes in NOD.B6$^{Il2\text{-}Tshb}$ Mice. The patterns of diabetes resistance observed in the NOD.B6$^{Il2\text{-}Tshb}$, NOD.B6^{Il2}, and NOD.B6^{Tshb} strains led us to hypothesize that at least two linked loci on chromosome 3 accounted for the profound diabetes resistance of the NOD.B6$^{Il2\text{-}Tshb}$ congenic strain. Resistance alleles from the B6.PL-*Thy1^a* strain at both loci were required for the near-complete resistance to diabetes. The reduced, but significant resistance to disease observed in the NOD.B6^{Il2} and NOD.B6^{Tshb} strains could then be explained by the fact that each of these strains expresses diabetes resistance via only one of the two loci active in the NOD.B6$^{Il2\text{-}Tshb}$ strain. To address this hypothesis, complementation studies were performed to determine if a combined activity of the putative resistance alleles could be observed.

The frequency of diabetes in (NOD.B6^{Il2} × NOD.B6^{Tshb})F_1 mice was compared with those of (NOD.B6^{Il2} × NOD)F_1, (NOD.B6^{Tshb} × NOD)F_1 and (NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F_1 mice (Fig. 2 *A*). Remarkably, the frequency of diabetes in (NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F_1 was equivalent to the frequency observed in (NOD.B6^{Il2} × NOD.B6^{Tshb})F_1 mice; both of these F_1 strains were protected significantly more than either the (NOD.B6^{Il2} × NOD)F_1 or (NOD.B6^{Tshb} × NOD)F_1 strains (p <0.006 for all comparisons). This observation supports the hypothesis outlined above and suggests that (NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F_1 and (NOD.B6^{Il2} × NOD.B6^{Tshb})F_1 mice both have one dose of the B6.PL-*Thy1^a*–derived protective allele at each of the two loci (*Idd3* for the centromeric locus and *Idd10* for the distal locus). In (NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F_1 mice, the protective alleles are *cis* whereas in the (NOD.B6^{Il2} × NOD.B6^{Tshb})F_1, the protective alleles are *trans*.

Observations made with additional F_1 strains support the two-gene hypothesis for chromosome 3 (Fig. 2 *B*). When two doses of the B6.PL-*Thy1^a* allele at *Idd3* were present, as in the NOD.B6^{Il2} strain, 23/81 females developed diabetes. However, when one dose of the B6.PL-*Thy1^a* allele at *Idd10* was present in addition to the two doses of the B6.PL-*Thy1^a* allele at *Idd3*, as in the (NOD.B6$^{Il2\text{-}Tshb}$ × NOD.B6^{Il2})F_1, fewer females developed disease (6/57, p = 0.0116). Similarly, the NOD.B6^{Tshb} strain, which has two doses of the protective allele at *Idd10*, was less protected from diabetes than (NOD.B6$^{Il2\text{-}Tshb}$ × NOD.B6^{Tshb})F_1 mice, which have a single dose of the protective allele at *Idd3* in addition to two doses of the protective allele at *Idd10* (50/152 vs. 9/61, p = 0.007). It is interesting to note that in female (NOD.B6$^{Il2\text{-}Tshb}$ × NOD.B6^{Tshb})F_1 mice, a single dose of the diabetes-resistance allele at *Idd3* decreased the incidence of diabetes when it was expressed in the context of resistance at *Idd10*. A protective effect from a single dose of this same *Idd3* allele was not observed in the context of the NOD background in females (see [NOD.B6^{Il2} × NOD]F_1 vs. NOD in Fig. 1 *C*).

Localization of Idd3 and Idd10. The lack of complete protection in the presence of one or two doses of the protective B6.PL-*Thy1^a* allele at *Idd3* or *Idd10* makes fine-mapping of either locus by backcross analysis impossible. We therefore selected a strategy in which informative recombination events were fixed on the NOD background and a sufficient number of homozygous mice were examined for their incidence of diabetes. In this way, protection from diabetes by *Idd3* and *Idd10* could be localized to smaller regions of chromosome 3. The NOD.B6^{D3Nds1} strain is an example of such a congenic (Table 1). This mouse was derived from the NOD.B6^{Il2} strain after an outcross to NOD and subsequent backcrossing to the NOD parent. A recombination event occurred between *D3Nds1* and *D3Mit6* during a backcross meiosis. The event was fixed by backcrossing the recombinant mouse to the NOD strain once again in order to produce male and female mice possessing the recombination event. Intercrossing such heterozygous mice produced the homozygous founders of the NOD.B6^{D3Nds1} strain in which B6.PL-*Thy1^a*–derived alleles are present at *D3Nds1*, *D3Mit22*, and *D3Mit51*.

It is interesting to note that the NOD.B6^{D3Nds1} strain was not protected from diabetes (Table 2). This result places *Idd3* centromeric of *D3Nds1* and *Idd10* telomeric of *D3Mit51*. We next took advantage of the results from NOD mice congenic for regions on chromosome 3 derived from the B10 strain to localize further *Idd3* and *Idd10* (Table 1). It is highly likely that the B10 and B6.PL-*Thy1^a* strains have the same alleles at both *Idd3* and *Idd10* since the B6 and B10 strains diverged only within the past 40 yr. In addition, the two strains have been shown to be identical at 101 of the 102 microsatellite sequences tested (32). Moreover, the frequency of diabetes is indistinguishable between the NOD.B10$^{Il2\text{-}Tshb}$ strain and the NOD.B6$^{Il2\text{-}Tshb}$ strain (Table 2). Similarly, the frequency of diabetes in NOD.B10^{Tshb} and NOD.B6^{Tshb} mice is not significantly different (Table 2).

Since the NOD.B10$^{Il2\text{-}Tshb}$ strain has fixed a recombination event between *D3Nds6* and *D3Nds12*(Table 1), but still remains profoundly diabetes resistant, *Idd3* must be telomeric of *D3Nds12*. Similarly, the protection from diabetes observed in NOD.B10^{Tshb} mice places *Idd10* telomeric of *D3Mit51*. Taking advantage of the mapping information from all the strains listed in Table 1, *Idd3* is located in a region of ~10 cM between (but not including) *D3Nds12* and *D3Nds1*, and *Idd10* is located in a region of ~34 cM between (but not including) *D3Mit51* and *D3Nds9*.

Resistance to Diabetes in NOD.B6$^{Il2\text{-}Tshb}$ Mice Segregates with Chromosome 3. To ensure that regions outside of chromosome 3 were not contributing to the results reported, we performed a segregation analysis with the NOD.B6$^{Il2\text{-}Tshb}$ congenic strain. (NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F_1 mice were backcrossed to NOD mice and the resulting progeny were monitored for diabetes. The incidence of disease was 52% for females (72/139) and 22% for males (32/147) at 6 mo of age, which is only slightly less than the 6 mo incidence

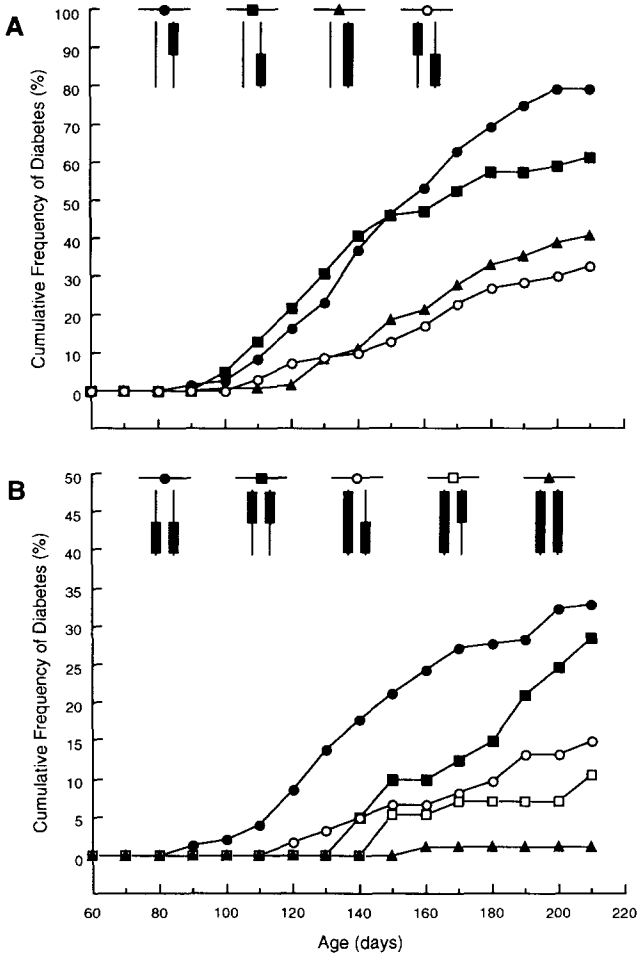

Figure 2. At least two genes on chromosome 3 influence the development of diabetes. (*A*) 73 (NOD.B6^{Il2} × NOD)F_1 (●), 78 (NOD.B6^{Tshb} × NOD)F_1 (■), 118 (NOD.B6$^{Il2\text{-}Tshb}$ × NOD)F_1 (▲), and 70 (NOD.B6^{Il2} × NOD.B6^{Tshb})F_1 (○) female mice were monitored for the development of diabetes. (*B*) 62 NOD.B6^{Tshb} (●), 81 NOD.B6^{Il2} (■), 61 (NOD.B6$^{Il2\text{-}Tshb}$ × NOD.B6^{Tshb})F_1 (○), 57 (NOD.B6$^{Il2\text{-}Tshb}$ × NOD.B6^{Il2})F_1 (□), and 159 NOD.B6$^{Il2\text{-}Tshb}$ (▲) female mice were observed for diabetes. Schematics representing the chromosomal regions contributed by the B6.PL-*Thy1*^{a} strain are shown.

of diabetes in our NOD colony, 74% for females and 28% for males (Fig. 1). Although a control group of 36 backcross mice showed the expected distribution of F_1 and NOD genotypes of *Il2* (17 F_1 and 19 NOD types at *D3Ndss6*) and *Tshb* (15 F_1 and 21 NOD types at *D3Ndss8*), the diabetic mice were enriched for NOD homozygosity at *Il2* and *Tshb*. Only slight enrichment of NOD homozygosity was observed among 49 female diabetics genotyped if *Il2* and *Tshb* loci were considered separately; 30 of the diabetics were NOD at *Tshb* (61%) and 28 were NOD at *Il2* (57%). However, consistent with the presence of two diabetes resistance genes on chromosome 3, only 13 of the 49 female diabetics typed were F_1 at both *Il2* and *Tshb* (27%). Among the 19 male diabetics analyzed, a greater enrichment of NOD homozygosity was observed as compared with the female diabetics; 15 of the diabetics were NOD at *Tshb* (79%) and 16 were NOD at *Il2* (84%). Only 2 of the 19 diabetic backcross males were F_1 at both *Il2* and *Tshb*. The high incidence of diabetes in this backcross generation and the results of the genotypic analysis support the hypothesis that B6.PL-*Thy1*^{a}–derived alleles located on chromosome 3 are the primary (or only) source of diabetes resistance in the NOD.B6$^{Il2\text{-}Tshb}$ congenic strain.

Insulitis and Cyclophosphamide-induced Diabetes in NOD.B6$^{Il2\text{-}Tshb}$, NOD.B6^{Il2}, and NOD.B6^{Tshb} Mice. Pancreata obtained from female and male NOD.B6$^{Il2\text{-}Tshb}$, NOD.B6^{Il2}, *and NOD.B6^{Tshb}* mice were examined at 2 and 5 mo of age for the presence of insulitis (Fig. 3, *A* and *B*). Despite the profound resistance to diabetes, insulitis was observed in the

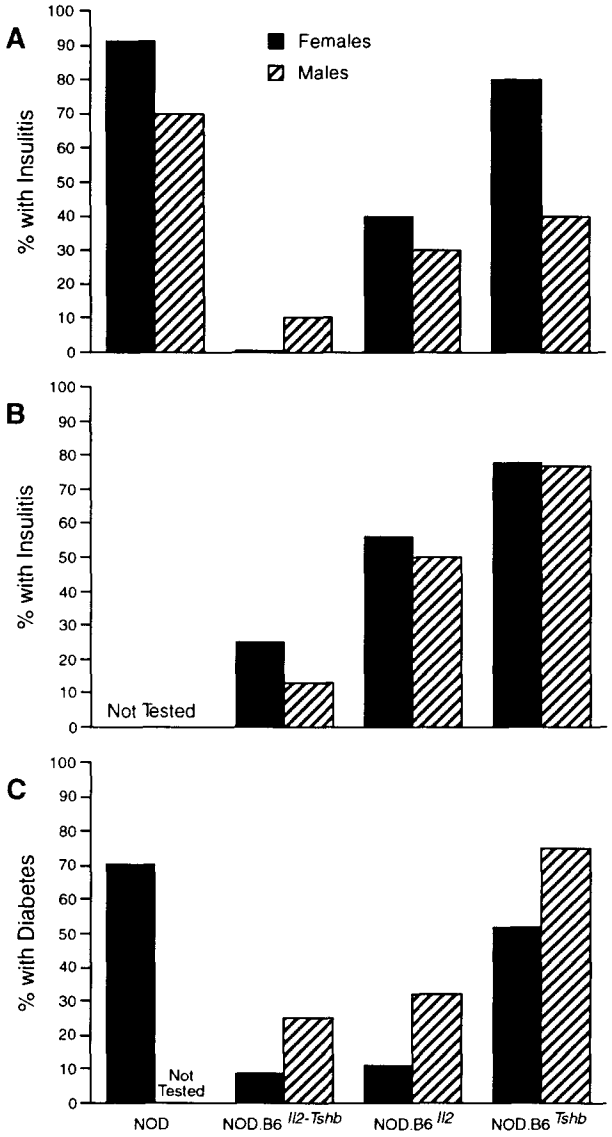

Figure 3. Incidence of insulitis and cyclophosphamide-induced diabetes in chromosome 3 congenic mice. The percentages of females and males (9–16 mice per group) with mild to severe insulitis at 2 and 5 mo of age are shown in *A* and *B*, respectively. In *C*, nondiabetic mice >7 mo of age (17–35 mice per group) were treated with cyclophosphamide as detailed in Materials and Methods and monitored for the development of cyclophosphamide-induced diabetes.

NOD.B6$^{Il2\text{-}Tshb}$ congenic strain. Insulitis was observed in only 1/10 males and 0/10 females examined at 2 mo of age as compared with 7/10 ($p = 0.02$) and 10/11 ($p < 10^{-4}$) age- and sex-matched NOD mice. With time, however, more NOD.B6$^{Il2\text{-}Tshb}$ mice developed insulitis; at 5 mo of age, 4/16 females and 2/16 males had insulitis (Fig. 3 *B*). The insulitis appeared to have β cell–specific cytotoxic potential since treatment of NOD.B6$^{Il2\text{-}Tshb}$ mice at 7 mo of age with cyclophosphamide caused diabetes in 3/35 females and 7/28 males (Fig. 3 *C*).

Since the NOD.B6^{Il2} and NOD.B6^{Tshb} strains present a higher frequency of diabetes than the NOD.B6$^{Il2\text{-}Tshb}$ strain (Fig. 1), it was not unexpected that more NOD.B6^{Il2} and NOD.B6^{Tshb} mice developed insulitis by 2 and 5 mo than did NOD.B6$^{Il2\text{-}Tshb}$ mice (Fig. 3). Consistent with the expression of insulitis, was the observation that the NOD.B6^{Il2} and NOD.B6^{Tshb} strains developed cyclophosphamide-induced diabetes (Fig. 3 *C*).

The results with the NOD.B6^{Il2} and NOD.B6^{Tshb} strains, expressing resistance alleles at *Idd3* and *Idd10*, respectively, suggest that resistance alleles at each of these two *Idd* loci reduce the frequency of insulitis. However, when resistance alleles are present at both loci, as they are in the NOD.B6$^{Il2\text{-}Tshb}$ strain, the reduction of insulitis is even more profound. This suggests that *Idd3* and *Idd10* act early in the disease process, during the development of insulitis. However, since some NOD.B6$^{Il2\text{-}Tshb}$ mice develop insulitis and a small number develop spontaneous diabetes, the initiation of the autoimmune response to the β cells can occur in the absence of NOD alleles at both *Idd3* and *Idd10*.

Discussion

Several years ago at the beginning of the present study, we expected to find three or four dominant-acting and fully penetrant protective alleles from the B10 and B6.PL-*Thy1*^{a} backgrounds. Previous segregation analyses with the B10 (18) and B6 (41) strains had demonstrated potent protective influences from these normal mice, consistent with the existence of what appeared to be fully recessive NOD-derived diabetes susceptibility loci. Our initial strategy to identify protective B10 or B6.PL-*Thy1*^{a} alleles at non-MHC–linked diabetogenic loci was to backcross (B10 × NOD)F_1 or (B6.PL-*Thy1*^{a} × NOD)F_1 mice to the NOD parental strain and select for pedigrees demonstrating a low frequency of diabetes. We hypothesized that each dominant diabetes-resistant allele derived from B10 or B6.PL-*Thy1*^{a} would reduce diabetes by 50%. We bred 23 different pedigrees to ensure that each of the proposed dominant protective alleles would be responsible for disease protection in at least one of the lines. However, with this strategy, we were unable to demonstrate the existence of any single dominant allele from the B10 or B6.PL-*Thy1*^{a} strains that protected against diabetes. Indeed, the eventual development of the congenic lines described in the present study, which were initiated with the above strategy, relied primarily on the fortuitous observation that male mice were protected from diabetes. In retrospect, this protection was dependent on the pedigrees not recombining between the two linked *Idd* loci on chromosome 3, *Idd3* and *Idd10*. Unfortunately, by the time we realized that many *Idd* loci were segregating in the (B10.*H-2*^{g7} × NOD) × NOD and (B6.PL-*Thy1*^{a} × NOD) × NOD backcrosses (33), only two pedigrees remained, eventually becoming the NOD.B10$^{Il2\text{-}Tshb}$ and NOD.B6$^{Il2\text{-}Tshb}$ strains, the other pedigrees were discarded because diabetes resistance could not be maintained.

The development of *Idd3* and *Idd10* congenic strains of mice has provided a number of insights into the contribution of non-MHC–linked genes to autoimmune diabetes in the NOD mouse. The first, and perhaps most striking finding revealed by the congenic strains, was that two independent *Idd* loci exist on chromosome 3. Our initial mapping studies pointed to a single locus located near *D3Nds1*, termed *Idd3* (33). In that study, there was less than a 1 in 1,000 likelihood of finding *Idd3* near *Il2*. We now realize that the initial localization of *Idd3* to *D3Nds1* was caused by the combined effects of two *Idd* loci, *Idd3* and *Idd10*, flanking *D3Nds1*. In fact, a *D3Nds1* NOD congenic strain is not protected from diabetes (Table 2). A second striking finding from the *Idd3* and *Idd10* congenic strains was that one or two doses of either resistant allele only partially protects from diabetes. Thus, at least in the case of *Idd3* and *Idd10*, NOD alleles at these loci contribute to, but are not absolutely required for, disease development. This is in contrast to NOD strains congenic for resistant alleles at *Idd1* where complete resistance to diabetes occurs with two doses of the resistant MHC allele and virtually complete resistance with one dose (24, 25, 28, 29).

Our goal of identifying the gene products of *Idd3* and *Idd10* is made more difficult by the observation that the presence of the NOD allele at either locus is not absolutely required for diabetes. Thus, fine-mapping these two *Idd* loci using conventional segregation analyses is not possible. We have therefore begun to fine-map *Idd3* and *Idd10* by identifying potentially informative recombination events and developing new *Idd3* and *Idd10* congenic strains with smaller introgressed regions. Such studies are ongoing and the strategy has been successful at defining smaller chromosomal regions that contain *Idd3* and *Idd10* (our unpublished observations).

There are several candidate genes for *Idd3* and *Idd10*. One candidate gene for *Idd3*, *Glut2*, has been ruled out in the current study since a congenic strain expressing the NOD allele at *Glut2* is resistant to diabetes (Tables 1 and 2). It is interesting to note that *Il2*, which is polymorphic between NOD and the B10 and B6 strains (35, 42), remains a candidate gene for *Idd3*. However, despite this polymorphism in the structural gene, no functional polymorphism of the lymphokine has been detected (42). *Fcgr1*, which encodes the high affinity Fc receptor and for which the NOD strain has a rare mutated allele encoding a defective receptor (36), remains a candidate gene for *Idd10*. Other noteworthy candidate genes for *Idd10* are *Csfm* and *Cd53*.

We speculate that, for several of the *Idd* loci that contribute to disease, the diabetes-susceptibility allele will often be found among inbred strains of mice because it alone does not confer disease susceptibility. These diabetogenic allelic variants could alter lymphocyte homing, the physiology of beta cells within

the islets, the establishment of peripheral tolerance, Th1/Th2 balance, or other aspects of the autoimmune response. This model predicts that overlapping, but different sets of *Idd* loci will be found to segregate depending on the strain that is used to outcross with the NOD. Each nondiabetic inbred strain tested would have a different pattern of alleles at the loci that contribute to the development of diabetes. For example, in an (SWR × NOD) × NOD backcross analysis, no linkage of diabetes to chromosome 3 was detected (Leiter, E., personal communication).

Finally, the development of *Idd* congenic strains allows gene interactions to be identified. For example, we found that the simultaneous presence of *Idd3* and *Idd10* confers more resistance from diabetes and insulitis than would be expected from the protection provided by either locus alone. Thus, protection caused by these two *Idd* loci appears to be synergistic rather than additive. It is interesting that a statistical evaluation of the contribution of *Idd* loci to the development of diabetes in the NOD mouse demonstrated that a multiplicative epistasis model provides the best description of the observed disease frequency (43). The molecular basis for the interaction between *Idd3* and *Idd10* must await the identification of these two genes and their biologic function in autoimmunity.

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Received for publication 12 April 1994 and in revised form 11 July 1994.

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