I-E$^+$ Nonobese Diabetic Mice Develop Insulitis and Diabetes

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Summary

The development of type I diabetes in the nonobese diabetic (NOD) mouse is under the control of multiple genes, one or more of which is linked to the major histocompatibility complex (MHC). The MHC class II region has been implicated in disease development, with expression of an I-E transgene in NOD mice shown to provide protection from insulitis and diabetes. To examine the effect of expressing an I-E$^+$ or I-E$^-$ non-NOD MHC on the NOD background, three I-E$^+$ and three I-E$^-$ NOD MHC congenic strains (NOD.*H-2^{i5}*, NOD.*H-2^k*, and NOD.*H-2^{h2}*, and NOD.*H-2^{h4}*, NOD.*H-2^{i7}*, and NOD.*H-2^b*, respectively) were developed. Of these strains, both I-E$^+$ NOD.*H-2^{h2}* and I-E$^-$ NOD.*H-2^{h4}* mice developed insulitis, but not diabetes. The remaining four congenic strains were free of insulitis and diabetes. These results indicate that in the absence of the NOD MHC, diabetes fails to develop. Each NOD MHC congenic strain was crossed with the NOD strain to produce I-E$^+$ and I-E$^-$ F_1 mice; these mice thus expressed one dose of the NOD MHC and one dose of a non-NOD MHC on the NOD background. While a single dose of a non-NOD MHC provided a large degree of disease protection to all of the F_1 strains, a proportion of I-E$^+$ and I-E$^-$ F_1 mice aged 5–12 mo developed insulitis and cyclophosphamide-induced diabetes. When I-E$^+$ F_1 mice were aged 9–17 mo, spontaneous diabetes developed as well. These data are the first to demonstrate that I-E$^+$ NOD mice develop diabetes, indicating that expression of I-E in NOD mice is not in itself sufficient to prevent insulitis or diabetes. In fact, I-E$^-$ F_1 strains were no more protected from diabetes than I-E$^+$ F_1 strains, suggesting that other non-NOD MHC–linked genes are important in protection from disease. Finally, transfer of NOD bone marrow into irradiated I-E$^+$ F_1 recipients resulted in high incidences of diabetes, indicating that expression of non-NOD MHC products in the thymus, in the absence of expression in bone marrow–derived cells, is not sufficient to provide protection from diabetes.

The nonobese diabetic (NOD)[1] mouse spontaneously develops autoimmune diabetes (1–4), and is considered an appropriate model for examining the etiology of human type I diabetes. As in human diabetes, the murine disease is associated with lymphocytic infiltration of pancreatic islets (insulitis) (1, 5), the appearance of autoantibodies directed against β cell proteins (6–13), the T cell–mediated destruction of β cells (14–17), and the presence of both MHC-linked (18–24) and non-MHC-linked (25–30) disease susceptibility genes.

As analyzed in an outcross with the C57BL/10SnJ strain, the development of diabetes in the NOD mouse is under polygenic control (20). At least three non-MHC-linked genes, located on chromosomes 1, 3, and 11 (25, 26), as well as one or more genes in the MHC (18, 20–22) contribute to disease progression and onset. The MHC class II region has been implicated in disease susceptibility, with the NOD strain expressing a unique I-A β chain (22) and no surface I-E molecules due to a lack of I-E α chain production (18). Evidence supporting the involvement of NOD class II products in diabetogenesis comes in part from studies using the cataract Shionogi (CTS) mouse. The CTS strain is identical to the NOD strain at the class II region of the MHC, but differs at the K and D class I loci (31). When two doses of the CTS MHC were expressed on the NOD background, the resulting congenic mice developed insulitis and diabetes (32). A second approach, using transgenic technology to express non-NOD class II products in NOD mice, has provided perhaps the

[1] *Abbreviation used in this paper:* NOD, nonobese diabetic.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/09/0793/11 $2.00
Volume 178 September 1993 793–803

strongest evidence for the involvement of class II molecules in diabetogenesis. In these studies, expression of I-E (33–36) and non-NOD I-A transgenes (37, 38) in the NOD strain provided a high degree of protection from insulitis and diabetes.

Two groups have demonstrated that expression of I-E after introduction of an I-E α^d transgene inhibits the development of insulitis and diabetes in NOD mice (33–36). Such complete protection from disease progression was not observed, however, in NOD.*H-2*$^{g7/nb1}$ mice (the NOD *H-2*^{g7} complex is K^d $I\text{-}A^{nod}$ $I\text{-}E^{null}$ D^b; the *H-2*^{nb1} complex from the nonobese nondiabetic [NON] strain is K^{non} $I\text{-}A^{non}$ $I\text{-}E^{non}$ D^b [31, 39]); although no NOD.*H-2*$^{g7/nb1}$ mice developed diabetes, one dose of I-E failed to prevent the development of mild insulitis in 37% of animals, and severe insulitis in 16% of animals (40).

To determine the extent of disease protection provided by the expression of I-E in NOD mice, various $I\text{-}E^+$ and $I\text{-}E^-$ MHC haplotypes were bred onto the NOD background, and the resulting NOD MHC congenic animals crossed with the NOD strain to produce the F_1 generation. Both $I\text{-}E^+$ and $I\text{-}E^-$ F_1 mice developed insulitis and diabetes, indicating that I-E expression in NOD mice is not in itself sufficient to prevent the development of diabetes.

Materials and Methods

Animals. NOD/MrkTacfBR (NOD) mice were obtained from Taconic Farms (Germantown, NY). C57BL/10SnJ (B10), B10.D2 (R107)/EgDvEg (B10.D2 [R107]), B10.A(5R)/SgSnJ (B10.A[5R]), B10.A(4R)/SgDvEg (B10.A[4R]), B10.A(2R)/SgSnJ (B10.A[2R]) and B10.BR/SgSnJ (B10.BR) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice at Taconic Farms and Merck Research Laboratories were housed under sterile, specific pathogen-free conditions, and do not harbor any known viral, bacterial, or parasitic pathogens.

NOD mice were outcrossed to the B10, B10.D2(R107), B10.A(5R), B10.A(4R), B10.A(2R), and B10.BR strains, and the progeny repetitively backcrossed to the NOD strain. Breeders were selected on the basis of heterozygosity at the MHC, after detection of class I and II MHC products on PBMC using the mAbs listed below. At the fifth (N6) generation, male and female MHC heterozygotes were intercrossed, and MHC homozygous progeny inbred, in order to fix each MHC haplotype of the above strains on the NOD background.

Unless otherwise specified, all experiments used female mice. Animals were tested biweekly for elevated urinary glucose using Tes-Tape (Eli Lilly and Co., Indianapolis, IN), and classified as diabetic after exhibiting glucose values of ≥2+.

MHC Typing. Dissociated spleen cells or PBMC were incubated with one of the following FITC-conjugated mouse mAbs (PharMingen, San Diego, CA): anti-K^d (SF1-1.1), anti-K^b (AF6-88.5), anti-K^k (AF3-12.1), anti-$I\text{-}A^b$ (AF6-120.1), anti-$I\text{-}A^k$ (11-5.2), anti-$I\text{-}A^{k,r,f,s}$ (11-3.25), which is reactive with $I\text{-}A^{nod}$ and $I\text{-}A^k$ but not $I\text{-}A^b$, anti-$I\text{-}E^{d,k,p,r}$ (AMS-16), and anti-D^d (AF4-62.4). All mAbs were titrated and used at optimal concentrations. After incubation with mAbs at 4°C for 20 min, the cells were washed and then analyzed by flow cytometry on the FACS IV® or FACStar PLUS® (Becton Dickinson & Co., Mountain View, CA). Propidium iodide was added to exclude dead cells from the analysis.

All strains of mice were tested with the eight class I– or class II–specific mAbs listed above. Cells from the NOD strain bound only the mAbs specific for K^d and $I\text{-}A^{k,r,f,s}$. Cells from each parental strain and its corresponding MHC congenic strain bound the following mAbs: B10 and NOD.*H-2*^{b}, anti-K^b and anti-$I\text{-}A^b$; B10.D2(R107) and NOD.*H-2*^{i7}, anti-K^b, anti-$I\text{-}A^b$, and anti-D^d; B10.A(5R) and NOD.*H-2*^{i5}, anti-K^b, anti-$I\text{-}A^b$, anti-$I\text{-}E^{d,k,p,r}$, and anti-D^d; B10.A(4R) and NOD.*H-2*^{h4}, anti-K^k, anti-$I\text{-}A^k$, and anti-$I\text{-}A^{k,r,f,s}$; B10.A(2R) and NOD.*H-2*^{h2}, anti-K^k, anti-$I\text{-}A^k$, anti-$I\text{-}A^{k,r,f,s}$, and anti-$I\text{-}E^{d,k,p,r}$; and B10.BR and NOD.*H-2*^{k}, anti-K^k, anti-$I\text{-}A^k$, anti-$I\text{-}A^{k,r,f,s}$, and anti-$I\text{-}E^{d,k,p,r}$.

Histology. Pancreata were fixed in 10% buffered formalin and processed for paraffin embedding. Tissue sections (5 μm) were stained with hematoxylin and eosin and microscopically evaluated for the presence of mononuclear cell infiltration. Two noncontiguous sections of each pancreas (45–50 μm between sections) were examined. Histology scores used were: normal, no infiltrating mononuclear cells observed in the pancreas; PV/PD, infiltrating cells observed only in perivascular and/or periductal locations of the pancreas; mild insulitis, mononuclear cell infiltration of islet tissue is limited to less than half of the islets in the two tissue sections; and extensive insulitis, mononuclear cells permeate most islets and β cell necrosis is seen.

Cyclophosphamide Treatment. Lyophilized cyclophosphamide (Cytoxan; Mead Johnson Oncology Products, Evansville, IN) was prepared immediately before use by adding sterile distilled water to give a final concentration of 20 mg/ml. Nondiabetic mice received 200 mg/kg by intraperitoneal injection on days 0 and 14, and were monitored for diabetes up to 28 d after the first injection.

Preparation and Analysis of Bone Marrow Chimeras. Bone marrow cells were harvested from 7-wk-old NOD and 7–11-wk-old (NOD × NOD.*H-2*^{k})F_1 and (NOD × NOD.*H-2*^{i5})F_1 female donors. To eliminate mature T cells, the bone marrow cells were incubated at 4°C for 30 min with a mixture of the following mAbs: anti-Thy-1.2 (HO-13-4) (41) (TIB 99; American Type Culture Collection, Rockville, MD), anti-Lyt-1.2 (C3PO.13) (42), and anti-Lyt-2 (3.155) (43) (TIB 211; American Type Culture Collection). The cells were then incubated with absorbed guinea pig complement at 37°C for 30 min. 6-wk-old NOD and 7–14-wk-old (NOD × NOD.*H-2*^{k})F_1 and (NOD × NOD.*H-2*^{i5})F_1 female recipients were irradiated with 1,000 rad from a ^{137}Cs source (Gammacell 40; Atomic Energy of Canada, Ltd., Ottawa, Ontario) and injected intravenously with 10–20 × 10^6 bone marrow cells. Chimeras are designated as bone marrow donor → irradiated recipient.

Chimeras were monitored for the development of diabetes for 6–7 mo after bone marrow reconstitution. To detect the percentage of contaminating cells originating from irradiated recipients, spleen cells from diabetic chimeras were typed with the class I–specific mAbs described above. In a group of 13 NOD → (NOD × NOD.*H-2*^{k})F_1 chimeras typed, the percentage of residual K^k-positive (NOD × NOD.*H-2*^{k})F_1 cells ranged from 1.9 to 5.2%, with a mean of 3.7%. In a group of three NOD → (NOD × NOD.*H-2*^{i5})F_1 chimeras typed, the percentage K^b-positive (NOD × NOD.*H-2*^{i5})F_1 cells ranged from 0.3 to 0.7%, with a mean of 0.5%. Thus, in all chimeric mice tested, ≥95% of the spleen cells expressed the class I and II MHC products of the bone marrow donor.

Results and Discussion

Establishment of $I\text{-}E^+$ and $I\text{-}E^-$ NOD MHC Congenic Strains. To establish the NOD.*H-2*^{b}, NOD.*H-2*^{i7}, NOD.*H-2*^{i5}, NOD.*H-2*^{h4}, NOD.*H-2*^{h2}, and NOD.*H-2*^{k} strains, NOD mice were outcrossed to B10 (*H-2*^{b}), B10.D2(R107) (*H-2*^{i7}), B10.A(5R) (*H-2*^{i5}), B10.A(4R) (*H-2*^{h4}), B10.A(2R) (*H-2*^{h2}),

Table 1. *Incidence of Spontaneous and Cyclophosphamide-induced Diabetes in NOD MHC Congenic Mice*

	MHC product					
	K	I-A	I-E	D		
		α β	β α		Cyclophosphamide	No. Diabetic/no. observed (%)
NOD	d	d nod	nod –	b	No	41/50 (82)
					Yes	8/9 (89)
NOD.*H-2*^{b}	b	b b	b –	b	No	0/21 (0)
					Yes	0/11 (0)
NOD.*H-2*^{i7}	b	b b	b –	d	No	0/40 (0)
					Yes	0/14 (0)
NOD.*H-2*^{i5}	b	b b	b k	d	No	0/39 (0)
					Yes	0/13 (0)
NOD.*H-2*^{h4}	k	k k	b –	b	No	0/54 (0)
					Yes	0/11 (0)
NOD.*H-2*^{h2}	k	k k	k k	b	No	0/38 (0)
					Yes	0/13 (0)
NOD.*H-2*^{k}	k	k k	k k	k	No	0/31 (0)
					Yes	0/4 (0)

All mice represented in this table were females. NOD and NOD MHC congenic mice were monitored for spontaneous diabetes for 7 and 5–13 mo, respectively. The groups indicated received 200 mg/kg of cyclophosphamide intraperitoneally on days 0 and 14, and were monitored for diabetes up to 28 d after the first injection. NOD and NOD MHC congenic mice were treated with cyclophosphamide at 6 and ⩾5 mo of age, respectively, and were normoglycemic at the initiation of treatment.

and B10.BR (*H-2*^{k}) mice, respectively, and repetitive backcrosses to the NOD were performed using progeny expressing the MHC haplotypes of the strains above. To fix these MHC haplotypes on the NOD background, mice at the N6 generation were intercrossed, and the resulting congenic strains (Table 1) maintained by brother–sister mating.

Examination of female NOD MHC homozygotes at the N6F$_1$ generation revealed a 74% incidence of diabetes by 5–7 mo of age (Table 2), consistent with the 65–82% incidence observed in 5–7-mo-old female NOD mice in our colony (Table 1) (25). This suggests that the non-MHC-linked diabetogenic genes are fixed for the NOD allele in each of the congenic strains. Thus, any influence on the diabetogenic process in NOD MHC congenic mice, as compared with the NOD strain, is the result of the non-NOD MHC.

Incidence of Insulitis and Diabetes in NOD MHC Congenic Strains. In the NOD mouse, the autoimmune destruction of pancreatic β cells begins as a perivascular and periductal accumulation of lymphocytes (PV/PD), evident by 3 wk of age. Lymphocytic infiltration of the islets can be observed as early as 4 wk of age, and by 3 mo, overt diabetes begins to occur (1–4). In our colony, 82% of females (Table 1) (25) and 45% of males (n = 49) develop diabetes by 7 mo of age, and nearly all females and males develop insulitis (11, 44). Treatment with cyclophosphamide, an agent that increases the rapidity and incidence of diabetes in the NOD mouse (45), causes 89% of female (Table 1) (11) and 73% of male (11) 6-mo-old nondiabetic NOD mice to develop diabetes. Cyclophosphamide fails to induce insulitis or diabetes in nondiabetic strains of mice (44–46), suggesting that cyclophosphamide induces diabetes only if a sufficient degree of insulitis has developed before treatment.

Table 2. *Incidence of Spontaneous Diabetes in N6F$_1$ Mice*

	NOD.*H-2*$^{g7/g7}$	NOD.*H-2*$^{g7/non\text{-}g7}$	NOD.*H-2*$^{non\text{-}g7/non\text{-}g7}$
NOD.*H-2*^{b}	7/7	0/5	0/9
NOD.*H-2*^{i7}	1/3	0/13	0/9
NOD.*H-2*^{i5}	6/8	0/10	0/10
NOD.*H-2*^{h4}	2/5	0/13	0/6
NOD.*H-2*^{h2}	6/8	0/5	0/2
NOD.*H-2*^{k}	3/3	0/15	0/6
Total	25/34 (74%)	0/61 (0%)	0/42 (0%)

MHC heterozygous mice at the fifth (N6) backcross generation were intercrossed and the MHC type of the resulting female progeny was determined. Intercross progeny were monitored for diabetes for 5–7 mo.

Examination of the NOD MHC congenic strains showed that each of the strains developed perivascular and periductal lymphocytic infiltration spontaneously by 7–10 mo of age (Table 3). Mice ranging from 5 to 13 mo of age that were treated with cyclophosphamide exhibited perivascular and periductal infiltrates as well. Four of the six congenic strains failed to develop insulitis (Table 3) or diabetes (Table 1), either spontaneously or after cyclophosphamide treatment. In contrast, both I-E$^+$ NOD.*H-2*^{h2} mice and I-E$^-$ NOD.*H-2*^{h4} mice developed insulitis (Table 3 and Fig. 1). These mice failed, however, to develop diabetes, either spontaneously or after treatment with cyclophosphamide (Table 1).

The importance of the MHC in the development of diabetes is supported by the observation that expression of two doses of a non-NOD MHC on the NOD background provides complete protection from disease. The fact that both the I-E$^+$ NOD.*H-2*^{h2} and the I-E$^-$ NOD.*H-2*^{h4} strains developed insulitis suggests that I-E does not play a critical role in this process. It is of interest, however, that both of these strains express I-A^k and D^b (Table 1). Given that the NOD.*H-2*^{k} strain, which expresses I-A^k but not D^b, and the NOD.*H-2*^{b} strain, which expresses D^b but not I-A^k, fail to develop insulitis, it is possible that the combination of I-A^k and D^b (or genes closely linked to these loci) confers susceptibility to lymphocytic infiltration of the islets, but not to the development of diabetes.

Incidence of Insulitis and Diabetes in F_1 Mice Not Expressing I-E. Each NOD MHC congenic strain was crossed with the NOD strain to produce the F_1 generation; thus, in F_1 mice, one dose of the NOD MHC and one dose of a non-NOD MHC is expressed on the NOD background. Examination of the pancreata of nondiabetic female mice within the three I-E$^-$ F_1 strains revealed the presence of perivascular and periductal infiltrates in both untreated (age, 6–10 mo) and cyclophosphamide-treated (age, 5–12 mo) mice (Table 4). The (NOD × NOD.*H-2*^{h4})F_1 and (NOD × NOD.*H-2*^{b})F_1

Table 3. *Pancreatic Histology of Nondiabetic NOD MHC Congenic Mice*

Age	No. observed	Cyclophosphamide	Histology Score (%)			
			Normal	PV/PD	Mild insulitis	Extensive insulitis
NOD.*H-2*^{b}						
mo						
7–10	10	No	3 (30)	7 (70)	0 (0)	0 (0)
6–11	11	Yes	6 (55)	5 (45)	0 (0)	0 (0)
NOD.*H-2*^{i7}						
9	15	No	9 (60)	6 (40)	0 (0)	0 (0)
5–11	14	Yes	7 (50)	7 (50)	0 (0)	0 (0)
NOD.*H-2*^{i5}						
7–10	13	No	10 (77)	3 (23)	0 (0)	0 (0)
5–9	13	Yes	9 (69)	4 (31)	0 (0)	0 (0)
NOD.*H-2*^{h4}						
8–10	17	No	4 (24)	8 (47)	3 (17)	2 (12)
5–13	11	Yes	3 (30)	6 (60)	1 (10)	0 (0)
NOD.*H-2*^{h2}						
9	15	No	0 (0)	10 (67)	4 (27)	1 (6)
10–12	13	Yes	4 (31)	9 (69)	0 (0)	0 (0)
NOD.*H-2*^{k}						
7–8	21	No	6 (29)	15 (71)	0 (0)	0 (0)
6–7	4	Yes	3 (75)	1 (25)	0 (0)	0 (0)

All mice represented in this table were nondiabetic females. The groups indicated received 200 mg/kg of cyclophosphamide intraperitoneally 28 and 14 d before histological examination. Two noncontiguous sections of pancreas were stained with hematoxylin and eosin and microscopically evaluated for the presence of mononuclear cell infiltration. Histology scores used were: normal, no infiltrating mononuclear cells observed in the pancreas; PV/PD, infiltrating cells observed only in perivascular and/or periductal locations of the pancreas; mild insulitis, mononuclear cell infiltration of islet tissue is limited to less than half of the islets in the two tissue sections; extensive insulitis, mononuclear cells permeate most islets and β cell necrosis is seen.

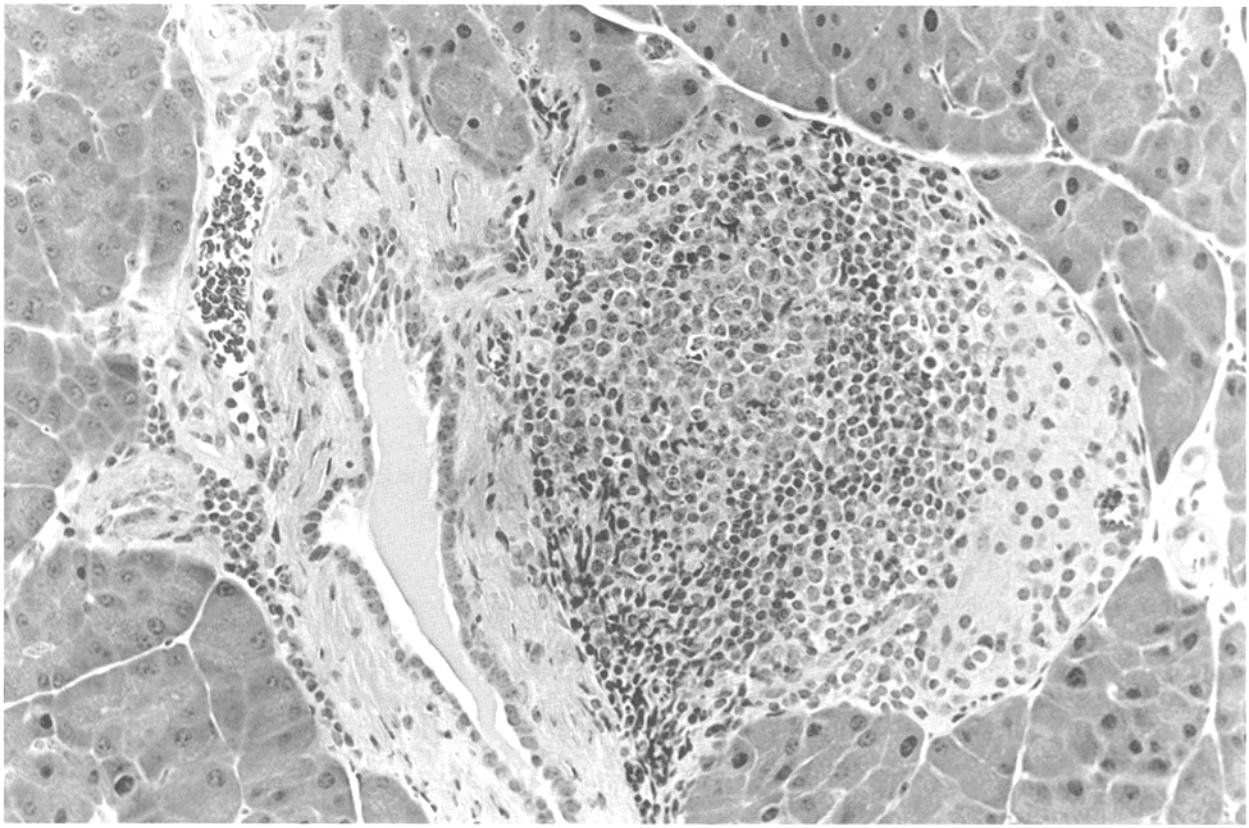

Figure 1. Pancreatic histology of a female NOD.*H-2*^{h2} mouse, showing extensive insulitis. Hematoxylin and eosin (×200).

Table 4. *Pancreatic Histology and Diabetes Incidence in I-E*$^-$ *F*$_1$ *Mice*

	Pancreatic histology of nondiabetic mice						Diabetes incidence	
			Histology score (%)					
Cyclophosphamide treatment	Age	No. observed	Normal	PV/PD	Mild insulitis	Extensive insulitis	Age	No. diabetic/ no. observed (%)
	mo						*mo*	
(NOD × NOD.*H-2*^{h4})F$_1$								
No	7–10	15	2 (13)	4 (27)	2 (13)	7 (47)	5–11	0/37 (0)
Yes	5–11	11	4 (37)	3 (27)	0 (0)	4 (36)	5–11	11/22 (50)
(NOD × NOD.*H-2*^{i7})F$_1$								
No	8	3	1 (33)	2 (67)	0 (0)	0 (0)	5–8	0/17 (0)
Yes	5–7	11	7 (64)	4 (36)	0 (0)	0 (0)	5–7	3/14 (21)
No*	5–6	15	10 (66)	1 (7)	4 (27)	0 (0)		
(NOD × NOD.*H-2*^{b})F$_1$								
No	6–8	30	1 (3)	13 (43)	5 (17)	11 (37)	6–9	0/56 (0)
Yes	5–12	33	9 (27)	16 (49)	4 (12)	4 (12)	6–9	13/56 (23)

Pancreatic histology and diabetes incidences were performed as described for Tables 3 and 1, respectively.
* Pancreata from nondiabetic male mice were examined for the presence of mononuclear cell infiltration. All other mice represented in this table were females.

Table 5. *Pancreatic Histology and Diabetes Incidence in I-E$^+$ F$_1$ Mice*

Cyclophosphamide treatment	Pancreatic histology of nondiabetic mice						Diabetes incidence	
			Histology score (%)					
	Age	No. observed	Normal	PV/PD	Mild insulitis	Extensive insulitis	Age	No. diabetic/ no. observed (%)
	mo						*mo*	
(NOD × NOD.*H-2^{i5}*)F$_1$								
No		ND					5–7	0/118 (0)
Yes	5–7	10	7 (70)	3 (30)	0 (0)	0 (0)	5–7	0/10 (0)
No	10–16	31	11 (36)	19 (61)	0 (0)	1 (3)	10–17	3/108 (3)
Yes	10–17	32	15 (47)	16 (50)	0 (0)	1 (3)	10–17	4/72 (6)
(NOD × NOD.*H-2^{k}*)F$_1$								
No	7	15	5 (33)	6 (40)	3 (20)	1 (7)	6–7	0/93 (0)
Yes	6–7	11	1 (9)	6 (55)	2 (18)	2 (18)	6–7	4/15 (27)
No	9–17	13	0 (0)	5 (38)	5 (38)	3 (24)	9–17	3/62 (5)
Yes	11–17	29	4 (14)	14 (48)	2 (7)	9 (31)	11–17	2/43 (5)
(NOD × NOD.*H-2^{h2}*)F$_1$								
No	7–8	10	3 (30)	2 (20)	1 (10)	4 (40)	6–8	0/15 (0)
Yes	6–7	4	0 (0)	3 (75)	0 (0)	1 (25)	6–7	1/5 (20)

Pancreatic histology and diabetes incidences were performed as described for Tables 3 and 1, respectively. Spontaneous diabetes in the (NOD × NOD.*H-2^{i5}*)F$_1$ mice occurred at 10, 11, and 13 mo of age, and in the (NOD × NOD.*H-2^{k}*)F$_1$ mice at 12, 14, and 15 mo of age.

strains displayed insulitis under both conditions as well. Due to the small number of female (NOD × NOD.*H-2^{i7}*)F$_1$ mice observed, pancreata from 15 untreated male (NOD × NOD.*H-2^{i7}*)F$_1$ mice were also examined. 4 of the 15 males (27%) exhibited mild insulitis, indicating that the (NOD × NOD.*H-2^{i7}*)F$_1$ strain is susceptible to the development of insulitis as well.

Although no (NOD × NOD.*H-2^{h4}*)F$_1$, (NOD × NOD.*H-2^{i7}*)F$_1$, or (NOD × NOD.*H-2^{b}*)F$_1$ mice spontaneously developed diabetes by 5–11 mo of age, cyclophosphamide induced disease in each of the three strains (Table 4). Examination of the pancreata of these diabetic mice revealed extensive insulitis, consistent with the onset of diabetes.

The development of insulitis and diabetes in I-E$^-$ *H-2$^{g7/nong7}$* mice has been previously observed. In female (NOD × SWR/J)F$_1$ × NOD MHC heterozygotes (47) and (NOD × B10)F$_1$ × NOD N3-8 MHC heterozygotes (48), insulitis and a low incidence of spontaneous diabetes developed, consistent with our finding in this study that a single dose of the NOD MHC is sufficient to mediate insulitis and cyclophosphamide-induced diabetes.

Incidence of Insulitis and Diabetes in F$_1$ Mice Expressing I-E. I-E$^+$ F$_1$ mice were aged 5–8 or 9–17 mo, and analyzed for the development of perivascular and periductal infiltration, insulitis, and diabetes. Perivascular and periductal infiltrates were observed in each age group of untreated and cyclophosphamide-treated (NOD × NOD.*H-2^{i5}*)F$_1$, (NOD × NOD.*H-2^{k}*)F$_1$, and (NOD × NOD.*H-2^{h2}*)F$_1$ mice (Table 5). Insulitis was present in untreated and cyclophosphamide-treated 6–8-mo-old (NOD × NOD.*H-2^{k}*)F$_1$ and (NOD × NOD. *H-2^{h2}*)F$_1$ mice (Fig. 2), as well as in 9–17-mo-old (NOD × NOD.*H-2^{k}*)F$_1$ mice. Cyclophosphamide-treated 5–7-mo-old (NOD × NOD.*H-2^{i5}*)F$_1$ mice did not exhibit insulitis, although a small percentage of mice aged 10–17 mo did display insulitis with and without cyclophosphamide treatment.

While 5–8-mo-old (NOD × NOD.*H-2^{i5}*)F$_1$, (NOD × NOD.*H-2^{k}*)F$_1$ and (NOD × NOD.*H-2^{h2}*)F$_1$ mice failed to develop diabetes spontaneously, treatment with cyclophosphamide induced diabetes in both the (NOD × NOD.*H-2^{k}*)F$_1$ and (NOD × NOD.*H-2^{h2}*)F$_1$ strains (Table 5). In addition, a small percentage of (NOD × NOD.*H-2^{i5}*)F$_1$ and (NOD × NOD.*H-2^{k}*)F$_1$ mice aged 9–17 mo developed diabetes spontaneously, as well as after cyclophosphamide treatment. Examination of the pancreata of these mice revealed extensive insulitis, consistent with the onset of diabetes.

This is the first report of I-E$^+$ NOD mice developing diabetes. Insulitis, but no diabetes, was previously observed in I-E$^+$ NOD.*H-2$^{g7/nb1}$* mice (40). It is of interest that the NOD.*H-2$^{g7/nb1}$* mice were aged to 10 mo; by this age only one of the six spontaneously diabetic I-E$^+$ F$_1$ mice in our study had developed disease (Table 5). Thus, the kinetics of disease onset may be an important consideration in determining the role of I-E in diabetogenesis.

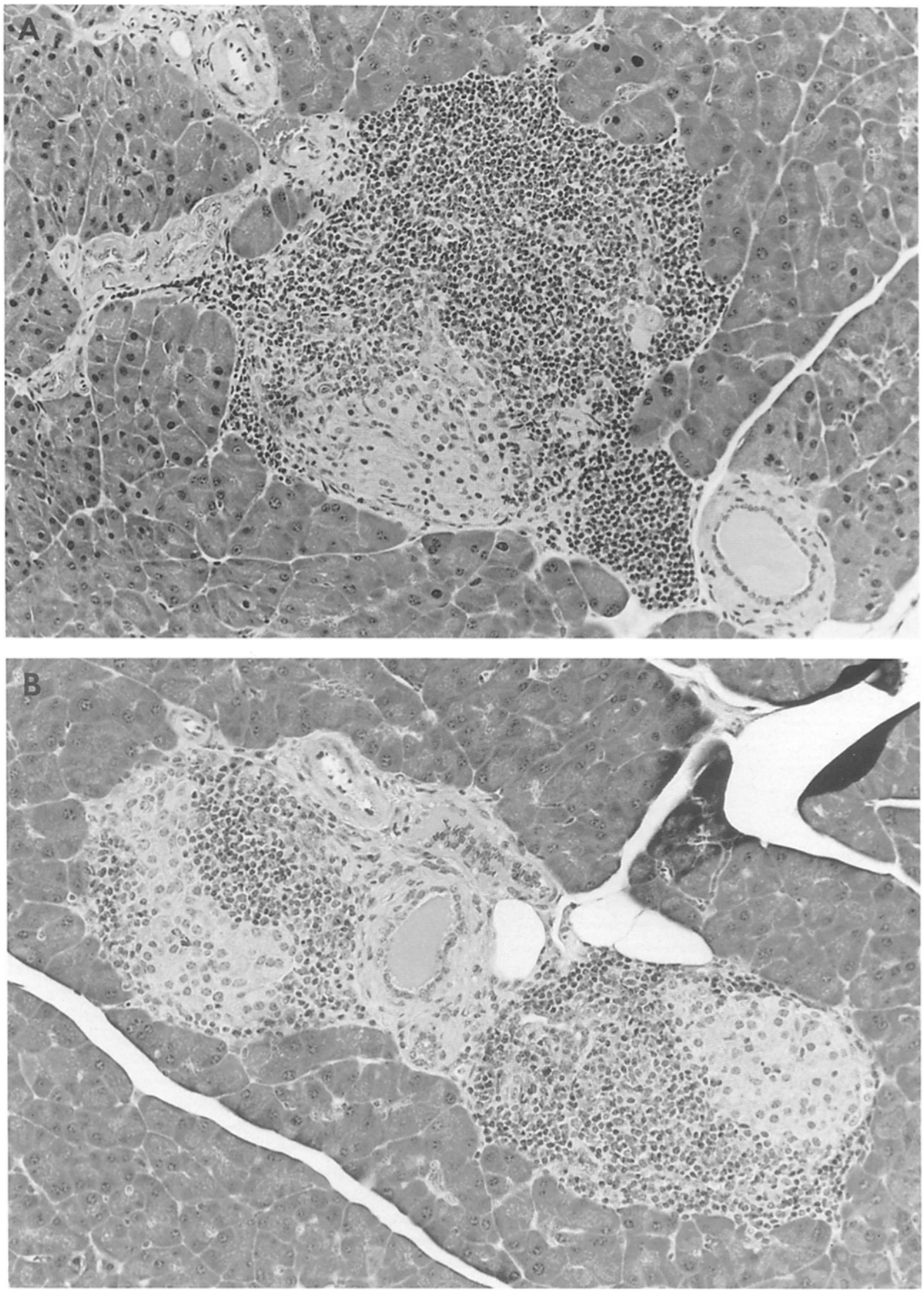

Figure 2. Pancreatic histology of female (NOD × NOD.*H-2*^{k})F_1 (*A*) and (NOD × NOD.*H-2*^{h2})F_1 (*B*) mice, showing extensive insulitis. Hematoxylin and eosin (×125).

In contrast to the results detailed above, expression of an I-E α^d transgene in NOD mice was found to provide complete protection from diabetes (33–36). In these studies, C57BL/6 mice expressing an I-E α^d transgene were outcrossed to the NOD strain, and I-E$^+$ progeny repetitively backcrossed to the NOD (33, 34), or an I-E α^d transgene was introduced directly into NOD mice (34–36). In both cases, I-E expression protected mice from the development of insulitis and diabetes, even after treatment with cyclophosphamide (35, 36). Interestingly, expression of an I-E α^k transgene in second backcross (to NOD) progeny provided incomplete protection from disease progression, with mild insulitis, but no diabetes, developing in 1 of 18 mice (49). One possible interpretation of these results is that I-E $\alpha^k\beta^{nod}$ is more permissive for disease development than I-E $\alpha^d\beta^{nod}$. It should be noted, however, that the sequences of I-E α^k and I-E α^d are identical in the peptide binding α1 domain, and differ by only three amino acids in the α2 domain (50). Thus, other experimental differences may account for the variability in disease protection provided by the expression of an I-E α transgene.

One explanation for the differing degrees of protection observed in I-E$^+$ NOD mice is the variable environments in which the mice are maintained. It is well established that environmental factors affect the incidence of diabetes in NOD colonies (51–53). In addition, infection with viruses, such as mouse hepatitis virus, affects the diabetogenic process, significantly decreasing the incidence of disease (54). Such environmental influences may be of even greater consequence when acting upon less diabetogenic strains, such as NOD mice that express non-NOD MHC products. For instance, in our high incidence colony, >95% of NOD mice developed insulitis as compared with 50% of (NOD × NOD.*H-2^b*)F$_1$ mice, with insulitis in the latter developing at a significantly slower rate (11). Similarly, the incidence of diabetes in female animals decreased from 82% in the NOD strain to 3% in the (NOD × NOD.*H-2^b*)F$_1$ strain. Had these animals been maintained in a lower incidence colony, it is possible that the prevalence of insulitis and diabetes in (NOD × NOD.*H-2^b*)F$_1$ mice would have been decreased to an extent that little insulitis and no diabetes could be detected. Thus, as a result of environmental influences, quantitative variability among mice maintained in different environments may appear instead as qualitative differences. The fact that the effects of expressing I-E in diabetogenic mice maintained in different environments has ranged from complete protection from insulitis and diabetes (33–36), to mild insulitis in a low percentage of mice (49), to more severe insulitis in a higher percentage of mice (40), to the development of insulitis as well as spontaneous and cyclophosphamide-induced diabetes (Table 5) suggests that environmental factors may influence diabetogenesis in I-E$^+$ NOD mice.

Alternatively, or in addition to environmental influences, the variability in disease protection observed in I-E$^+$ NOD mice may be the result of the different non-NOD MHC–linked genes present in the mice, and thus a consequence of the approach used to express I-E on the NOD background. While the transgenic NOD mice expressed either I-E $\alpha^d\beta^{nod}$ (33–36) or I-E $\alpha^k\beta^{nod}$ (49), the NOD.*H-2$^{g7/nb1}$* mice expressed both I-E $\alpha^{non}\beta^{non}$ and I-E $\alpha^{non}\beta^{nod}$ (40). Similarly, our I-E$^+$ F$_1$ strains expressed two I-E $\alpha\beta$ pairs, with (NOD × NOD.*H-2^{i5}*)F$_1$ mice expressing I-E $\alpha^k\beta^b$ and I-E $\alpha^k\beta^{nod}$, and (NOD × NOD.*H-2^{h2}*)F$_1$ mice and (NOD × NOD.*H-2^k*)F$_1$ mice expressing I-E $\alpha^k\beta^k$ and I-E $\alpha^k\beta^{nod}$. Thus, transgenic technology produced only one I-E molecule of either the $\alpha^d\beta^{nod}$ or $\alpha^k\beta^{nod}$ type, while conventional genetic approaches produced two I-E types in each animal. In the studies presented here, I-E $\alpha^k\beta^b$ or $\alpha^k\beta^k$, as well as I-E $\alpha^k\beta^{nod}$, were expressed in each I-E$^+$ mouse. Whether such differences are significant in determining disease susceptibility remains to be determined. It is possible that I-E $\alpha^{d,k}\beta^{nod}$ is more protective than I-E $\alpha^k\beta^b$ or $\alpha^k\beta^k$, and that conventional genetic approaches result in reduced levels of I-E $\alpha^{d,k}\beta^{nod}$ due to the concomitant expression of I-E $\alpha^k\beta^b$ or $\alpha^k\beta^k$, thereby rendering animals more susceptible to the development of diabetes. It is only when transgenic technology and conventional genetic approaches are used under the same environmental conditions that the roles of genetics versus the environment in I-E protection can be delineated.

Each F$_1$ strain in our study was provided some degree of protection through the expression of a single dose of a non-NOD MHC. It should be noted that while subtle differences in the level of protection were apparent amongst the strains, this variability was not significant. Of interest is the fact that the I-E$^+$ F$_1$ strains were no more protected from disease than the I-E$^-$ F$_1$ strains. Age-related groups of I-E$^+$ and I-E$^-$ F$_1$ mice exhibited similar incidences of perivascular and periductal infiltration, insulitis, and eventually spontaneous or cyclophosphamide-induced diabetes. Thus, the protective effect provided by one dose of a non-NOD MHC was not augmented by the expression of I-E.

A possible explanation for this observation is that I-E and non-NOD I-A provide protection from diabetes via the same mechanism, and that maximal protection by class II molecules can be achieved through the expression of either I-E or I-A alone. Since each F$_1$ strain expressed a non-NOD I-A, the concomitant expression of I-E in the (NOD × NOD.*H-2^{i5}*)F$_1$, (NOD × NOD.*H-2^k*)F$_1$, and (NOD × NOD.*H-2^{h2}*)F$_1$ strains did not increase the degree of protection in these mice as compared with the I-E$^-$ F$_1$ strains. In accordance with this theory, the expression of an I-A^k transgene in NOD mice has been found to markedly reduce insulitis (37, 38), and completely prevent the development of diabetes (37).

Incidence of Diabetes in Bone Marrow Chimeras. To determine the diabetogenic potential of NOD hematopoietic stem cells educated in an I-E$^+$ F$_1$ environment, lethally irradiated (NOD × NOD.*H-2^k*)F$_1$ and (NOD × NOD.*H-2^{i5}*)F$_1$ mice were reconstituted with NOD bone marrow. Use of irradiated NOD mice as bone marrow recipients resulted in an 83% incidence of diabetes (Table 6), nearly identical to the incidence seen in unmanipulated NOD mice (Table 1). This indicates that the experimental procedure itself does not prevent disease development in chimeric mice. In addition, (NOD

Table 6. *Incidence of Spontaneous Diabetes in Chimeric Mice*

Donor → Recipient	No. diabetic/ no. observed (%)	Mean age of onset ± SD
		d
NOD → NOD	5/6 (83)	124 ± 8
NOD → (NOD × NOD.*H-2*^{k})F_1	20/30 (61)	131 ± 19
NOD → (NOD × NOD.*H-2*^{i5})F_1	4/9 (44)	136 ± 33
(NOD × NOD.*H-2*^{k})F_1 → (NOD × NOD.*H-2*^{k})F_1	1/18 (6)	126
(NOD × NOD.*H-2*^{i5})F_1 → (NOD × NOD.*H-2*^{i5})F_1	0/9 (0)	–

All mice represented in this table were females. Chimeras are designated as bone marrow donor → irradiated recipient. Recipients were 7–11 wk of age at the time of bone marrow reconstitution, and were monitored for diabetes for 6–7 mo after chimera construction. The mean age of onset represents the number of days between chimera construction and onset of diabetes.

× NOD.*H-2*^{k})F_1 → (NOD × NOD.*H-2*^{k})F_1 and (NOD × NOD.*H-2*^{i5})F_1 → (NOD × NOD.*H-2*^{i5})F_1 bone marrow chimeras showed low incidences of diabetes (Table 6), similar to those of unmanipulated (NOD × NOD.*H-2*^{k})F_1 and (NOD × NOD.*H-2*^{i5})F_1 mice (Table 5). Thus, irradiation and bone marrow reconstitution does not artificially induce diabetes.

Reconstitution of irradiated (NOD × NOD.*H-2*^{k})F_1 and (NOD × NOD.*H-2*^{i5})F_1 mice with NOD bone marrow resulted in diabetes incidences of 61 and 44%, respectively, by 6–7 mo after chimera construction (Table 6). In comparison, unmanipulated (NOD × NOD.*H-2*^{k})F_1 and (NOD × NOD.*H-2*^{i5})F_1 mice failed to develop spontaneous diabetes by 5–7 mo of age, and showed incidences of 5 and 3%, respectively, by 9–17 mo of age (Table 5). Thus, NOD bone marrow–derived cells retain their ability to induce a high incidence of diabetes in (NOD × NOD.*H-2*^{k})F_1 and (NOD × NOD.*H-2*^{i5})F_1 mice, despite being educated in the presence of an I-E$^+$ non-NOD MHC.

These results indicate that the high degree of disease protection provided by one dose of a non-NOD MHC requires expression of the non-NOD MHC products in bone marrow–derived cells; expression of these products in the thymus alone fails to provide protection from diabetes. A similar conclusion was reached by Böhme et al. (49), who found that the incidence of insulitis in I-E$^+$ NOD transgenic mice was greatly reduced when I-E was expressed in both the thymus and in peripheral lymphoid cells, but not when I-E was expressed in the thymus alone. A possible explanation for the importance of peripheral MHC expression in disease protection is that non-NOD class II molecules reduce or prevent binding of diabetogenic peptides to the NOD class II molecule, resulting in a reduction or lack of autoantigen presentation to the immune system (55, 56). Alternatively, immune responses restricted by non-NOD class II products may expand the islet-reactive Th2 population, which in turn could downregulate β cell–specific immune destruction (57).

In summary, the studies presented here indicate that I-E expression in NOD mice is not sufficient to prevent insulitis or diabetes. The expression of two doses of an I-E$^+$ or I-E$^-$ non-NOD MHC on the NOD background can be permissive for insulitis, although diabetes fails to develop in the absence of the NOD MHC. NOD mice expressing one dose of an I-E$^+$ or I-E$^-$ non-NOD MHC and one dose of the NOD MHC exhibit a high degree of disease protection, although insulitis and diabetes do develop in a percentage of these animals. Interestingly, I-E$^+$ MHC heterozygous mice are no more protected from diabetes than are I-E$^-$ MHC heterozygotes. Construction of bone marrow chimeras indicates that expression of non-NOD MHC products in the thymus, in the absence of expression in bone marrow–derived cells, fails to provide protection from diabetes.

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Received for publication 12 April 1993 and in revised form 17 May 1993.

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