GENETIC CONTROL OF DIABETES AND INSULITIS IN THE NONOBESE DIABETIC (NOD) MOUSE

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Autoimmune, insulin-dependent diabetes mellitus in man is an inherited disease. Many studies have demonstrated (reviewed in references 1, 2) that genes linked to the MHC of man contribute to the genetic susceptibility to diabetes. >90% of Caucasian patients suffering from type 1 diabetes express the DR3 and/or DR4 antigens as compared with a 60% expression in the total population. Interestingly, DR3/DR4 heterozygotes are particularly susceptible to the development of diabetes since up to 50% of type 1 diabetics possess this genotype as compared with only 5% of the general population. The fine specificity of the association of DR3 and DR4 with diabetes has recently been defined using HLA restriction endonuclease fragment length polymorphisms (3–5) and human T lymphocyte clones that define DR subtypes (6, 7).

The autoimmune response in type 1 diabetes is characterized by insulitis, which is an inflammatory infiltrate affecting the islets of Langerhans. In a study of 60 recent-onset type 1 diabetics, insulitis was present in 78% of patients (8). A persistence of memory cells specific for insulin-producing β cells is suggested by the observation that long-term insulin-dependent patients receiving a pancreas transplant from an identical twin will still reject the islet tissue even though the original antigenic stimulus has been absent for years (9).

Recently, two animal models exhibiting spontaneous diabetes mellitus have been identified. The BB rat (10, 11) and the nonobese diabetic (NOD)[1] mouse (12–16) both evidence the destructive autoimmune pancreatic insulitis that is characteristic of human type 1 diabetes. In the BB rat model, diabetes can be adoptively transferred with Con A–activated splenic lymphocytes obtained from diabetic BB rats (17, 18). Further evidence supporting the autoimmune etiology of diabetes in the BB rat is that treatment of BB rats with a combination of immunosuppressive agents, which included cyclosporine A, glucocorticoids, and

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

antiserum to rat lymphocytes, reduced the severity of spontaneous diabetes and lowered the frequency of diabetes in susceptible animals (19). It has been shown (20–22) that at least two independent genes or gene complexes are necessary for the inheritance of diabetes in the BB rat, one gene that is closely linked to the MHC and a second gene that is linked to the locus that controls the T cell lymphopenia that is observed in the BB rat (20–22). However, recent data from Like et al. (23) demonstrate that lymphopenia is not obligatory for the development of diabetes in some lines of BB rats.

In the NOD mouse (12–16), the pancreatic inflammatory process begins at 4–6 wk of age as a periductal and perivascular accumulation of lymphocytes. At 6–8 wk of age, these lymphocytes begin to invade the islets and specifically destroy insulin-producing β cells. Overt diabetes is observed beginning at 3 mo of age, and by 7 mo of age, 70% of females and 40% of males have become diabetic. As in the BB rat, this disease process appears to be immune based since T cells obtained from diabetic mice induce diabetes in young, nondiabetic NOD mice within 2–3 wk after transfer (24, 25). Unlike the BB rat, acute T cell lymphopenia is not observed in the NOD mouse (26).

Recently, in crosses of NOD mice with normal C3H mice, Hattori et al. (27) detected a recessive diabetogenic gene linked to the MHC of the NOD. The development of overt diabetes depended on homozygosity of the NOD MHC in addition to at least two other diabetogenic genes. It was also shown in this study that the NOD fails to express an I-E product. The authors suggested that the presence of the C3H I-E product in MHC heterozygous F_2 and first-backcross mice might prevent the development of diabetes. In a separate study, Leiter et al. (28) have suggested that three recessive genes, including one located on chromosome 9 and one linked to the MHC on chromosome 17, control the development of diabetes in the NOD. Thus, an MHC-linked gene has been implicated in the susceptibility to diabetes in man and in both animal models of type 1 diabetes.

The present study was undertaken to define the minimum number of genes or gene complexes that control the development of overt diabetes and insulitis in the NOD mouse. We have specifically asked whether there is any influence on either process by the MHC. To accomplish these goals, the NOD and C57BL/10 (B10) strains were bred to produce the F_1, F_2, and $F_1 \times$ NOD first-, second-, and third-backcross generations in which segregation of diabetes, insulitis, and MHC-encoded products was monitored. The B10 strain was chosen for these studies since it does not develop overt diabetes or insulitis and, as observed in the NOD strain, fails to express an I-E product (29). We have found that the development of diabetes and insulitis is under partially overlapping but distinct genetic controls. Diabetes appears to be controlled by at least three independent genes or gene complexes, one of which is linked to the MHC. In contrast, the initiation of insulitis is determined by a single gene not linked to the MHC; however, the severity of insulitis is greatly influenced by an MHC-linked gene.

Materials and Methods

Animals. A breeding nucleus of NOD mice (K^d, I-A^{NOD}, D^b) was kindly provided by Dr. Yoshihiro Tochino (Aburabi Laboratories, Shionogi and Co., Osaka, Japan). C57BL/10 (B10) (K^b, I-A^b, D^b) and NZB/B1NJ (NZB) (K^d, I-A^d, D^d) mice were obtained

from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained under specific pathogen-free conditions and did not display antibody titers to Sendai or Mouse Hepatitis viruses. Animals were tested biweekly for urinary glucose using Tes-Tape (Eli Lilly and Co., Indianapolis, IN) and were classified as diabetic after producing consistent Tes-Tape values of ≥1+.

MHC Typing. Dissociated spleen cells (10^6) from test mice were incubated with the following mAbs to determine the MHC phenotype of the animals: anti-I-$A^{b,d}$, H-$2^{p,q}$ (34-5-3S [30]; Litton Bionetics, Charleston, SC), which is not reactive with the NOD I-A product, and anti-I-$A^{k,r,f,s,u}$ (10-2.16 [31, 32], American Type Culture Collection, Rockville, MD), which is reactive with the NOD I-A product but not the B10 I-A product. The cells were washed once and then incubated with FITC-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) that had been absorbed with Sepharose-conjugated mouse IgM protein, MOPC 104E (Litton Bionetics). Cells were analyzed by flow cytometry on the FACS IV (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Histology. Pancreata obtained after killing mice were fixed either in formalin or Bouin's solution and processed for paraffin embedment. Tissue sections (4 μm) stained with H and E were scrutinized for evidence of mononuclear cell inflammation. Fig. 1 illustrates representative histological abnormalities of the pancreas that were encountered and the classification scheme used to categorize the inflammatory lesions. Histology scores used were: 0, no inflammatory cells observed in the pancreas; 1, infiltrating cells observed in periductal and/or perivascular locations; 2, relatively small numbers of islet-associated inflammatory cells are observed at the islet periphery; and 3, moderate to severe inflammation of the islet in which infiltrating mononuclear cells permeate islet tissues and evidence of β cell necrosis is seen.

Histological classification of pancreatic tissues was made using the most severe inflammatory lesions detected. For example, an animal displaying both periductal inflammation (score of 1) and severe insulitis (score of 3) received a score of 3.

Statistical Analysis. Statistical significance was assessed by χ^2 analysis.

Results

Diabetes and Insulitis in NOD, B10, and (NOD × B10)F_1 Mice. It has been previously shown (12–14) that the NOD strain develops insulitis and overt diabetes. In our NOD colony, 72% of females and 40% of males become diabetic by 7 mo of age (Table I). Histological examination of representative animals exhibiting diabetes revealed the presence of islets undergoing active mononuclear cell inflammation and β cell destruction (data not shown). In addition, end-stage islets characterized by an absolute loss of component β cells and regression of insulitis were frequently observed. Pancreata from 16 female and male NOD mice that had not become diabetic by 7 mo of age were histologically examined and all received scores of 3 (Table I). In contrast, B10 mice did not develop diabetes, and pancreata obtained from B10 animals did not display insulitis. Only 1 of 16 B10 mouse pancreata showed a small focal perivascular lymphocytic infiltrate. We bred (NOD × B10)F_1 mice so we could monitor the inheritance of diabetes and insulitis. None of the 200 F_1 mice observed developed diabetes, which is consistent with a recessive inheritance of this phenotype (Table I). Insulitis was observed in only 1 of 64 (NOD × B10)F_1 mice. However, 17 of the 54 female F_1 mice examined had small pancreatic lymphoid infiltrations of the exocrine pancreas that were associated with ductular and vascular elements but not with the islets. Since one F_1 animal did develop insulitis and many others appeared to exhibit inflammatory lesions mimicking the early stages of histological abnormalities observed in young NOD mice, these data suggest that insulitis

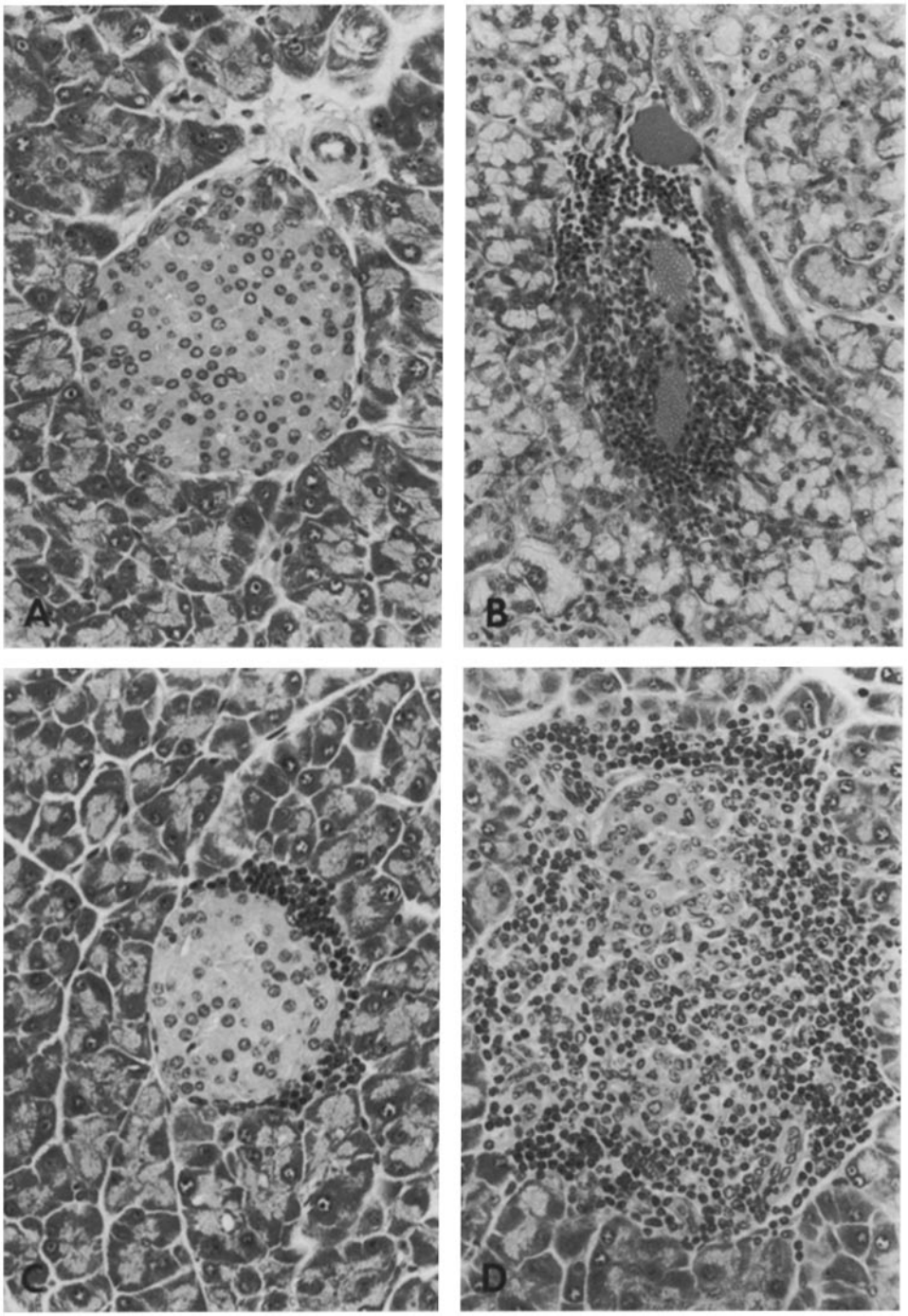

FIGURE 1. Range of histological abnormalities used in the scoring of mouse pancreata (see Materials and Methods). (*A*) Normal islet morphology (grade 0); (*B*) periductular and perivascular mononuclear cell inflammation (grade 1); (*C*) early stage insulitis. Small numbers of islet-associated inflammatory cells are peripherally located (grade 2); (*D*) severe insulitis. Inflammatory cells surround and permeate the islet. β cell necrosis is seen (grade 3). H and E staining. All figures approximately × 600.

TABLE I

Prevalence of Diabetes and Insulitis in NOD, B10, and (NOD × B10)F_1 Mice

Strain	Sex	Pancreas histology					Diabetes	
		Number observed	Histology score				*n*	Percent
			0	1	2	3		
			%					
NOD	F	7	0	0	0	100	78/108	72
NOD	M	9	0	0	0	100	30/76	40
B10	F	11	91	9	0	0	0/50	0
B10	M	5	100	0	0	0	0/50	0
F_1	F	54	67	31	2	0	0/100	0
F_1	M	10	90	10	0	0	0/100	0

Ages of mice exceeded 7 mo at the time of analysis. The severity of pancreatic inflammation was scored as described in the Materials and Methods section. The 16 NOD mice histologically examined were not diabetic.

TABLE II

Prevalence of Diabetes in (NOD × B10)F_2 and First and Second (F_1 × NOD) Backcross Mice

Strain	Sex	MHC type	Diabetes		Histology score (percent of total examined)			
			n	Percent	0	1	2	3
F_2	F	NOD	0/21	0	0	33	43	24
		NOD/b	0/27	0	26	22	37	15
		b	0/13	0	46	15	31	8
	M	NOD	0/10	0	20	40	40	0
		NOD/b	0/32	0	37	41	22	0
		b	0/12	0	67	25	0	8
First BC	F	NOD	4/32	12.5	6	16	25	53
		NOD/b	0/29	0	21	31	34	14
	M	NOD	1/20	5	30	15	20	35
		NOD/b	0/31	0	45	23	26	6
Second BC	F	NOD*	41/84	48.8	13	0	7	80
		NOD/b	1/21	4.8	19	19	38	24
	M	NOD*	16/79	20.3	0	0	0	100
		NOD/b	0/16	0	25	50	12.5	12.5
Third BC	F	NOD	8/11	72.7	0	0	9	91
		NOD/b	0/27	0	8	15	44	33

Mice exhibited diabetes or were >7 mo old at the time of analysis.

* All mice were histologically examined, except those in these groups in which 15 of 84 second-backcross NOD MHC homozygous females and 10 of 79 males were examined.

is controlled by an incompletely dominant gene that appears to have low penetrance in the F_1.

Segregation of Diabetes and Insulitis in the (NOD × B10)F_2 Generation. Because of the known association of the MHC with type I diabetes both in man (1–7) and the BB rat (21, 22), spleen cells from members of the (NOD × B10)F_2 generation were typed at the *I-A* region. As described in Table II, none of the 61 female (<1.6%) or 54 male (<1.9%) F_2 mice became diabetic by 7 mo of age. These

data are consistent with the hypothesis that overt diabetes is controlled by more than one gene in the NOD (see complete analysis in Table III). In contrast to the lack of diabetes, most F_2 mice displayed pancreatic inflammatory lesions including mild to severe insulitis. This high frequency of mononuclear cell infiltration in the F_2 suggests that pancreatic inflammation and insulitis are controlled by a single dominant or incompletely dominant gene. These data obtained from the F_2 generation indicate that fewer genes are required for the expression of pancreatic inflammation and insulitis as compared with the expression of diabetes.

In female F_2 mice, grade 2 or 3 insulitis was observed in 67% of mice homozygous for the NOD MHC, 52% of NOD/B10 MHC heterozygotes, and 39% of B10 MHC homozygotes. A similar pattern of insulitis was observed in male F_2 mice, since 40% of NOD MHC homozygotes, 22% of NOD/B10 MHC heterozygotes, and 8% of B10 MHC homozygotes had lymphocytic infiltration associated with islets. Although the MHC appeared to influence the development of insulitis in the F_2 generation, the differences in the incidences of insulitis in both the female and male MHC homozygous and heterozygous groups were not statistically significant. Of particular interest is the appearance of islet-associated inflammation in B10 MHC homozygotes, which suggests that insulitis in the NOD mouse does not require a gene linked to the NOD MHC.

Segregation of Diabetes and Insulitis in the [(NOD × B10)F_1 × NOD] First-Backcross Generation. In the first backcross of (NOD × B10)F_1 mice to NOD mice, 4 of 61 females and 1 of 51 males were overtly diabetic by 7 mo of age (Table II). This low incidence of diabetes in the first-backcross generation strongly suggests that multiple genes or gene complexes control the development of diabetes in the NOD. It is likely that one of the genes necessary for the development of diabetes is linked to the NOD MHC since 5 of 52 NOD MHC homozygotes became diabetic as compared with 0 of 60 NOD/B10 MHC heterozygotes ($p < 0.05$).

Insulitis was observed in both NOD MHC homozygotes and NOD/B10 MHC heterozygotes in the first-backcross generation. Insulitis among first-backcross females was more frequent in NOD MHC homozygotes (78%) as compared with NOD/B10 MHC heterozygotes (48%) ($p < 0.05$). In addition, fewer female heterozygotes (14%) exhibited grade 3 inflammation as compared with homozygotes (53%) ($p < 0.005$). Similarly, severe insulitis (grade 3) among first-backcross male mice was observed in 35% of the NOD MHC homozygotes and in only 6% of the NOD/B10 MHC heterozygotes ($p < 0.025$). Thus, the MHC appears to influence both the incidence and severity of insulitis in the first-backcross generation since two doses of the NOD MHC allowed for a more aggressive autoimmune response than that observed in NOD/B10 MHC heterozygotes.

Diabetes and Insulitis in the [(NOD × B10)F_1 × NOD] Second-Backcross Generation. In the second-backcross generation, a large increase in the number of mice becoming diabetic was observed (Table II). Among the backcross mice that were homozygous for the NOD MHC, 49% of the females and 20% of males became diabetic, approximately half the incidence observed in the NOD parental strain. The influence of sex on the development of diabetes in the NOD strain

was evident in the second-backcross generation since there was a significant difference in the incidence of diabetes in the two sexes ($p < 0.001$).

One of 21 (<5%) NOD/B10 MHC heterozygous females became diabetic, demonstrating that although MHC heterozygosity protects most animals from diabetes ($p < 0.001$), NOD MHC homozygosity is not obligatory for the development of diabetes. Alternatively, a crossover may have occurred on chromosome 17 between the *I-A* region and the MHC-linked gene essential for the expression of diabetes. We favor the former possibility since 24% of the NOD/B10 MHC heterozygous females in the second backcross exhibited grade 3 insulitis, indicating that a vigorous immune response can occur in the MHC heterozygotes.

Insulitis in both male and female second-backcross mice was greatly influenced by the MHC. 80% of female NOD MHC homozygotes and 100% of the males displayed severe insulitis (score of 3), as compared with only 24% of female ($p < 0.005$) and 12.5% of male ($p < 0.001$) MHC heterozygotes. However, as mentioned above, NOD MHC homozygosity increased the incidence of severe insulitis over that observed in the NOD/B10 MHC heterozygotes but was not obligatory for the development of inflammatory lesions.

Diabetes and Insulitis in the Third-Backcross Generation. In the third-backcross generation, analysis of diabetes and insulitis was limited to females (Table II). The prevalence of diabetes in NOD MHC homozygous third-backcross mice (73%, $n = 11$) reached that of parental NOD female mice (72%, Table I). In addition, 91% of the homozygotes received a score of 3 for pancreatic histology. In contrast, all mice that were heterozygous at the MHC failed to develop diabetes ($n = 27$), confirming the previous conclusion that NOD MHC homozygosity is usually required for the expression of diabetes ($p < 0.001$). Although 77% of the MHC heterozygotes developed insulitis, only 33% developed grade 3 insulitis, which is much lower than the 91% incidence of severe insulitis observed in the NOD MHC homozygotes ($p < 0.005$). Thus, as seen in the first- and second-backcross generations, the development of diabetes depended on NOD MHC homozygosity whereas the initiation of insulitis did not. Since >70% of the NOD homozygotes became diabetic and nearly all scored a 3 in pancreatic histology, the data strongly suggest that permissive alleles are present at the non–MHC-linked diabetogenic loci in most of the third-backcross mice, including the MHC heterozygotes. Therefore, we can conclude that the presence of a non-NOD MHC haplotype in heterozygotes at the MHC attenuates the pancreatic inflammatory process such that most heterozygotes do not develop the severe insulitis that ultimately results in widespread β cell destruction observed of NOD MHC homozygotes.

Estimate of the Minimum Number of Genes Required for the Expression of Diabetes in the NOD. The development of diabetes in the NOD is clearly linked to one known genetic locus, the MHC (Table II). This locus acts primarily in a recessive fashion since only 1 of the 71 first-, second-, and third-backcross male and female mice becoming diabetic in this study was heterozygous at the MHC (1 of 124 MHC heterozygotes, 70 of 226 MHC homozygotes; $p < 0.0001$). The recessive nature of the MHC-linked diabetogenic gene is derived from the ability of NOD MHC homozygotes to develop both a high incidence and severity of insulitis, whereas NOD/B10 MHC heterozygotes exhibit less severe abnormalities.

TABLE III

Estimation of the Number of Genes Required for the Expression of Diabetes in the NOD

Strain	Sex	MHC type	Overt diabetes		χ^2
			Actual	Expected	
F_2	F	NOD	0/21	0.92/21	ND*
		NOD/b	0/27		
		b	0/13		
	M	NOD	0/10	0.25/10	ND*
		NOD/b	0/32		
		b	0/12		
First BC	F	NOD	4/32	5.60/32	0.554
		NOD/b	0/29		
	M	NOD	1/20	2.00/20	0.556
		NOD/b	0/31		
Second BC	F	NOD	41/84	32.9/84	3.278
		NOD/b	1/21		
	M	NOD	16/79	17.7/79	0.210
		NOD/b	0/16		
Third BC	F	NOD	8/11	5.9/11	1.612
		NOD/b	0/16		

Expected values have been calculated assuming that two recessive genes in addition to NOD MHC homozygosity are needed to produce overt diabetes and that the diabetes phenotype has a penetrance of 0.7 in females and 0.4 in males. Goodness of fit was assessed using the χ^2 test. For all χ^2 values determined, $p > 0.05$, which indicates that expected and observed values were not significantly different.

* The expected values in the F_2 generation were too small for χ^2 analysis.

Since NOD MHC homozygosity appears to be nearly essential for the expression of diabetes, the incidence of overt diabetes was subjected to χ^2 analysis only in the NOD MHC homozygotes of the first-, second-, and third-backcross generations (Table III); expected values in the F_2 generation were too small for valid analysis. In determining the expected values in this analysis, it was assumed that diabetes had a 0.7 penetrance in females and 0.4 in males. Penetrance values were based on the disease incidence in the NOD parental strain of 72% for females and 40% for males (Table I). No significant differences were found between observed and expected incidences of diabetes in the first-, second-, and third-backcross generations when it was assumed that two recessive unlinked genes were necessary for the development of diabetes in NOD MHC homozygotes. Although a χ^2 value was not determined for the F_2 generation, the lack of diabetes in the F_2 is consistent with the hypothesis that two recessive diabetogenic genes in addition to the MHC control diabetes. Significant differences between observed and expected incidences of diabetes were observed in the backcross generations when it was assumed that 1 or 3 recessive genes controlled the development of diabetes in NOD MHC homozygotes (calculations not shown).

Based on this analysis, we hypothesize that three functionally recessive genes or gene complexes control the development of diabetes, one linked to the MHC of the NOD and two that are not linked to the MHC. Although there may in

TABLE IV

Segregation of Severe (Grade 3) Insulitis and Diabetes in NOD MHC Homozygous Females

Generation	Mice with grade 3 insulitis		Expected*		Grade 3 mice with diabetes		Expected‡	
	n	Percent	*n*	Percent	*n*	Percent	*n*	Percent
F_2	5/21	24	5.25/21	25	0/5	<20	0.88/5§	17.6
BC1	17/32	53	16/32	50	4/17	23.5	5.95/17	35.0
BC2	67/84	80	63/84	75	41/67	61.2	35.2/67	52.5
BC3	10/11	91	9.6/11	87.5	8/10	80.0	6.1/10	61.0

* Expected values for grade 3 insulitis were calculated assuming that a single recessive gene controlled this phenotype. Goodness of fit was determined using the χ^2 test. All χ^2 values were <0.889, $p > 0.25$.

‡ Expected values for mice that expressed grade 3 insulitis to become diabetic were calculated assuming that a single recessive gene controlled this phenotype. Penetrance of the diabetes phenotype was assumed to be 0.7. All χ^2 values were <2.01, $p > 0.10$.

§ The expected value in the F_2 generation was too small for χ^2 analysis.

fact be a more complex genetic control of diabetes in the NOD, the three-recessive-genes hypothesis is the simplest model consistent with the data obtained. In addition, our hypothesis represents a minimal estimate of the number of genes involved since the analysis would not detect additional dominant genes or genes linked to those observed in this study.

Analysis of Genes Influencing the Development of Insulitis and Diabetes. From our analysis of the backcross generations we found that the MHC significantly influenced both the incidence and severity of insulitis (Table II). The animals in each generation expressing the most severe levels of insulitis were female NOD MHC homozygotes. Since the penetrance of the gene controlling the insulitis phenotype appeared to be the greatest in this group (assumed to be 100% in the following analysis), the genetic control of insulitis was studied in this subset of mice (Table IV).

Analysis of female NOD MHC homozygotes for grade 3 insulitis revealed that 24% of F_2, 53% of BC1, 80% of BC2, and 91% of BC3 mice expressed this phenotype. χ^2 analysis of these data showed no significant difference between observed and expected frequencies of grade 3 insulitis when it was assumed that two doses of the NOD-derived allele were required for the development of severe insulitis. Ideal expected values for grade 3 insulitis using these assumptions are 25, 50, 75, and 87.5% for the F_2, BC1, BC2, and BC3 generations, respectively. Whereas two doses of the NOD-derived allele appear to determine the development of severe insulitis, the presence of one dose of the permissive allele allows for a less severe autoimmune response in the pancreas (Table II). Therefore, this gene that influences the development of insulitis appears to be incompletely dominant and is most likely one of the non–MHC-linked, functionally recessive genes required for the expression of diabetes discussed in the previous section. It is possible that this gene which controls severe insulitis is the same incompletely dominant gene discussed earlier in the Results section that controls the initiation of pancreatic inflammation in the NOD.

Since severe insulitis is required for the development of diabetes we considered whether all mice with severe insulitis become diabetic or only a subset of such mice develop diabetes. Therefore, NOD MHC homozygous female mice express-

ing severe (grade 3) insulitis in the F_2 and backcross generations were assessed for the prevalence of overt diabetes. The percentage of mice expressing grade 3 insulitis that had become diabetic was <20% in F_2, 23.5% in BC1, 61.2% in BC2, and 80.0% in BC3 mice. Assuming a penetrance for diabetes of 0.7, as in the parental female NOD, and that a single recessive gene controls the development of diabetes in mice expressing severe insulitis, the expected values for diabetes are 17.6%, 35.0%, 52.5%, and 61.0% for the F_2, BC1, BC2, and BC3 generations, respectively. By χ^2 analysis, no significant differences were found between the expected and observed frequencies of diabetes in the backcross generations. The expected values were too small for χ^2 analysis of the F_2 generation data. Thus, these data support the hypothesis that a recessive gene is important in the development of overt diabetes from severe insulitis.

Lack of Diabetes and Insulitis in the (NOD × NZB)F_1. Since diabetes is not observed in F_1 mice bred from the NOD and normal mouse strains such as B10 (the current study), B6 (13), C3H (27), or NON (28), F_1 progeny were produced with the NZB, a murine strain that exhibits autoimmune disorders, in an attempt to determine if diabetes and insulitis are recessive even in the presence of autoimmune alleles contributed by the NZB. It has been demonstrated (33) that the NZB mouse spontaneously develops autoimmune hemolytic anemia, immune complex glomerulonephritis, and abnormal proliferation of B lymphocytes. In addition, mononuclear cell infiltrates have been identified (34) in the lung, liver, kidney, salivary gland, mesentery, and pancreas of adult NZB mice.

Overt diabetes was not found in any of the 75 (NOD × NZB)F_1 mice that were observed for a period of 1 yr. Analysis of pancreata examined from 7 female NZB and 25 female (NOD × NZB)F_1 mice that were >7 mo old revealed that 6 of 7 NZB and 24 of 25 (NOD × NZB)F_1 mice displayed inflammatory lesions. The histology observed in the F_1 resembled that of the NZB parent since mononuclear cell infiltrates were found in the exocrine parenchyma rather than in the islets as seen in the NOD. The pattern of inflammation observed has been previously described by Seemayer and Colle (34) and was distinct from that found in NOD mice. Thus, even when the NOD is crossed with another autoimmune strain such as the NZB, it appears that severe insulitis, which culminates in nearly complete β cell destruction and diabetes, is a recessive phenotype.

Discussion

In this study we have presented data that are consistent with the hypothesis that a minimum of three functionally recessive genes or gene complexes control the development of diabetes in the NOD mouse. One of these three diabetogenic genes was shown to be linked to the MHC region of the NOD mouse. In contrast to the three-gene control for overt diabetes, insulitis appears to be determined by a single, incompletely dominant gene or gene complex that is not linked to the MHC. Since the development of diabetes in the NOD is always preceded by the appearance of severe insulitis and β cell destruction, our data suggest that two of the three functionally recessive diabetogenic genes are accounted for by a non–MHC-linked, incompletely dominant gene controlling the development of severe insulitis and an MHC-linked gene that modulates the incidence and severity of this insulitis. The third diabetogenic gene appears to act after the

development of severe insulitis and may control a protective suppressor T cell response to the autoimmune process. The incompletely dominant gene observed in F_1 and F_2 animals that controls the initiation of pancreatic inflammation and insulitis is likely to be identical to the incompletely dominant gene responsible for the development of severe insulitis in NOD MHC homozygotes in the F_2 and backcross generations. We are currently fixing the gene that appears to initiate insulitis onto the B10 background to address this question.

In our analysis of the segregation of the diabetes and insulitis phenotypes, it became apparent that many animals did not express insulitis but did show abnormal pancreatic inflammatory lesions that resembled those observed in young NOD mice. This was particularly apparent in F_2 mice and in MHC heterozygotes in the backcross generations in which animals displayed histological abnormalities in their pancreata ranging from mild periductular infiltrates to severe insulitis (Table II). It is likely that this wide range of abnormalities results from the same genetically controlled initiating event and that other genes (such as the MHC) influence the progression of the autoimmune response. In addition, this histological evidence indicates the the genetic ability to develop insulitis is not equivalent to the genetic ability to develop diabetes. Additional genes apparently determine whether the insulitis proceeds to a point at which most β cells are destroyed and diabetes results.

A surprising result in light of the extensive pancreatic inflammation and insulitis observed in the F_2 generation was the low incidence of these abnormalities in the (NOD × B10)F_1. Only 33% of female and 10% of male (NOD × B10)F_1 mice displayed pancreatic lymphocytic infiltration (Table I) as compared with 74% of female ($p < 0.005$) and 63% of male ($p < 0.025$) F_2 mice heterozygous at the MHC (Table II). These data suggest that additional loci influence the penetrance of the gene that initiates pancreatic inflammation and insulitis.

The single exception to recessive MHC-linked genetic control of diabetes was observed in the second-backcross generation in which an $H\text{-}2^{NOD}/H\text{-}2^{b}$ heterozygote became diabetic (Table II). Either this heterozygote represents a crossover event between the NOD *I-A* region and the MHC-linked NOD diabetogenic gene, or this animal demonstrates that in rare cases the severe insulitis observed in some MHC heterozygotes can proceed to overt diabetes. We favor the latter possibility since it is likely that the MHC-linked gene controlling diabetes is *I-A*. We have recently discovered (Wicker, L. S. and B. J. Miller, unpublished observations) a diabetic fifth-backcross female mouse that was heterozygous at both the *K* and *I-A* regions. An analysis of the prevalence of diabetes in the progeny of this animal will support only one of the above hypotheses. A high incidence of diabetes in the MHC heterozygous progeny of this diabetic MHC heterozygous female will suggest a crossover event, whereas a low incidence will support the hypothesis that in rare cases MHC heterozygotes can become diabetic.

The functions of the individual diabetogenic genes in the NOD that have been identified in this analysis remain to be defined. The stimulus that initiates pancreatic lymphocytic infiltration does not appear to come from the external environment since diabetes is observed in gnotobiotic NOD mice (Wicker, L. S.,

and B. J. Miller, unpublished observations). The reaction to the antigenic event(s) appears to be controlled by an incompletely dominant, non–MHC-linked gene.

After the initiating event responsible for pancreatic inflammation, a gene (or genes) linked to the MHC influences the progression of the pancreatic inflammation to insulitis. It has been suggested (35, 36) that antigen-presenting Ia molecules, which are encoded by the *I-A* region in the MHC, influence the immune response by determining whether helper or suppressor cells will dominate in response to a given antigen. It is possible that helper cells dominate in backcross animals that are homozygous for the NOD MHC, whereas suppressor cells are dominant in MHC heterozygotes.

It is also within this balance of help and suppression that the non–MHC-linked recessive gene controlling the development of overt diabetes from insulitis in the NOD (Table IV) might function. This gene may be important in setting the response level of the immune system to the presentation of foreign or self antigens by I-A gene products. For example, in the immune response to the defined protein hen egg-white lysozyme (HEL), most $H\text{-}2^b$ strains are nonresponders due to the induction of suppressor cells (37). However, the BALB.B, which is $H\text{-}2^b$, responds to HEL. Genetic analysis of recombinant inbred lines has defined a non–MHC-linked gene, *Ir-2* on chromosome 2, which appears to tip the balance of the immune response from suppression to help (38, 39).

In addition to the three diabetogenic genes or gene complexes that were defined in this study, there is also an effect of sex on the development of diabetes since twofold more female NOD and NOD MHC homozygous backcross mice become diabetic as compared with males. This sex difference appears to be caused by the differential effects of male and female sex hormones on the immune system with male hormones possibly augmenting the suppressor arm of the immune response (40, 41). The importance of immune suppression in the NOD mouse has been suggested by studies (42) in which treatment with cyclophosphamide (which is thought to preferentially eliminate suppressor T cells) induced diabetes. Thus it is tempting to postulate that a balance of suppression and help exists within NOD mice. When suppressor cells are partially eliminated or impaired by cyclophosphamide treatment this balance shifts in favor of helper functions that may in turn induce increased activity of cytotoxic effector cells.

Our study is in agreement with the recent reports by Hattori et al. (27) and Leiter et al. (28) demonstrating the importance of the MHC in the development of overt diabetes in the NOD. Hattori et al. (27) analyzed F_2 and first-backcross mice of the (NOD × C3H) cross. Since the C3H strain expresses an Ia antigen encoded by the *I-E* region, it was possible that the failure of MHC heterozygotes to become diabetic was due to the presence of an I-E product. Our study demonstrates that this is unlikely since when NOD is crossed with B10, a strain that does not express an I-E product, $H\text{-}2^{NOD}/H\text{-}2^b$ heterozygotes still fail to become diabetic. Hattori et al. (27) indicated that their data were consistent with the hypothesis that two or more diabetogenic genes, in addition to an MHC-linked recessive gene, are required for the expression of diabetes. However, histological data describing the segregation of insulitis were not presented in their study.

Our data underscore the importance of MHC-linked and non–MHC-linked diabetogenic genes in the NOD model of diabetes. The specific initiating events

of the autoimmune insulitis as well as the mechanisms of action of the diabetogenic genes that control the progression of insulitis to diabetes remain to be elucidated.

Summary

Genetic analysis of the development of diabetes and insulitis has been performed in the nonobese diabetic (NOD) mouse strain, a model of insulin-dependent (type I) diabetes mellitus. (NOD × C57BL/10)F_1, F_2, and (F_1 × NOD) first-, second-, and third-backcross generations were studied. The data obtained were consistent with the hypothesis that diabetes is controlled by at least three functionally recessive diabetogenic genes, or gene complexes, one of which is linked to the MHC of the NOD. In contrast, pancreatic inflammation leading to insulitis was found to be controlled by a single incompletely dominant gene.

One of the two diabetogenic loci that is not linked to the MHC appears to be essential for the development of severe insulitis. This diabetogenic gene may be identical to the gene that controls the initiation of the autoimmune response that progresses to insulitis. Although this gene appears to be functionally recessive in its control of diabetes, it is incompletely dominant in its control of insulitis. The MHC-linked diabetogenic gene, although not required for the development of insulitis, apparently influences the progression of the autoimmune response since NOD MHC homozygotes in the backcross generations displayed the highest incidence and most severe cases of insulitis. Interestingly, we have found two MHC heterozygous backcross females that have become diabetic, suggesting that either the MHC-linked diabetogenic gene is not strictly recessive or that a recombination event has occurred between the diabetogenic gene and the *K* or *I-A* regions of the MHC. The third diabetogenic locus appears to influence the progression of severe insulitis to overt diabetes. In animals homozygous at this locus, diabetes may result from a decreased ability to develop a protective suppressor response to the autoimmune process.

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