Major Histocompatibility Complex Class I–Deficient NOD-*B2m*^{null} Mice Are Diabetes and Insulitis Resistant

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Specific allelic combinations within the class II region of the major histocompatability complex (MHC) represent a major genetic component for susceptibility to autoimmune insulin-dependent diabetes mellitus (IDDM) in humans (1,2). We produced and used a stock of NOD/Lt mice congenic for a functionally inactivated β2-microglobulin (*B2m*^{null}) locus to assess whether there was an absolute requirement for MHC class I expression and/or CD8$^+$ T-cells in diabetogenesis. These NOD-*B2m*^{null} mice do not express cell surface MHC class I molecules or produce detectable levels of CD8$^+$ T-cells and are diabetes and insulitis resistant. Previous results from transgenic mouse models indicated that intracellular accumulation of MHC class I molecules negatively affects pancreatic β-cell function and can result in the development of nonautoimmune insulin-dependent diabetes mellitus (IDDM). MHC class I molecules have been shown to accumulate intracellularly in the presence of a disrupted *B2m* locus, but this mutation does not negatively affect plasma insulin levels in either NOD/Lt mice or in those of a mixed 129 and C57BL/6 genetic background. Interestingly, 14% of the male mice in this mixed background did develop hyperinsulinemia (>1,500 pM) independent of the disrupted *B2m* locus, suggesting that these mice could conceivably develop insulin-resistant diabetes. However, none of these mice became diabetic at up to 22 months of age. Thus, elimination of cell surface MHC class I expression with a disrupted *B2m* gene blocks autoimmune diabetes in NOD/Lt mice, without engendering a separate, distinct form of glucose intolerance. *Diabetes* 43:505–509, 1994

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HLA, human leukocyte antigen; MHC, major histocompatibility complex; *B2m*, β2-microglobulin; IDDM, insulin-dependent diabetes mellitus; PBL, peripheral blood leukocytes; PCR, polymerase chain reaction; FACS, fluorescence-activated cell sorter; APC, antigen-presenting cell.

Specific combinations of HLA-DQ and HLA-DR (human leukocyte antigen) alleles within the major histocompatibility complex (MHC) class II region represent a major component of susceptibility to autoimmune insulin-dependent diabetes mellitus (IDDM) in humans (1–2). Similarly, genes within the MHC class II region of the unusual *H-2*^{g7} haplotype (K^d, I-A^{g7}, I-E^{null}, D^b) clearly represent a major genetic component for susceptibilty of NOD mice to T-cell–mediated autoimmune diabetes (3). Because a major component of diabetes susceptibility maps to the MHC class II region of *H-2*^{g7}, it is not surprising that CD4$^+$ T-cells contribute to autoimmune β-cell destruction in NOD mice (4). However, it remains controversial whether β-cell destruction can be initiated by CD4$^+$ T-cells alone, because results from other studies indicate that the common class I alleles (e.g., K^d and D^b) of the *H-2*^{g7} haplotype also may play a diabetogenic role in NOD mice by mediating the selection and targeting of β-cell autoreactive CD8$^+$ T-cells (5–7).

β2-microglobulin (*B2m*) is required for the transport of MHC class I molecules to the cell surface (8). Chimeric mice derived from embryonic stem cells in which the *B2m* locus was functionally inactivated by homologous recombination do not express MHC class I molecules on the cell surface, but instead accumulate them intracellularly (9–10). Hence, these mice are unable to positively select MHC class I–restricted CD8$^+$ T-cells (11–12). Faustman et al. (13) have reported that such mice with a mixed 129 and C57BL/6 genetic background develop autoimmune diabetes, and that NOD mice are characterized by diminished levels of constituitive MHC class I expression on splenocytes. Hence, it was proposed that "MHC class I ablation, in of itself," is sufficient to induce a loss of immunological tolerance to β-cells (14). To provide an unambiguous means for determining the role of MHC class I expression and CD8$^+$ T-cells in the

pathogenesis of autoimmune IDDM, we developed a stock of NOD mice congenic for the functionally inactivated *B2m* locus (designated here as NOD-*B2m*^{null}) previously described by Koller and Smithes (9). This stock was used to determine whether the autoimmune diabetic syndrome characteristic of NOD/Lt mice could develop in the absence of MHC class I expression and β-cell autoreactive CD8$^+$ T-cells. However, studies using transgenic mice have demonstrated that excessive intracellular accumulation of MHC class I molecules in pancreatic β-cells can result in sequestering of peptides involved in insulin synthesis or secretion and subsequently lead to development of nonautoimmune IDDM (15). This syndrome could be reversed if the β-cells were made to coexpress a *B2m* transgene (16). Thus, we also monitored plasma insulin levels in NOD-*B2m*^{null} mice and *B2m*^{null} mice on a mixed 129 and C57BL/6 genetic background to determine whether an inability to transport MHC class I molecules to cell surfaces impairs insulin dynamics in such a way that nonautoimmune diabetes could develop in either of these stocks.

RESEARCH DESIGN AND METHODS

Development of NOD-*B2m*^{null} mice. NOD/Lt mice have been maintained in our research colony by brother-sister mating since being obtained from Dr. George Eisenbarth (Joslin Diabetes Center, Boston, MA) at the 32nd generation of inbreeding. Currently, diabetes develops in 85% of female and 37% of male NOD/Lt mice by 30 weeks of age. A stock of mice on a mixed 129/Ola and C57BL/6 genetic background, in which the *B2m* locus on chromosome 2 has been functionally inactivated by insertion of a neomycin resistance gene (*neo*^{r}) into the second exon (9), was generously provided by Drs. Beverly Koller and Oliver Smithes (University of North Carolina, Chapel Hill, NC). The disrupted *B2m* locus (officially designated *B2m*^{tm1Unc}) from these mice was backcrossed to the NOD/Lt background for the indicated number of generations. To identify heterozygous breeders in each backcross generation, DNA isolated from peripheral blood leukocytes (PBL) was typed for the disrupted *B2m* locus by polymerase chain reaction (PCR) using the oligonucleotide primer set 5′-GCTATTCGGCTATGACTGGG-3′ and 5′-GAAGGCGATAGAAGGCGATG-3′, which generates a 706–base pair product from within the *neo*^{r} insert. Each of 35 amplification cycles on a 9,600 Thermal Cycler (Perkin-Elmer/Cetus, Norwalk, CT) consisted of denaturation at 94°C for 10 s, primer annealing at 60°C for 10 s, and extension at 72°C for 80 s. At the indicated backcross generations, mice identified as *B2m*^{null}/*B2m*$^+$ heterozygotes by PCR were intercrossed to generate F1 and F2 segregants that were homozygous for the intact or disrupted *B2m* locus. PBL from the segregants also were typed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA) for MHC class I expression with the rat monoclonal M1/42 that recognizes all mouse class I alleles, while the presence of CD8$^+$ and CD4$^+$ T-cells was assessed with the rat monoclonal antibodies 53–6.72 and GK-1.5, respectively. To assure that the diabetogenic *H-2*^{g7} haplotype was fixed to homozygosity in *B2m* deficient and intact segregants, PBL were typed by fluorescence-activated cell sorter (FACS) with the monoclonal antibody M5/114. This antibody recognizes the I-A^b MHC class II gene product common to both the 129/Ola and C57BL/6 genetic backgrounds, but does not recognize the unusual I-A^{g7} gene product of NOD (17). After the 10th backcross generation, the NOD-*B2m*^{null} congenic stock will be made available as a genetic resource from the Jackson Laboratory.

Assessment of diabetes and insulitis development. Female mice with an intact or disrupted *B2m* locus were maintained under specific pathogen-free conditions with unlimited access to food (diet 96W, Emory Morse, Guilford, CT) and acidified drinking water and monitored weekly for the development of glycosuria with Tes-Tape (supplied by Lilly, Indianapolis, IN). Mice were diagnosed as diabetic when glycosuria was ≥3 and plasma glucose concentrations were >16.7 mM as measured with Glucose Analyzer II (Beckman, Palo Alto, CA). As indicated, pancreases from *B2m* intact and deficient segregants that remained normoglycemic were fixed in Bouin's solution, sectioned at three nonoverlapping levels, and stained with aldehyde fuchsin for histological analysis of insulitis development. Islets (at least 25/mouse) were individually scored as follows: 0, no lesions; 1, islet associated with perivascular, periductal leukoctic infiltrates only; 2, <25% islet destruction; 3, >25% islet destruction; 4, complete islet destruction. An insulitis score for each mouse was obtained by dividing the total score for each pancreas by the number of islets examined. Data are represented as the mean insulitis score ± SE for the number of mice examined in each segregation class.

Plasma insulin determinations. Plasma insulin concentrations of the indicated mice were determined using a rodent insulin radioimmunoassay kit (Linco, St. Louis, MO) using rat insulin as a standard.

RESULTS

NOD-*B2m*^{null} mice do not express cell surface MHC class I, produce CD8$^+$ T-cells, and are diabetes resistant. At the N5 and N8 generations of backcrossing to the NOD/Lt genetic background, mice that were homozygous for the diabetogenic *H-2*^{g7} haplotype and heterozygous for the disrupted *B2m* locus were intercrossed. As expected, ~25% of F1 segregants generated from the N5 (9 of 35) and N8 intercross (3 of 10) did not express MHC class I molecules on PBL or produce detectable levels of CD8$^+$ T-cells (Fig. 1). These mice were scored as homozygous for the disrupted *B2m* locus. The remaining F1 segregants expressed MHC class I at high levels on PBL and generated CD8$^+$ T-cells (Fig. 1). MHC class I–expressing F1 segregants were scored as homozygous for the intact wild type *B2m* locus if the *neo*^{r} insert was not detected by PCR. F1 segregants that contained the *neo*^{r} insert but continued to express MHC class I on PBL were scored as *B2m*^{null}/*B2m*$^+$ heterozygotes. CD4$^+$ T-cells were present in mice from all three groups regardless of the presence or absence of MHC class I expression (Fig. 1).

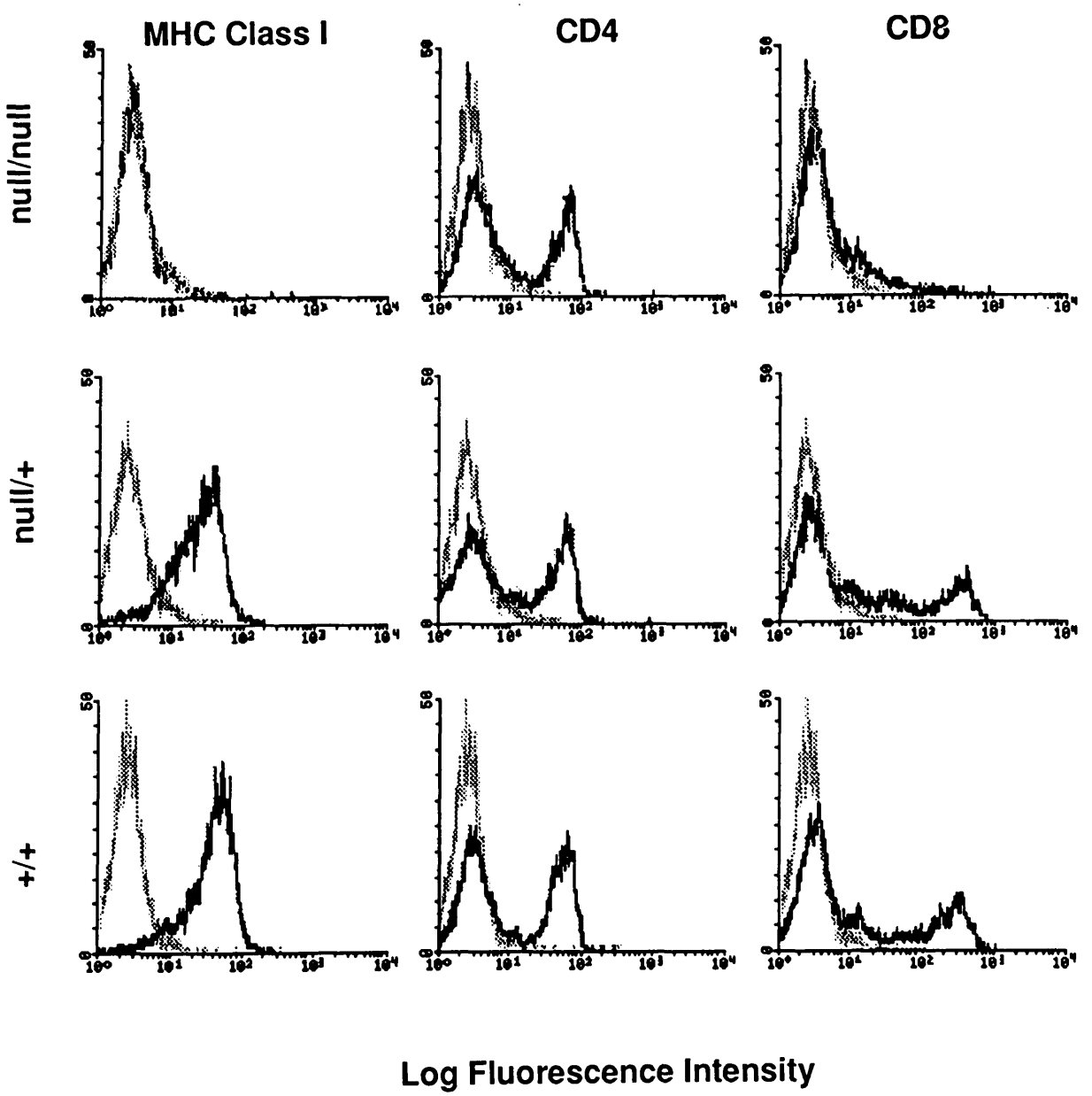

FIG. 1. NOD-*B2m*^{null} mice fail to express cell surface MHC class I or generate detectable levels of CD8$^+$ T-cells, but continue to produce CD4$^+$ T-cells. PBL from N8 segregants either homozygous (null/null) or heterozygous (null/+) for the disrupted *B2m* locus and from segregants homozygous for the intact *B2m* locus (+/+) were typed by FACS for MHC class I expression and presence of CD4$^+$ and CD8$^+$ T-cells with the fluorescein isothiocyanate–conjugated monoclonal antibodies described in METHODS. Light gray depicts background staining; black represents specific antibody staining.

F1 segregants in the N5 and N8 generations typed as homozygous for either the wild type or disrupted *B2m* locus were both intercrossed to produce MHC class I expressing and nonexpressing F2 mice. The MHC class I expressing and nonexpressing F1 and F2 female mice were monitored for diabetes development. This analysis clearly demonstrated that diabetogenesis in NOD mice requires cell surface MHC class I expression and/or CD8$^+$ T-cells. As shown in Table 1, 11 of 15 of the MHC class I–expressing segregants in the N8 generation became diabetic by 4 months of age. In contrast, 0 of 8 of the non–class I–expressing N8 segregants became diabetic over the same period of time. The incidence of diabetes in MHC class I–expressing females in the N8 generation (73.3%) was equivalent to that of standard NOD/Lt females. This indicated that by the N8 generation, most if not all of the non-MHC linked polygenes required for the development of T-cell–mediated autoimmune diabetes had been fixed as NOD type in the NOD-*B2m*^{null} congenic stock. The lower incidence of overt diabetes in MHC class I–expressing segregants from the N5 generation (18%) indicated that multiple non-MHC linked susceptibility modifiers were still segregating. However, a diabetogenic requirement for the combination of MHC class I expression and CD8$^+$ T-cells could still be demonstrated in the N5 females that remained normoglycemic, because significant levels of insulitis could be detected in class I–expressing seg-

TABLE 1
Autoimmune diabetes fails to develop in the absence of MHC class I and $CD8^+$ T-cells

	MHC class I positive		MHC class I negative	
	Diabetic (%)	Insulitis score (*n*)	Diabetic (%)	Insulitis score (*n*)
N5-F1, F2 females	3 of 17 (17.6)	1.62 ± 0.46 (10)	0 of 10 (0)	0.12 ± 0.04 (5)*
N8-F1, F2 females	11 of 15 (73.3)	—	0 of 8 (0)†	—
Total	14 of 32 (43.8)	—	0 of 18 (0)†	—

Data are means ± SE for insulitis score. MHC class I–expressing and nonexpressing segregants in the N5 and N8 generation were monitored for diabetes development through 40 and 16 weeks of age, respectively.
*$P < 0.035$ significantly less by Student's *t* test than MHC class I–expressing segregants at 40 weeks of age.
†$P < 0.005$ significantly less by χ^2 analysis than MHC class I–expressing segregants.

regants (mean insulitis score = 1.62 ± 0.46), whereas the non–class I–expressing segregants were virtually insulitis-free (mean insulitis score = 0.12 ± 0.04). Histological examination of islets from the insulitis-resistant NOD-*B2m*^{null} homozygotes revealed the presence of intact, well-granulated β-cells.

A disrupted *B2m* locus does not affect circulating insulin levels. To test the hypothesis that failure to transport MHC class I molecules to cell surfaces in NOD-*B2m*^{null} homozygotes might alter circulating insulin such that nonautoimmune IDDM could develop, we compared circulating plasma insulin levels in MHC class I–expressing and –nonexpressing segregants that were still normoglycemic between 14 and 40 weeks of age. Plasma insulin levels did not statistically differ between MHC class I–expressing (334.5 ± 46.5 pM) and –nonexpressing (481.5 ± 160.5 pM) female segregants (Table 2). Similarly, although mean levels were higher than in females, no significant difference in plasma insulin levels was observed between MHC class I–expressing (840.0 ± 157.5 pM) and –nonexpressing (948.0 ± 106.9 pM) male segregants.

Faustman et al. (13) have reported that homozygous *B2m*^{null} mice segregating for 129 and C57BL/6 background genes develop autoimmune IDDM. Therefore, we also monitored plasma insulin and glucose levels between 3 and 22 months of age in a stock of *B2m*^{null} homozygotes with a similar genetic background (9,11). None of these mice (9 females and 30 males) were hyperglycemic. However, plasma insulin levels of >1,500 pM were detected in 3 of 21 male mice tested at 3–4 months of age. Thus, the occasional development of hyperinsulinemia in this stock appears to be a function of genes in the mixed 129/Ola and C57BL/6 background, rather than resulting from the disrupted *B2m* locus and the absence of cell surface MHC class I expression. This conclusion is also supported by the data described above, demonstrating that plasma insulin levels in the NOD-*B2m*^{null} congenic stock are equivalent to that of MHC class I–expressing segregants with an intact *B2m* locus.

TABLE 2
Plasma insulin levels are not affected by lack of MHC class I expression

	Plasma insulin concentration (pM)	
	MHC class I positive (*n*)	MHC class I negative (*n*)
N5, N8 females	334.5 ± 46.5 (11)	481.0 ± 160.5 (5)
N5, N8 females	840.0 ± 157.5 (10)	948.0 ± 106.9 (8)

Data are means ± SE. Plasma was obtained from N5 segregants remaining normoglycemic at 40 weeks of age and from normoglycemic N8 segregants at 14–28 weeks of age. Student's *t* test revealed that plasma insulin levels did not differ significantly between class I–expressing and nonexpressing female ($P = 0.2$) or male ($P = 0.6$) segregants.

DISCUSSION

These results demonstrate conclusively that the development of autoimmune IDDM in NOD/Lt mice requires MHC class I expression and/or the presence of $CD8^+$ T-cells. This would support previous findings indicating that in addition to genes in the unusual class II region, the common MHC class I alleles (e.g., K^d and D^b) of the *H-2*^{g7} haplotype also contribute to diabetogenesis in NOD mice by mediating the selection and targeting of β-cell autoreactive $CD8^+$ T-cells. These include the findings that NOD islets express high constitutive levels of MHC class I (18–19); that autoreactive MHC class I–restricted $CD8^+$ T-cells can be isolated from NOD islets (20–21); that treatment of standard prediabetic NOD mice with anti-MHC class I monoclonal antibodies blocks the development of disease (22); and that both $CD4^+$ and $CD8^+$ T-cells must be transferred from prediabetic NOD donors to initiate β-cell destruction in T and B lymphocyte deficient NOD-*scid* recipients (7). Last, the incidence of diabetes is reduced in NOD mice congenic for the MHC haplotype of CTS, an NOD-related strain, which shares class II but not class I alleles with the *H-2*^{g7} haplotype (23).

Whereas diabetes and insulitis resistant NOD-*B2m*^{null} mice fail to express MHC class I molecules or generate $CD8^+$ T-cells, they produce equivalent numbers of $CD4^+$ T-cells as do the MHC class I–expressing and diabetes susceptible segregants. Thus, $CD4^+$ T-cells by themselves are unable to initiate autoimmune destruction of pancreatic β-cells in NOD mice. However, note that initiation of β-cell necrosis by a process requiring the presence of MHC class I–restricted $CD8^+$ T-cells results in the subsequent activation and amplification of a number of immunological effectors. These include $CD4^+$ T-cells that are able to mediate subsequent β-cell destruction in a non-MHC restricted fashion (4,7,24).

Although NOD mice do not develop autoimmune IDDM in the total absence of MHC class I expression, it cannot be excluded that aberrant regulation of MHC class I expression on antigen-presenting cells (APC) may contribute to diabetogenesis. In contrast to the report of Faustman et al. (13), we found previously that levels of MHC class I expression on unstimulated macrophages from NOD mice were equivalent or higher than that of macrophages from diabetes-resistant control strains (25). However, because of transcriptional factor defects, MHC class I expression is aberrantly downregulated in NOD macrophages, but not pancreatic β-cells, exposed to interferon-γ for 6 days (25). Conceivably, this could impair the ability of these APCs to mediate negative selection of autoreactive $CD8^+$ T-cells that could then be efficiently targeted to NOD β-cells expressing high levels of MHC class I (19).

The failure of otherwise genetically susceptible NOD/Lt mice to develop autoimmune diabetes when made MHC class I deficient with a disrupted *B2m* locus indicates that the hyperglycemia that reportedly develops in $B2m^{null}$ mice segregating for 129 and C57BL/6 background genes (13) is unlikely to be of an autoimmune eitiology. However, hyperinsulinemia does occasionally develop in mice from the mixed 129 and C57BL/6 genetic background independently of the disrupted *B2m* locus. Thus, it is possible that an occasional mouse in this stock develops hyperglycemia associated with insulin resistance rather than insulin dependence. However, hyperglycemia was not observed in any of these mice up to 22 months of age.

In conclusion, whereas the unusual MHC class II region of the $H\text{-}2^{g7}$ haplotype clearly contributes to the pathogenesis of autoimmune IDDM in NOD mice, the absence of disease in the NOD-$B2m^{null}$ congenic stock conclusively demonstrates that the initiation of β-cell destruction also requires cell surface expression of MHC class I molecules. Studies are currently being conducted to determine whether diabetogenesis is dependent on MHC class I expression on β-cells as well as on cell types involved in T-cell selection and activation. These studies may guide the analysis of similar epistatic interactions between class I and class II alleles in MHC haplotypes associated with diabetes development in humans.

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