A Mechanism for the Major Histocompatibility Complex–linked Resistance to Autoimmunity

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Summary

Certain major histocompatibility complex (MHC) class II haplotypes encode elements providing either susceptibility or dominant resistance to the development of spontaneous autoimmune diseases via mechanisms that remain undefined. Here we show that a pancreatic beta cell–reactive, I-A\(^{g7}\)–restricted, transgenic TCR that is highly diabetogenic in nonobese diabetic mice (H-2\(^{g7}\)) undergoes thymocyte negative selection in diabetes-resistant H-2\(^{g7/b}\), H-2\(^{g7/k}\), H-2\(^{g7/q}\), and H-2\(^{g7/nb1}\) NOD mice by engaging antidiabetogenic MHC class II molecules on thymic bone marrow–derived cells, independently of endogenous superantigens. Thymocyte deletion is complete in the presence of I-A\(^{b}\), I-A\(^{k}\), I-E\(^{k}\), or I-A\(^{nb1}\)/I-E\(^{nb1}\) molecules, partial in the presence of I-A\(^{q}\) or I-A\(^{k}\) molecules alone, and absent in the presence of I-A\(^{m}\) molecules. Mice that delete the transgenic TCR develop variable degrees of insulitis that correlate with the extent of thymocyte deletion, but are invariably resistant to diabetes development. These results provide an explanation as to how protective MHC class II genes carried on one haplotype can override the genetic susceptibility to an autoimmune disease provided by allelic MHC class II genes carried on a second haplotype.

Insulin-dependent diabetes mellitus (IDDM), a prototype of organ-specific autoimmune diseases, results from selective destruction of pancreatic beta cells by a T lymphocyte–dependent autoimmune process in genetically predisposed individuals (1). Genetic susceptibility and/or resistance to most autoimmune disorders, including IDDM, is associated with highly polymorphic genes of the MHC complex and, to a lesser extent, with polygenic modifiers on other chromosomes (2).

Population and animal studies have suggested that the MHC class II–linked susceptibility and resistance to IDDM are inherited as dominant traits with incomplete penetrance (2, 3). In humans, the MHC–associated IDDM susceptibility and resistance are predominantly, but not exclusively, determined by polymorphisms at the human leukocyte antigen (HLA) DQ\(\beta\)1 locus. Alleles encoding DQ\(\beta\) chains with serine, alanine, or valine at position 57 provide susceptibility, whereas those encoding DQ\(\beta\) chains with aspartic acid at position 57 provide resistance with differing degrees of dominance (1, 2). In mice, susceptibility and resistance to spontaneous IDDM are also linked to the MHC (H-2). The diabetes-prone nonobese diabetic (NOD) mouse, which spontaneously develops a form of diabetes closely resembling human IDDM, is homozygous for a unique H-2 haplotype (H-2\(^{g7}\)). This haplotype carries a nonproductive I-E\(\alpha\) gene and encodes an I-A\(^{g7}/\)I-A\(^{b}\) heterodimer in which the histidine and aspartic acid found at positions 56 and 57 in most murine I-A\(^{b}\) chains (the counterpart of human DQ\(\beta\) chains) are replaced by proline and serine, respectively (4, 5). Studies of congenic NOD mice expressing non-NOD MHC haplotypes, and of NOD mice expressing I-E\(\alpha\), modified I-A\(^{b}\), I-A\(^{k}\)/I-A\(^{k1}\), or I-A\(^{nb1}\) transgenes, have demonstrated that MHC class II molecules encoded by H-2 haplotypes derived from NOD or IDDM–resistant mice play a direct role in providing either susceptibility or resistance to spontaneous IDDM, respectively (6–18).

The precise mechanisms through which MHC genes provide autoimmune disease susceptibility and resistance, however, remain mysterious. MHC molecules are cell-surface receptors that present short fragments of self and for-
eign proteins to T lymphocytes and play a pivotal role in
instructing T lymphocytes maturing in the thymus how to
discriminate between self- and nonself-antigens (19, 20).
Thymocytes bearing TCR's capable of recognizing self-pep-
tide-MHC complexes with high affinity/avidity die or are
rendered unresponsive to antigenic stimulation (21–27). In
contrast, thymocytes bearing TCR's capable of engaging
self-peptide-MHC complexes with intermediate affinity/
avidity survive and are exported to the peripheral lymphoid
organs as cells capable of recognizing foreign antigens bound
to self-MHC molecules (25–30). On the basis of some of
this knowledge, a number of authors hypothesized that
MHC molecules providing resistance to autoimmune dis-
orders, i.e., by removing certain highly pathogenic au-

Materials and Methods

Generation of TCR Transgenes. The TCR-α and -β cDNAs of
NY.4.1 were cloned by anchored PCR and multiple recombi-
nants sequenced as described (34). The cDNAs were then ampli-
fied by PCR using primers containing L-V and J-C intron se-
quences, the corresponding splice donor and acceptor sites
and variable restriction sites, cloned into pBS-SK+ (Stratagene, La
Jolla, CA), and sequenced. Inserts with the expected sequences
were released from the vector by digestion with Clal and NotI
(TCR-β) or XhoI and NotI (TCR-α). The 4.1-VDβ sequence
was subcloned into a TCR-β shuttle vector carrying the endoge-

Thymocyte deletion or anergy of autoreactive T cells (8, 11, 31). Studies
instructing T lymphocytes maturing in the thymus how to
discriminate between self- and nonself-antigens (19, 20).

Contrary to what we expected, the thymocyte de-
letion in these mice was not mediated by endog-
enous superantigens, since it was absent in single-chain
hybrid mice lacking endogenous superantigens binding to
the transgenic TCR. In NOD mice, T cells expressing the
transgenic TCR underwent positive thymic selection and
triggered a dramatic acceleration of the onset of IDDM. In
certain F1 hybrid strains, however, the same cells under-
went negative selection and the mice did not develop
IDDM. Contrary to what we expected, the thymocyte
deletion and IDDM resistance observed in these mice coseg-
regated as a single locus trait with MHC haplotypes known
to provide dominant resistance to IDDM in MHC-trans-
gated as a single locus trait with MHC haplotypes known
and/or -congenic NOD mice (H-2k/b). H-2k/b-congenic NOD mice revealed that this highly
pathogenic TCR was also deleted in the presence of I-αq
and I-A/I-Enb1 molecules, which also provide dominant re-

NOD mice. Our unexpected results provide an explanation
as to how protective MHC class II molecules may provide
resistance to spontaneous T cell-mediated autoimmune dis-
orders, i.e., by removing certain highly pathogenic au-
toreactive T cells.

The studies presented here were initiated to test the hy-
pothesis that diabetes-resistant genetic backgrounds express
non-MHC-linked genetic elements other than endoge-
nous superantigens that are tolerogenic for diabeticogenic
T cells. To that end, we followed the fate of an NOD idet-
derived, beta cell–specific, I-Aqβ-restricted transgenic TCR
in diabetes-prone NOD mice, and in diabetes-resistant F1
hybrid mice lacking endogenous superantigens binding to
the transgenic TCR. In NOD mice, T cells expressing the
transgenic TCR underwent positive thymic selection and
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toreactive T cells.
A CD8+ T cell-depleted islet-derived CD4+ T cells from diabetic 4.1-NOD mice (5 × 10^6 cells/mouse) were transfused into the tail veins of sid-NOD/Lt (Jackson Laboratory) in 200 µl of PBS, pH 7.2. Transfused mice were followed for development of IDDM by monitoring blood glucose levels with Glucostix and a glucometer (Miles Canada, Etobicoke, Ontario). Mice were killed at IDDM onset for flow cytometry and immunopathological studies.

Bone Marrow Chimeras. Bone marrow chimeras were generated following standard protocols (36). In brief, bone marrow suspensions (5–10 × 10^6 cells) from donor mice (transgenic NOD or [N × B] F1 mice) were injected into the tail vein of recipient mice (nontransgenic NOD, [N × B] F1 or [N × S] F1 mice) treated with two doses of 500 rads 3 h apart from a 137Cs source (Gammaccell; Atomic Energy of Canada, Ottawa, Ontario). Chimeric mice were killed 5–6 wk after bone marrow transplantation.

Statistical Analyses. Statistical analyses were performed using Mann-Whitney U and χ2 tests.

Results

Generation of Beta Cell-specific, I-A^q-restricted TCR-αβ-transgenic NOD Mice. A CD4+ T cell clone (N Y 4.1) that was derived from pancreatic islets of a diabetic NOD mouse and that recognized a putative beta cell autoantigen in the context of I-A^q (37) was chosen as donor of the TCR transgenes. This T cell clone transcribed one functional TCR-β rearrangement, carrying Vβ11 and Jβ2.4 sequences and one functional TCR-α rearrangement, carrying a novel Vα gene (Vαx4.1) and the Jα33 element (These sequence data are available from EMBL/GenBank/DDJB under accession numbers U80816 and U80817). These TCR rearrangements were subcloned into genomic TCR-transgenic NOD mice, respectively.

In 90% CD4+ T cells from diabetic 4.1-NOD mice (5 × 10^6 cells/mouse) were transfused into the tail veins of sid-NOD/Lt (Jackson Laboratory) in 200 µl of PBS, pH 7.2. Transfused mice were followed for development of IDDM by monitoring blood glucose levels with Glucostix and a glucometer (Miles Canada, Etobicoke, Ontario). Mice were killed at IDDM onset for flow cytometry and immunopathological studies.

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Figure 1. Expression of the TCR-α/β transgenes in 4.1-NOD mice. (A and B) CD4, CD8, and Vβ11 profiles of thymocytes (A) and splenic cells (B) from transgenic and nontransgenic mice. (Top) CD4 versus CD8 dot plots of cell suspensions stained with anti–CD8–PE, anti–Vβ11–FITC, and anti–CD4–biotin plus Streptavidin-PerCP. (Bottom) Vβ11 fluorescence histograms of each T cell subset after electronic gating. Numbers indicate the average percentage of cells (top) or the average number of Vβ11+ cells (bottom) in each subset. Data correspond to 6–10 3–5-wk-old mice/group. DP, double-positive cells; DN, double-negative cells.

(C) In vitro proliferation of splenic CD4+ T cells in response to islet cells. Cultures of 2 × 10^4 splenic CD4+ T cells from TCR-α/β-transgenic, TCR-β-transgenic, and nontransgenic NOD mice were incubated with γ-irradiated NOD islet cells and splenocytes (10^5/well) for 3 d, pulsed with [3H]thymidine, and harvested. Bars show the standard error of the means.
α/β (as determined by staining with an anti-C β mAb) than CD4⁺CD8⁻ thymocytes from TCR-β-transgenic NOD mice (mean fluorescence intensities 59 ± 12 versus 31 ± 3, respectively; P < 0.001), suggesting early TCR-α chain expression (23, 38, 39). Second, thymocyte development in 4.1-NOD mice, but not TCR-β-transgenic NOD mice, was skewed towards the CD4⁺CD8⁻ subset (Fig. 1A), compatible with TCR-α transgene-dependent positive selection of 4.1-CD4⁺ thymocytes. Third, skewing of thymocytes into the CD4⁺CD8⁻ subset occurred in transgenic mice expressing the selecting I-A^77 molecule, but not in transgenic mice expressing only nonselecting I-A molecules (i.e., I-A^b, see below), as seen with other MHC class II-restricted TCR-α/β-transgenic models (38, 40). Fourth, 37 out of 37 4.1-NOD TCR-α cDNA sequences, generated from splenic CD4⁺T cell-derived RNA by anchored PCR, were TCR-α transgene-derived. Finally, splenic CD4⁺ T cells from 4.1-NOD, but not TCR-β-transgenic or nontransgenic, NOD mice proliferated in a dose-dependent manner in response to irradiated NOD islet cells (Fig. 1C). The islet cell–induced proliferation of 4.1- and control CD4⁺ T cells was quite variable between experiments, perhaps due to variability in the quality of the islet cell preparations; however, the differences within individual experiments were reproducible. Taken together, these results provide strong evidence that in 4.1-NOD mice, the 4.1-TCR specificity is expressed appropriately, and that, in the presence of the selecting I-A^77 molecule, 4.1-TCR-α/β transgene expression fosters the positive selection of beta cell–reactive CD4⁺ T cells.

To investigate the mechanisms underlying disease acceleration in 4.1-NOD mice, we then followed the progression of insulitis in prediabetic and diabetic mice. Histopathological studies of pancreata from 3- and 6-wk-old prediabetic 4.1-NOD mice showed that acceleration of diabetes in these mice was a result of faster progression, but not earlier onset, of islet inflammation (Fig. 2B). As expected, the insulitis lesions of diabetic 4.1-NOD mice contained more CD4⁺ and fewer CD8⁺ T cells (but similar numbers of B cells and macrophages not shown), than those of diabetic nontransgenic NOD mice (Fig. 2C). Islet-derived CD4⁺ T cells from 4.1-NOD mice expressed high levels of the transgene-encoded V₃¹⁺ chain (Fig. 2D), proliferated in response to NOD islet cells in vitro (Fig. 2E), and transcribed messenger RNA for IL-2 and IFN-γ, but not IL-4 (data not shown). These data indicate that these cells were transgenic, beta cell reactive, and of the Th1 type, as expected. Moreover, purified islet-derived CD4⁺T cells from three different diabetic 4.1-NOD mice were able to transfer IDDM into three different scid-NOD mice shortly after transfusion (36 ± 12 d) in the absence of CD8⁺ T cells in the inflamed islets (Fig. 2F). We thus conclude that expression of the 4.1-TCR in the NOD background promotes the selection of highly diabetogenic CD4⁺ Th1 cells and their accelerated recruitment into pancreatic islets, leading to massive beta cell destruction and IDDM within the first few weeks of life.

Thymocyte Deletion and Insulitis and IDDM Resistance in 4.1-F1 Hybrid Mice. The exquisite pathogenicity of the 4.1-TCR provided us with a powerful tool with which to test our initial hypothesis that diabetes-resistant backgrounds encode non-MHC-linked elements other than endogenous mouse mammary tumor virus superantigens (vSAgs) that are tolerogenic for diabetogenic T cells. To investigate this, we crossed 4.1-NOD mice (H-2⁰) with SJL/J (H-2s), C57BL/6 (H-2b), and C58/J mice (H-2k); F1 mice resulting from crosses of nontransgenic NOD mice with these strains express the diabetogenic I-A^77 molecule, but are diabetes-resistant, and do not delete V₃¹⁺ T cells (42). The lack of vSAg-mediated deletion of V₃¹⁺ or V₂⁺⁺ T cells in these backgrounds was confirmed by the fact that the thymocyte profiles of single-chain TCR-β or TCR-α-transgenic F1 mice and those of TCR-β- or TCR-α-transgenic NOD mice, respectively, were indistinguishable; as shown in Fig. 3, the percentages of thymic and splenic CD4⁺CD8⁻V₃¹⁺ cells in TCR-β-transgenic (Fig. 3A) or nontransgenic F1 mice (Fig. 3B) were virtually identical (if not greater than) those seen in TCR-β-transgenic or nontransgenic NOD mice, respectively.

The flow cytometric profiles of thymocytes (Fig. 4A) and splenocytes (Fig. 4B) from 4.1-(N × S)F1 mice (n = 8) were comparable to those seen in 4.1-NOD mice (Fig. 1), indicating that the 4.1-TCR specificity also underlies positive selection in H-2⁰/N mice. In contrast, all 4.1-(N × B)F1 mice (n = 22) and most 4.1-(N × C)F1 mice (n = 16/19) had only one-third to one-half the number of thymocytes seen in 4.1-NOD mice (and 4.1-(N × S)F1 mice), and displayed flow cytometric profiles of thymocytes and...
Figure 2. TCR-α/β-transgene expression and diabetogenesis. (A) Cumulative incidence of IDDM in female (25 transgenic and 114 nontransgenic) and male (15 transgenic and 59 nontransgenic) NOD mice. (B) Progression of insulitis in transgenic and nontransgenic NOD mice. Hematoxylin-eosin stained pancreatic sections of 3- and 6-wk-old mice (4–13 mice/age group) were scored for the degree of insulitis as described in Materials and Methods. (4
spleen sections were stained with anti-CD8 (53.6-7) or anti-CD4 (GK1.5) mAbs and FITC-labeled anti–rat IgG. Original magnification: 200. (C) Phenotype of idet-infiltrating T cells in transgenic (A) and nontransgenic (B) F1 hybrid mice. Data correspond to average values from 3–6 mice/group. T, thymocytes; S, splenocytes. *P < 0.02.

Figure 3. Absence of deletion of V\beta11-CD4+ T cells in TCR-\beta-transgenic (A) and nontransgenic (B) F1 hybrid mice. Data correspond to average values from 3–6 mice/group. T, thymocytes; S, splenocytes. *P < 0.02.
carrying these haplotypes results from deletion of diabeticogenic thymocytes.

I-A^b and I-A^x molecules as triggers of 4.1 T lymphocyte deletion. The data presented above suggested that the deletion of transgenic thymocytes and the IDDM resistance observed in 4.1-F1 and -F2 mice might be mediated by MHC class I and/or class II molecules encoded by the protective H-2 haplotypes. To determine whether 4.1 thymocyte deletion required the engagement of MHC class I molecules, we followed the maturation of 4.1 thymocytes in CD8-α- or β2m-deficient 4.1-(N × B) F1 mice (H-2^b/b), which either do not express the MHC class I-binding CD8

Figure 4. CD4, CD8, and Vβ11 profiles of thymocytes (A) and splenic cells (B) from transgenic F1 hybrid mice. See legend to Fig. 1 for details. Data shown are average values of 7–29 mice/group. In the text, transgenic NOD are referred to as 4.1-NOD; (NOD × SJL) F1-TG as 4.1-(N × SJL) F1; (NOD × B6) F1-TG as 4.1-(N × B) F1; and (NOD × C58) F1-TG as 4.1-(N × C) F1. When compared to 4.1-NOD mice, 4.1-(N × B) F1 and 4.1-(N × C) F1 mice had fewer CD4^+CD8^- thymocytes (P < 0.0002), fewer Vβ11^+CD4^+CD8^- thymocytes (P < 0.0002), and more CD4^+CD8^- thymocytes (P < 0.0002) (A). In the spleen (B), 4.1-NOD mice had more CD4^+ T cells (P < 0.0001) and more Vβ11^+CD4^+ T cells (P < 0.002) than 4.1-(N × B) F1 and 4.1-(N × C) F1 mice. All comparisons were done using the Mann-Whitney U test.
coreceptor on thymocytes, or lack MHC class I molecules, respectively. These mice deleted transgenic thymocytes as efficiently as wild-type 4.1-F1 mice (data not shown), thus indicating that deletion of transgenic thymocytes was not mediated by MHC class I molecules.

Since H-2^k/b mice do not express I-E molecules, we reasoned that deletion in these mice might be mediated by I-Ab. To test this notion, we followed the development of 4.1 thymocytes in I-A^b/b–deficient 4.1-(N^3B) F1 mice; except for the I-A^b/b mutation, I-A^b/b–deficient 4.1-(N^3B) F1 and 4.1-(N^3B) F1 mice are genetically identical. Selective abrogation of I-A^b/b expression in 4.1-(N^3B) F1 mice restored, at least in part, the positive selection of the transgenic TCR, as evidenced by (a) significant increases in the percentage of V_b11^+ thymocytes (Table 3), (b) significant increases in the ratio of CD4^+CD8^− to CD4^−CD8^+ T cells in the thymus and in the ratio of CD4^+ to CD8^+ T cells in the spleen (Table 3), (c) the reappearance of beta cell–reactive CD4^+ T cells in the spleen (Fig. 6, left), and (d) the reemergence of insulitis (Table 3). The overall positive selection of the 4.1-TCR in I-A^b/b–deficient 4.1-(N^3B) F1 mice, however, was less efficient than in 4.1-NOD mice; I-A^b/b–deficient 4.1-(N^3B) F1 mice had more CD4^+CD8^− thymocytes than, and half the splenic CD4^+ T cells of, age-matched (3–5-wk-old) 4.1-NOD mice (Table 3). Furthermore, the peripheral CD4^+ T cells of I-A^b/b–deficient 4.1-(N^3B) F1 mice proliferated less efficiently in response to NOD islet cells than the peripheral CD4^+ T cells of 4.1-NOD mice (Fig. 6, left). Finally, the insulitis lesions of I-A^b/b–deficient 4.1-(N^3B) F1 mice were milder than those seen in 4.1-NOD mice and did not lead to IDDM in any of the 15 mice that were followed (Table 3). Therefore, the I-A^b/b gene is not the only protective element present in (N^3B) F1 mice, but its expression is sufficient in and of itself to induce deletion of 4.1 thymocytes.

To investigate whether the H-2^k–dependent deletion of thymocytes in 4.1-(N^3C) F1 mice was also I-A mediated, we crossed 4.1-NOD mice with I-A^a/k/I-A^b/k (I-A^k)–transgenic NOD mice, to generate 4.1/I-A^k–NOD mice. Except for the presence of the I-A^k transgenes in 4.1/I-A^k–NOD mice, 4.1-NOD and 4.1/I-A^k–NOD mice have virtually identical genetic backgrounds. We found that 4.1-NOD mice had significantly more thymocytes (data not shown) and greater ratios of CD4^+CD8^− to CD4^−CD8^+ thymocytes and of CD4^+ to CD8^+ splenocytes than 4.1/
I-Ak–NOD mice (Table 3), results compatible with deletion of 4.1 thymocytes in 4.1/I-Ak–NOD mice. This deletion, however, was incomplete; unlike the splenic CD4+ T cells of deleting H-2b/c 4.1-F2 mice, the few CD4+ T cells that matured in 4.1/I-Ak–NOD mice expressed high levels of the transgenic TCR-β chain, and these cells proliferated in response to beta cells in vitro (Tables 2 and 3; and Fig. 6, middle). Furthermore, unlike deleting H-2b/c 4.1-F2 mice, 4.1/I-Ak–NOD mice developed moderate insulitis, suggesting that these cells were also responsive to antigen stimulation in vivo (Table 3). The insulitis lesions of these mice, however, were less severe than those of 4.1-NOD mice, and did not lead to diabetes in any of the nine mice that were followed (Table 3). It appears, then, that (a) deletion of thymocytes in H-2b/c 4.1 mice is triggered, at least in part, by I-Ak molecules, and (b) in addition to causing partial deletion of 4.1 thymocytes, I-Ak molecules may somewhat abrogate the diabetogenic potential of the 4.1 T cells that escape deletion.

Thymocyte Deletion in 4.1-NOD.H-2g7/q and 4.1-NOD.H-2g7/nb1 Mice. We next asked whether the 4.1-TCR could engage additional antidiabeticogenic MHC class II molecules during thymocyte development. We therefore crossed 4.1-NOD mice with NOD mice congenic for H-2q or H-2nb1 haplotypes, which provide dominant resistance to diabetes in nontransgenic NOD.H-2g7/q or nb1 mice (7, 15), and followed the fate of the 4.1-TCR in the TCR-transgenic offspring. As shown in Table 3 and Fig. 6 (right) 4.1-NOD.H-2g7/q and 4.1-NOD.H-2g7/nb1 mice had phenotypes compatible with partial or complete deletion of the 4.1-TCR, respectively. When compared to 4.1-NOD mice, 4.1-NOD.H-2g7/q mice had twofold fewer thymocytes (data not shown), increased percentages of CD4+CD8− thymocytes, and reduced percentages of thymic and splenic CD4+CD8− T cells (Table 3). The splenic CD4+ T cells of these mice proliferated in response to NOD islet cells in vitro, but did so less efficiently than those of 4.1-NOD mice (Fig. 6, right). Furthermore, these mice only developed very mild perinsulitis and never became diabetic (Table 3). In contrast, thymocyte deletion in 4.1-NOD.H-2g7/nb1 mice was complete; these mice had a three- to fourfold reduction in the absolute number of thymocytes (data not shown), dramatically increased percentages of CD4+CD8− thymocytes, and reduced percentages of thymic and splenic CD4+CD8− T cells (Table 3). These mice did not contain detectable beta cell–reactive CD4+ T cells in the spleen (Fig. 6, right) and developed neither diabetes nor insulitis (Table 3). Thymocyte deletion in these mice was not mediated by putative NOD vSAgs (i.e., mouse mammary tumor virus superantigen 17) binding to Vβ11 and H-2q or H-2nb1 MHC class II molecules, since the spleens of nontransgenic NOD.H-2g7/q and NOD.H-2b/c mice were not found to be insulin-free.

**Table 2.** Cosegregation of thymocyte deletion and resistance to insulitis and IDDM with H-2b and H-2k haplotypes in 4.1-transgenic mice.

<table>
<thead>
<tr>
<th>H-2</th>
<th>n</th>
<th>Delet</th>
<th>CD4+CD8− (Vβ11+)</th>
<th>CD4+CD8+</th>
<th>CD4−CD8−</th>
<th>CD4−CD8+</th>
<th>IDDM</th>
<th>Age at onset</th>
<th>Insulitis score ‡</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NOD × B6) F1</td>
<td>× NOD</td>
<td>H-2g7</td>
<td>15</td>
<td>−</td>
<td>31 ± 7a</td>
<td>44 ± 12</td>
<td>22 ± 7a</td>
<td>3 ± 1</td>
<td>7/15a</td>
<td>53 ± 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-2g7/b</td>
<td>16</td>
<td>+</td>
<td>15 ± 3b</td>
<td>49 ± 12</td>
<td>33 ± 10b</td>
<td>3 ± 1</td>
<td>0/16</td>
<td>−</td>
</tr>
<tr>
<td>(NOD × C58) F1</td>
<td>× NOD</td>
<td>H-2g7</td>
<td>9</td>
<td>−</td>
<td>35 ± 5c</td>
<td>43 ± 7</td>
<td>19 ± 4h</td>
<td>3 ± 2</td>
<td>5/9m</td>
<td>47 ± 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-2g7/k</td>
<td>6</td>
<td>-</td>
<td>35 ± 4d</td>
<td>45 ± 8</td>
<td>16 ± 6e</td>
<td>4 ± 2</td>
<td>0/6</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-2g7/k</td>
<td>9</td>
<td>+</td>
<td>17 ± 3e</td>
<td>45 ± 6</td>
<td>33 ± 6f</td>
<td>4 ± 3</td>
<td>0/9</td>
<td>−</td>
</tr>
</tbody>
</table>

*All mice were killed at IDDM onset or at 10 wk if nondiabetic. Flow cytometry was done as described in the legend to Fig. 1.

† Nondiabetic mice only.

Groups of mice include both male and female mice (~50% each). No differences in the incidence of IDDM nor in the degree of insulitis were noted between male and female mice within groups. Insulitis (15–30 i.eats/mouse) was scored as described in Maters and Methods.

a versus b,c versus e,d versus f, versus h; m versus j, versus l: P < 0.0002; k versus j: P < 0.0001; m versus n, versus o, s versus t: P < 0.002; p versus q: P < 0.0006; r versus s: P < 0.0007; t versus u: P < 0.01 (compared by χ²[t] and Mann-Whitney U test [t]).
Table 3. Influence of I-A<sup>a</sup>, I-A<sup>k</sup>, I-A<sup>q</sup>, and H<sup>2</sup>nb<sup>1</sup> MHC Class II Molecules on 4.1 Thymocyte Development

<table>
<thead>
<tr>
<th>n</th>
<th>Organ</th>
<th>Percentage of cells&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Ratios&lt;sup&gt;$&lt;/sup&gt;</th>
<th>IDDM&lt;sup&gt;$&lt;/sup&gt;</th>
<th>Insulitis&lt;sup&gt;$&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; (V&lt;sub&gt;b&lt;/sub&gt;11&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>x ± SD</td>
<td>CD4/&lt;sup&gt;+&lt;/sup&gt;DN</td>
<td>CD4/&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>NOD × B6 F1</td>
<td>29</td>
<td>T</td>
<td>15 ± 5&lt;sup&gt;*&lt;/sup&gt; (67 ± 7)</td>
<td>53 ± 10</td>
<td>27 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>12 ± 6 (78 ± 12)</td>
<td>-</td>
<td>84 ± 8</td>
</tr>
<tr>
<td>NOD × I-A&lt;sup&gt;a&lt;/sup&gt;-B6 F1</td>
<td>9</td>
<td>T</td>
<td>36 ± 5&lt;sup&gt;‡&lt;/sup&gt; (98 ± 3)</td>
<td>37 ± 9</td>
<td>23 ± 4&lt;sup&gt;p&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>12 ± 6 (86 ± 11)</td>
<td>-</td>
<td>86 ± 7</td>
</tr>
<tr>
<td>NOD</td>
<td>9</td>
<td>T</td>
<td>30 ± 7&lt;sup&gt;‡&lt;/sup&gt; (94 ± 2)&lt;sup&gt;v&lt;/sup&gt;</td>
<td>53 ± 9</td>
<td>13 ± 4&lt;sup&gt;p&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>24 ± 4&lt;sup&gt;‡&lt;/sup&gt; (88 ± 4)&lt;sup&gt;q&lt;/sup&gt;</td>
<td>-</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>NOD.H-2&lt;sup&gt;g&lt;/sup&gt;/I-A&lt;sup&gt;k&lt;/sup&gt;</td>
<td>7</td>
<td>T</td>
<td>25 ± 3 (93 ± 1)</td>
<td>49 ± 5</td>
<td>21 ± 3&lt;sup&gt;p&lt;/sup&gt;</td>
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<tr>
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<td></td>
<td>S</td>
<td>6 ± 1&lt;sup&gt;n&lt;/sup&gt; (90 ± 1)</td>
<td>-</td>
<td>92 ± 1</td>
</tr>
<tr>
<td>NOD.H-2&lt;sup&gt;g&lt;/sup&gt;/I-A&lt;sup&gt;q&lt;/sup&gt;</td>
<td>7</td>
<td>T</td>
<td>19 ± 3 (88 ± 3)</td>
<td>59 ± 5</td>
<td>19 ± 3</td>
</tr>
<tr>
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<td>S</td>
<td>11 ± 8 (67 ± 4)</td>
<td>-</td>
<td>86 ± 10</td>
</tr>
<tr>
<td>NOD.H-2&lt;sup&gt;g&lt;/sup&gt;/nb&lt;sup&gt;1&lt;/sup&gt;</td>
<td>11</td>
<td>T</td>
<td>11 ± 3&lt;sup&gt;n&lt;/sup&gt; (37 ± 9)</td>
<td>52 ± 7</td>
<td>34 ± 5&lt;sup&gt;p&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>6 ± 2&lt;sup&gt;n&lt;/sup&gt; (51 ± 16)&lt;sup&gt;n&lt;/sup&gt;</td>
<td>-</td>
<td>91 ± 4</td>
</tr>
</tbody>
</table>

Mice were studied at 3–5 (<sup>‡</sup>) or at 10 wk (<sup>§</sup>) of age. Flow cytometry was done as indicated in the legend to Fig. 1.

DN, double-negative cells (CD4<sup>+</sup>CD8<sup>+</sup>); T, thymus; S, spleen.

Statistics (Mann-Whitney U test): CD4<sup>+</sup>CD8<sup>+</sup>: a versus b, P < 0.0001; c versus f, P < 0.0001; e versus g, P < 0.0001; h versus f, P < 0.0001; i versus d, P < 0.0001; j versus f, P < 0.005; l versus p, P < 0.03; m versus f, P < 0.0002; n versus g, P < 0.0002; o versus p, P < 0.015; p versus q, P < 0.0002; q versus r, P < 0.0002; r versus s, P < 0.0002; s versus t, P < 0.0002; t versus u, P < 0.0002; u versus x, P < 0.0002; v versus z, P < 0.015; w versus u, P < 0.0002; x versus u, P < 0.0002; y versus z, P < 0.015; z versus aa, P < 0.002; ab versus aa, P < 0.0009; ac versus aa, P < 0.002; ad versus aa, P < 0.0002; IDDM: ad,ae,ah,ai,aj versus ag, P < 0.0001; Insulitis Score: al versus ak, P < 0.0006; ak versus am, P < 0.0005; al versus am, P < 0.005; an versus am, P < 0.03; ao versus am, P < 0.008; ap versus am, P < 0.006.
2g7/nb1 littermates harbored more Vβ11+ CD4+ T cells than nontransgenic NOD mice (20 ± 7 and 12 ± 2% versus 6 ± 1%, P < 0.008 and P < 0.04, respectively).

Thymocyte Deletion in H-2 Heterozygous 4.1 Mice Is Not Restricted by I-Ag7. To elucidate whether in vivo deletion of 4.1 thymocytes was “restricted” by I-Ag7 molecules, we next studied thymocyte maturation in 4.1 mice homozygous for either nonselecting/nondeleting (H-2s) or deleting (H-2b or H-2k) MHC haplotypes. As expected, 4.1 thymocytes underwent neither positive nor negative selection in H-2s 4.1 mice; these mice had significantly fewer CD4+CD8- thymocytes (but not more CD4-CD8+ thymocytes) than H-2g7/s 4.1 mice (compare Fig. 4, left and 7, left; P < 0.005). In contrast, the thymocyte profiles of H-2b and H-2k 4.1 mice were compatible with deletion; these mice had fewer thymocytes and greater percentages of CD4-CD8+ thymocytes than H-2s 4.1 mice (Fig. 7, middle and right). Therefore, unlike the positive selection of 4.1 T cells in selecting (I-Ag7+) mice, the I-Ab- and I-Ak-mediated deletion of 4.1 thymocytes in 4.1-F1 mice is not I-Ag7-restricted.

Thymocyte Deletion in 4.1-F1 Mice Is Preceded by Positive Selection and Is Mediated by Hematopoietic Cells. Previous studies have shown that the factors underlying the MHC-linked resistance to spontaneous IDDM predominantly reside in the bone marrow (17, 33, 44-48). To determine whether deletion of diabetogenic thymocytes in 4.1-F1 mice was mediated by hematopoietic cells or by thymic epithelial cells, we transfused bone marrow from deleting H-2g7/b- or selecting H-2g7-4.1 mice into lethally-irradiated nontransgenic NOD (H-2g7) or (N3B) F1 mice (H-2g7/b), respectively, and followed the fate of 4.1 thymocytes in the chimeras. As shown in Fig. 8, the mice that received marrow from H-2g7/b-4.1 mice (expressing I-Ab only on hematopoietic cells), but not those that received marrow from H-2g7-4.1 mice (expressing I-Ab only on thymic epithelial cells), had a phenotype compatible with deletion (low thymocyte CD4+CD8+/CD4-CD8+ ratios and small per-
percentages of V\(\beta\)11\(^+\)CD4\(^+\)CD8\(^-\) thymocytes. It thus appears that deletion of thymocytes in 4.1-F1 hybrid mice is preceded by their positive selection on radioresistant thymic epithelial cells, and is mediated by hematopoietic cells.

This finding raised one final question: if deletion is preceded by positive selection, why does it also occur in mice homozygous for the deleting H-2 haplotypes, which lack the selecting I-A\(^\beta\) molecule? We reasoned that the 4.1-TCR might actually perceive the deleting I-A molecules (i.e., I-Ab) expressed on thymic epithelial cells as selecting. To test this hypothesis, we followed the fate of 4.1 thymocytes arising from marrow of H-2\(^{a7}\)-4.1 mice in lethally irradiated H-2\(^{a7}\) mice, which express deleting (I-A\(^\beta\)) and nonselecting/nondeleting (I-A\(^\alpha\)), but not selecting (I-A\(^\alpha7\)), MHC class II molecules on thymic epithelial cells. As shown in Fig. 8 (right), the thymocyte profiles of recipient mice were indistinguishable from those of rats injected with H-2\(^{a7}\)-4.1 mice transplanted with marrow from H-2\(^{a7}\)-4.1 mice, which were nondeleting (Fig. 8, middle). It thus appears that in heterozygotes, the 4.1-TCR senses the I-A\(^\beta\) molecules expressed by thymic epithelial cells as selecting, rather than as deleting.

**Discussion**

The first striking observation of this study was the finding that positive selection of the 4.1-TCR in NOD mice caused a dramatic acceleration of the onset of IDDM (by ~3 mo). This was surprising to us considering the observations of Katz et al. in transgenic NOD mice expressing another beta cell–specific and I-A\(^\alpha7\)-restricted transgenic TCR (BDC-2.5) (32, 41). BDC-2.5 mice do not develop an accelerated onset of diabetes (32, 41) and, when housed under specific pathogen-free conditions (41), develop diabetes less frequently than 4.1-NOD mice (15 versus 74%). We are confident that beta cell destruction in 4.1-NOD mice was triggered and/or effected by CD4\(^+\) T cells expressing the 4.1-TCR since recombination activating gene 2–deficient 4.1-NOD mice, which cannot rearrange endogenous TCR genes, develop IDDM as early, and as frequently, as recombination activating gene-2 4.1-NOD mice (Verdaguer, J., D. Schmidt, B. Anderson, A. Amrani, and P. Santamaria, manuscript in preparation). Most surprising, however, was the observation that 4.1 thymocytes undergo deletion in diabetes-resistant H-2\(^{a7\beta}\)-, H-2\(^{a7k}\)–, H-2\(^{a7\alpha}\)–, and H-2\(^{a7\alpha\beta}\)– NOD mice by engaging anti-diabetogenic I-A (I-A\(^\alpha\), I-A\(^\beta\), I-A\(^\kappa\)) and, possibly, I-E molecules (I-E\(^\kappa\), I-E\(^\omega1\)) on thymic APCs, particularly since previous studies did not find evidence for deletion of autoreactive T cells in congenic or transgenic NOD mice expressing these MHC class II molecules (7, 15, 16, 32, 33, 44). This deletion was clearly not mediated by endogenous superantigens binding to V\(\beta\)11, since it was absent in TCR-\(\beta\)-transgenic and nontransgenic mice expressing the deleting MHC haplo-
was clearly more efficient than that observed in I-Ak–transgenic I-A\(b\) (N × B) F1 mice, which express the C57BL/6-derived I-A\(a\) gene, may be a result of engagement of I-\(\alpha\)b\(\beta\)1/I-A\(a\)\(\beta\) complexes by the 4.1-TCR with an affinity/avidity approaching the threshold for deletion, or perhaps, to other tolerogenic factors unique to the C57BL/6 background. Similarly, thymocyte deletion in H-2\(^{g7}\)k 4.1-F1 and -F2 mice, which express both I-A\(k\) and I-E\(k\) molecules, was clearly more efficient than that observed in I-A\(k\)–transgenic 4.1-NOD mice, raising the possibility that thymocyte deletion in the former also involves the engagement of I-E\(k\) and/or I-E\(\alpha\)\(\gamma\)/I-E\(\beta\)\(\beta\) molecules by the 4.1-TCR. An alternative, but not mutually exclusive, possibility is that the timing and levels of I-A\(k\) expression in I-A\(k\)–transgenic 4.1-NOD mice are different than the timing and levels of expression of endogenous I-A\(k\) molecules in H-2\(^{g7}\)k 4.1-F1 or -F2 mice. In this respect, it is worth noting that, unlike wild-type H-2\(^{k}\) mice, I-A\(^{k}\)-transgenic NOD mice do not express I-A\(k\) molecules on bone marrow cells (they do, however, express them on bone marrow–derived APCs), and that the thymic cortical epithelial cells of I-A\(^{k}\)-transgenic NOD mice express lower levels of I-A\(k\) than those of H-2\(^{k}\) mice (13). Whatever the explanation for these differences, the MHC-induced T cell tolerance in H-2\(^{g7}\)h 4.1-F1 and H-2\(^{g7}\kappa\) 4.1-F1 and -F2 mice was complete. Deletion of the 4.1-TCR was also observed in 4.1-NOD.H-2\(^{g7n}\)\(^{a1}\) mice (complete deletion), which express I-A\(^{n}\) and I-E\(^{n}\) molecules, and in 4.1-NOD.H-2\(^{g7q}\) mice (partial deletion), which express I-A\(^{q}\), but not I-E, molecules. It thus appears that, unlike many other MHC class II–restricted TCR specificities (32, 38, 40), the highly diabetogenic 4.1-TCR can engage several distinct MHC class II molecules in the thymus with totally different consequences, i.e., positive selection or dominant negative selection.

The extensive promiscuity of the 4.1-TCR raises a series of important questions. How can 4.1 thymocytes recognize so many different MHC class II molecules? Does the 4.1-TCR recognize each of these different molecules bound to specific peptides? Or to a common peptide? Or, does it bind to all of them regardless of the molecular nature of the bound peptides? Is there anything unique about the molecular structure of the deleting I-A molecule that makes them function as such? We do not yet have answers to these questions, but we have some clues. For example, we know that the diabetogenic 4.1-TCR is not a classic alloreactive TCR; the MHC molecules that mediate deletion of the 4.1-TCR when presented by thymic APCs did not trigger proliferation of naive and preactivated CD4\(^{+}\) T cells from 4.1-NOD mice when presented by peripheral APCs from deleting F1 backgrounds (our unpublished data). Since thymocyte tolerance is a more sensitive response than peripheral T cell activation (43), these results need not imply that the putative peptide–MHC class II complexes that mediate thymic deletion in our system are expressed solely by a specialized thymic APC subpopulation; they may also be present on peripheral APCs, but may not be able to trigger mature T cell proliferation. We also know that the I-\(\alpha\)B chains of all the deleting I-A molecules that we have tested so far (I-\(\alpha\)B, I-\(\alpha\)A, and I-\(\alpha\)q) share residues at positions 57 (aspartic acid; Asp) and 61 (tryptophan). Interestingly, I-E\(k\) and I-E\(\alpha\)\(\beta\)\(\beta\) molecules, which are encoded on the deleting H-2\(^{k}\) and H-2\(^{n}\)\(^{a1}\) haplotypes, respectively, have the same residues at these two positions (49). I-\(\alpha\)\(\beta\)\(d\), which is encoded on the deleting H-2\(^{n}\)\(^{a1}\) haplotype (but may or may not be engaged by the 4.1-TCR), is also Asp-57\(^{+}\) and, like I-\(\alpha\)B, I-\(\alpha\)A, and I-\(\alpha\)q, has a bulky hydrophobic residue at position 61 (phenylalanine). We are not certain as to whether the presence of aspartic acid at I-\(\beta\)3 and/or I-\(\alpha\)B position 57, which is not associated with human and murine IDDM (1, 2), is necessary to trigger deletion of the 4.1-TCR. However, we know it is not sufficient, since I-\(\alpha\)A is also Asp-57\(^{+}\), but it is nondeleting.

Whatever the specific residues involved, the unexpected promiscuity of the highly pathogenic 4.1-TCR raises the intriguing possibility that pathogenicity of autoreactive TCRs and their ability to cross-react with different MHC class II molecules are related phenomena. At the present time, it is difficult to envision how promiscuity may lead to pathogenicity. It is possible, however, that promiscuous autoreactive TCRs, like the 4.1-TCR, are primarily selected (positively and/or negatively) by reaction with MHC residues rather than with peptide residues, as recently proposed for alloreactive TCRs (50). Like the latter (50), these promiscuous autoreactive TCRs may then be able to engage a larger range of peripheral self-peptide–selecting MHC complexes above the affinity threshold required for mature T cell activation than nonpromiscuous autoreactive TCRs, resulting in increased chances for pathogenicity. This would predict that promiscuity would not be unusual among those autoreactive TCRs with the highest pathogenic potential (i.e., those that trigger diabetes), and that MHC molecules providing dominant resistance to a given autoimmune disease would do so predominantly by removing the most pathogenic autoreactive T cells rather than all autoreactive T cells, regardless of their pathogenicity (i.e., those recruited during amplification of the autoimmune response). This postulate would provide an explanation as to why the beta cell–reactive and I-\(\alpha\)\(\beta\)\(d\)-restricted BDC-2.5-TCR of Katz et al. (32), which does not accelerate IDDM onset in NOD mice (32, 41), did not undergo tolerance in H-2\(^{g7b}\) F1 mice or in I-E\(^{\text{tr}}\)-transgenic NOD mice (32). It would also account for the presence of mildly insulitogenic, but not diabetogenic, T cells in some congenic NOD.H-2\(^{g7}\), NOD.H-2\(^{g7q}\), or NOD.H-2\(^{g7n}\)\(^{a1}\) mice (7, 15, 16), I-A\(^{\text{d}}\)-transgenic NOD mice (18), NOD mice reconstituted with bone marrow from I-\(\text{E}^{-}\)-transgenic NOD mice (33), and I-A\(^{\text{A}}\)-transgenic NOD mice (12, 13).

In evaluating the relevance of our findings in 4.1 mice...
with respect to the MHC-linked susceptibility and resistance to spontaneous IDDM in non-TCR-transgenic mice, one should consider two additional aspects. The first aspect has to do with the pathological consequences of thymocyte selection in 4.1 mice. There was an absolute correlation between deletion of thymocytes in H-2\(g^{7/b}\) and H-2\(g^{7/k}\) 4.1-F2 mice and resistance to insulitis and diabetes; deleting offspring of the second backcross of 4.1-F1 mice to NOD mice never developed insulitis, whereas all their nondeleting littermates developed moderate to severe insulitis, and \(~50\%\) of those which inherited two H-2\(g^{7}\) haplotypes became diabetic (a significant percentage given the polygenic nature of murine IDDM; reference 2). The same was true for 4.1-NOD.H-2\(g^{7/a}\) and 4.1-NOD.H-2\(g^{7/nb1}\) mice, which developed mild periinsulitis or no insulitis, respectively, and did not become diabetic. It is noteworthy that the factors underlying the MHC-linked resistance to spontaneous IDDM predominantly reside in the bone marrow (17, 33, 44–48). Taken together, then, these data support the genetic susceptibility provided by MHC genes carried on one haplotype (e.g., I-Ab in mice and DQA1*0301/DQB1*0602 in humans) can over-ride the genetic susceptibility provided by MHC genes carried on a second haplotype (e.g., I-A\(g^{7}\) in mice and DQA1*0301/DQB1*0302 in humans).

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