Accelerated Diabetes in Rat Insulin Promoter–Tumor Necrosis Factor-α Transgenic Nonobese Diabetic Mice Lacking Major Histocompatibility Class II Molecules

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The major predisposing genetic component in type 1 diabetes maps to the major histocompatibility complex locus in both mice and humans. To verify the HLA class II association with disease pathogenesis, we adopted the transgenic approach. Expression of HLA-DQ8, the molecule showing the strongest association with human type 1 diabetes, in the diabetes-predisposing milieu of NOD mice in the absence of the endogenous class II molecule I-Ag7 did not render susceptibility to type 1 diabetes. To study if providing a local proinflammatory environment would lead to diabetes in these mice, AB.NOD.DQ8 were bred with C57BL/6 mice expressing tumor necrosis factor (TNF)-α in the β-cells of the islets of Langerhans. Surprisingly, although diabetes was evident in the F1 intercross expressing rat insulin promoter (RIP)-TNF, offspring lacking either endogenous or transgenic class II molecules developed accelerated diabetes with high frequency in both sexes. Moreover, expression of any functional class II molecule seemed to confer significant protection from diabetes in this model. Thus, neonatal expression of TNF-α in an islet-specific manner bypassed the requirement of CD4+ T-cells and resulted in diabetes that could be mediated by CD8+ T-cells. We also show for the first time that diabetes in NOD.RIP-TNF mice can occur independent of inheritance of NOD-derived idd1. Diabetes 52:342–347, 2003

Type 1 diabetes is an organ-specific autoimmune disease resulting from the interaction of several genetic elements and yet undetermined environmental factors. It is characterized by a predominately T-cell–mediated destruction of insulin-producing β-cells of the islets of Langerhans culminating in life-long insulin dependence. More than 18 genetic loci are implicated in the etiopathogenesis of type 1 diabetes, of which idd1 (in mice) or IDDM1 (in humans), which encompass the major histocompatibility complex (MHC) gene complex, has consistently shown the strongest association (1).

A significant majority of patients with type 1 diabetes express certain HLA class II molecules, such as HLA-DQ8/DR4 or DQ2/DR3 (2). However, the precise roles played by these MHC class II molecules in the pathogenesis of type 1 diabetes still remain speculative. Promiscuous peptide presentation by the disease-associated class II molecules resulting in improper negative selection of autoreactive T-cells remains as the favored hypothesis (3). A significant influence of class I molecules is also suspected because CD8+ T-cells also play a crucial role in the disease pathogenesis (4).

Much of the understanding of the immunopathogenesis of several autoimmune diseases, including diabetes, has been feasible only because of the availability of suitable animal models (5). Of the two models of spontaneous type 1 diabetes, NOD mice and BB rats, the former is widely used where the disease occurs with increased frequency and disease onset is earlier in females (6). The strong structural (7) and functional (8) similarity between the NOD class II molecule, I2-Ag7, and its human counterpart, HLA-DQ8, suggest a common etiological role. Spontaneous loss of tolerance to GAD65, one of the major autoantigens implicated in type 1 diabetes, in HLA-DQ8 transgenic mice on a diabetes-resistant B10 background lends support to this hypothesis (9,10). Although diabetes was seldom seen in these mice, mild insulitis in a small percentage of islets was a consistent finding (9). Absence of disease per se in the face of demonstrable autoreactivity in HLA-DQ8 transgenic mice indicated the requirement of additional factors/stimuli. Islet-specific expression of the costimulatory molecule B7 happens to be one such factor/stimulus, which can drive the nonpathogenic autoreactive T-cells to the pathogenic pathway in these mice (11,12). This effect was HLA-DQ8 specific because rat insulin promoter (RIP)-B7 mice transgenic for HLA-DQ6 were diabetes free (11). Even though HLA-DQ8 exhibited such strong pro-diabetogenic properties, transgenic expression of DQ8 in the diabetes-predisposing milieu of NOD mice lacking endogenous class II (I-Ag7) did not confer susceptibility to type 1 diabetes (13). Nevertheless, NOD mice transgenic for DQ8 were susceptible to spontaneous disease in the presence of I-Ag7, indicating that DQ8 is permissable to diabetes (14).

Similar to the situation where provision of local costimulation with transgenic B7 resulted in diabetes in mice expressing HLA-DQ8 (11), we envisaged that islet-specific expression of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α would also facilitate the study of
TABLE 1
List and genotypes of mice used in the present study

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<th>Aβ</th>
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B6.RIP-TNF mice were mated with Aβ⁺⁺.NOD.DQ8 (N10). The first generation offspring was intercrossed for two to three generations and was used in the study. Presence or absence of appropriate class I, class II, and RIP-TNF was determined by PCR and/or flow cytometry.

HLA-DQ8 association with type 1 diabetes. In this context, it is important to know that even though neonatal expression of TNF-α under the control of RIP resulted in extensive insulitis in diabetes-resistant B6 mice (15), it led to diabetes only in NOD mice (16), indicating that only the NOD genetic background is permissible for induction of diabetes by RIP-TNF (17). Therefore, in the present study, HLA-DQ8 transgenic mice on NOD background (Aβ⁺⁺.NOD.DQ8) were mated with the diabetes-resistant RIP-TNF mice on the B6 background.

RESEARCH DESIGN AND METHODS

Generation of HLA transgenic RIP-TNF mice. C57BL/6 mice expressing TNF-α in the β-cells of the islets of Langerhans under the control of RIP have been described earlier (15). Aβ⁺⁺.NOD.DQ8 mice, the 10th generation backcross between Aβ⁺⁺.HLA-DQA8 transgenic mice and NOD mice, homozygous for 15 NOD idd alleles (Aβ⁺⁺.NOD.DQ8), has been described elsewhere (13). B6.RIP-TNF and Aβ⁺⁺.NOD.DQ8 were mated to generate F1 offspring. Littermates obtained by intercrossing these first generation offspring were typed and used in the study. Independent segregation of genes would result in offspring with several different genotypes (Table 1). Presence or absence of appropriate MHC class I/class II molecules were determined by PCR and/or flow cytometry as described elsewhere (13). Presence of RIP-TNF was confirmed by PCR. Mice were bred and maintained at the Immunogenetics Mouse Colony at the Mayo Clinic (Rochester, MN).

Diabetes monitoring. The 4- to 6-week-old mice were moved from the barrier facility to the conventional facility after weaning, and their glycemic status was monitored weekly using a Glucometer (Bayer, Pittsburgh, PA). Diabetes was diagnosed when two consecutive random blood glucose levels were >13.9 mmol/l.

Histopathology and immunohistochemistry. Histopathology was performed as described earlier (13). For immunofluorescence staining, pancreata were embedded in an OCT compound (Tissue Tek; Sakura FineTek, Torrance, CA), immersed in chilled isopentane, and frozen immediately in liquid nitrogen. Thin sections made from frozen tissue were first air-dried, fixed in cold acetone, and subsequently treated with 1% paraformaldehyde. Sections were subsequently incubated with rat anti-mouse primary antibodies, CD4 (GK1.5), CD8 (Lyt 2.2), CD19 (Pharmingen, San Diego, CA), and CD11b (Pharmingen). A rat isotype control was also included. In the next step, sections were incubated with goat anti-rat secondary antibody (Jackson Immunoresearch Lab, West Grove, PA) followed by rabbit anti-goat IgG/fluorescein isothiocyanate conjugate (ICN, Costa Mesa, CA). Sections were examined under a fluorescent microscope.

Statistical analysis. The statistical analyses were performed using SAS software (Version 4.0.4, SAS Institute, Cary, NC).

RESULTS

NOD/B6.RIP-TNF mice develop diabetes, which is dependent on islet-specific expression of TNF. Previous studies have shown that the F1 intercrosses of B6.RIP-TNF mice and NOD mice seldom developed diabetes (15,17). This indicated that the presence of TNF did not overcome the dominant protective effects of B6-derived diabetes-resistant alleles when inducing diabetes in NOD F1 intercrosses. However, in the present study, diabetes developed in NOD/B6.RIP-TNF F1 intercrosses. Mice belonging to either sex were equally susceptible. However, diabetes only developed in littermates expressing TNF (males, 58/112, 49.15%; females, 38/106, 35.85%; overall, 96/218, 42.2%). Littermates lacking RIP-TNF were diabetes free during the entire observation period (males, 0/67; females, 0/69), confirming the prior reports that the presence of NOD background per se does not result in diabetes in NOD × B6 crosses (18). The possibility that transgenically expressed TNF could be directly toxic to the β-cells is unlikely, because if this were the case, the incidence of diabetes would have been similar in all the groups and would have occurred even in the B6.RIP-TNF founder line.

Diabetes in NOD/B6.RIP-TNF mice is independent of functional class II molecules. Aβ⁺⁺.NOD.DQ8 mice used for initial breeding lack endogenous class II molecules because the H2-αβ gene has been inactivated by gene targeting (19). The functional I-E molecule is also not expressed in these mice because of a natural mutation in the H2-Eα promoter region, which precludes the expression of the I-Eα chain (20). Therefore, these mice express only the transgenic HLA-DQ8. On the other hand, B6.RIP-TNF mice do express functional H2-A molecules. The littermates generated by crossing Aβ⁺⁺.NOD.DQ8 and B6.RIP-TNF can therefore express different combinations of class II molecules, as shown in Table 1, or may not express any class II molecules at all. Interestingly, littermates completely lacking any functional class II molecules were indeed susceptible to diabetes (males, 14/17, 82.35%; females, 12/18, 66.67%; overall, 26/35, 74.28%). The onset of diabetes was earliest in this group of mice, and the disease progressed rapidly. Most importantly, the incidence of disease was highest in this group (P < 0.001, χ² test; Fig. 1).

**FIG. 1.** Cumulative incidence of diabetes. Mice belonging to different groups were monitored weekly to assess their glycemic status. Diabetes was diagnosed when two consecutive random blood glucose levels were >13.9 mmol/l. Figures in parentheses indicate total number/group.

![Diagram](image-url)
1 and Table 2). However, diabetes in this group also depended on islet-specific expression of TNF because littermates lacking both class II and RIP-TNF were diabetes free (overall, 0/24; males, 0/7; females, 0/17). There were no significant differences in B220+ cells, macrophages, NK cells, or CD8+ T-cells in the secondary lymphoid organs between littermates of Aβ+.NOD.RIP-TNF mice with and without RIP-TNF, indicating that transgenic expression of TNF in the islets does not alter the development of immune components (data not shown). Similarly, there was no appreciable difference in these above parameters between euglycemic and hyperglycemic Aβ+.NOD.RIP-TNF mice (data not shown).

Expression of class II molecules downregulates diabetes. Compared with class II–deficient littermates, the incidence of diabetes in mice that expressed any single functional class II molecules (Aβ+/-,DQ8+.NOD.RIP.TNF+, Aβ+/-,DQ8-.NOD.RIP.TNF+, and Aβ+/+,DQ8-.NOD.RIP.TNF+) was lower. In this group, the onset of disease was delayed and the disease progression was slower; however, the incidence of disease did not differ significantly. There was also no significant difference in the incidence of diabetes in the HLA-DQ8+ mice that were homozygous or heterozygous for I-Aβ. Interestingly, mice homozygous for H2-Aβ and also expressing HLA-DQ8 (Aβ+/-,DQ8+.NOD.RIP.TNF+) had the lowest incidence of diabetes (P < 0.05 when compared with mice expressing any single class II molecule, and P < 0.001 when compared with mice devoid of all class II molecules; χ2 test; Fig. 1 and Table 2). This result indicated that the presence of functional class II molecules and therefore CD4+ T-cells downregulates disease incidence and severity in RIP-TNF mice. We also generated three lines of Aβ+.DQ8+.RIP.TNF+ mice lacking CD8, β2-microglobulin, or the classic class I molecules Kβ and Dβ. So far, the preliminary results indicate that these mice are diabetes free (data not shown). This result implies that CD8+ T-cells are most likely the effectors. We are currently generating these lines (CD8−/−, β2-microglobulin−/−, and KβDβ−/−) on the class II–deficient background to prove a similar role for CD8+ T-cells in class II–deficient RIP-TNF mice.

Put together, these results indicate that 1) diabetes in NOD.RIP-TNF mice can occur even in the absence of functional class II molecules and CD4+ T-cells, and 2) expression of functional class II molecules offers significant protection from diabetes.

Histopathology of islets. Infiltration of islets with mononuclear cells was evident as early as 3 weeks in RIP-TNF+ mice (Fig. 2), as shown in earlier studies (15,21), whereas the littermates lacking RIP-TNF did not show evidence of insulitis. The infiltrates were primarily composed of B-cells and macrophages. T-cells were also detected. No significant difference in the extent of insulitis or pattern of insulitis in different groups of mice based on the expres-
molecules, whereas CD8\(^+\) T-cells were present in islets from both groups (Fig. 3). B-cells and macrophages could be seen in islets from both groups; however, there seemed to be a difference in the distribution of B-cells and macrophages. Whereas the islets from mice expressing class II molecules had more macrophages, islets from mice lacking class II molecules had more B-cells (Fig. 3). Salivary glands, kidneys, and thyroid glands were devoid of any mononuclear cell infiltration (data not shown) as described previously (15).

**DISCUSSION**

During the normal course of diabetes in the NOD strain of mice, both CD4\(^+\) and CD8\(^+\) T-cells seem to be necessary for the development of diabetes (4). NOD mice deficient in MHC class I expression and thereby lacking CD8\(^+\) T-cells are protected from insulitis and type 1 diabetes (4). Similarly, NOD mice lacking the class II transactivator (CIITA\(^-\)), which have drastically reduced surface class II (therefore lacking CD4\(^+\) T-cells), show perinsulitis but are nevertheless protected from diabetes (22). However, CD8\(^+\) T-cells seem to be important in the early phase of the disease (4). NOD.RIP-TNF mice (with intact NOD idd1) lacking class I molecules (\(\beta_2\) microglobulin\(^-\)) were protected from diabetes (16). Susceptibility of RIP-TNF mice expressing class I molecules alone (without any class II) and the absence of disease in CD8 or \(\beta_2\) microglobulin null mice (data not shown) in the present study confirm the pathogenic role of CD8\(^+\) T-cells.

However, in an earlier study, abolishing the expression of class II molecules by mutating CIITA neither protected nor accelerated diabetes in NOD.RIP-TNF mice, implying that the class II molecules and CD4\(^+\) T-cells are not involved in disease pathogenesis in this model (22). Furthermore, residual class II expression and presence of CD4\(^+\) T-cells (albeit in reduced numbers) in CIITA\(^-\)/NOD.RIP-TNF mice (23) might complicate the conclusion drawn from CIITA\(^-\)/NOD.RIP-TNF mice. However, in the present study, the incidence of diabetes was significantly reduced in mice expressing any single competent class II molecule (H2-A\(b\) or DQ8) and was reduced more so when both class II molecules were present, indicating that expression of functional class II molecules (and therefore presence of CD4\(^+\) T-cells) is protective. The reason(s) is not clear as to why expression of H2-A\(b\) and DQ8 (as seen in the present study) protected RIP-TNF mice from diabetes, while H2-A\(a\) had no such modulatory role in an earlier study (16). Nevertheless, several nonmutually exclusive reasons could be put forth. It could be due to the anti-diabetogenic effects of the genes closely associated with H2-A\(b\) and the DQ8 transgene (positional effect), because both of these molecules are from a diabetes-resistant background. It could also be due to the intrinsic differences in class II molecules themselves. More importantly, we envisage a protective/regulatory role for CD4\(^+\)CD25\(^+\) T regulatory cells.

Several studies have shown that CD4\(^+\) T-cells expressing CD25, the IL-2R \(\alpha\)-chain, have an important immunoregulatory role (24). CD4\(^+\)CD25\(^+\) T regulatory cells have been shown to suppress the activity of both CD4\(^+\) and CD8\(^+\) T-cells. Because expression of class II molecules on the cortical epithelial cells seems to mediate the selection
of CD4+CD25+ immunoregulatory T-cells (25), these cells should therefore be absent in class II–deficient mice, and, hence, Aβ0.NOD.RIP-TNF mice have an increased incidence of disease. The role of CD4+CD25+ regulatory T-cells in spontaneous type 1 diabetes has been addressed earlier using the murine model of the disease (26). A recent human study has also shown that patients with type 1 diabetes also have low numbers of resting CD4+CD25+ T-cells (27). The recent demonstration by Green et al. (28) that CD4+CD25+ T-cells efficiently inhibit diabetes in RIP-B7.RIP-TNF double transgenic mice underscores the significance of these immunoregulatory T-cells in type 1 diabetes. However, a recent study has shown that absence of CD4+CD25+ T-cells per se is not sufficient to result in autoimmunity. Nevertheless, in an appropriate pro-inflammatory environment, deficiency of CD4+CD25+ T-cells can result in autoimmunity (29). The results of the present study correlate well with this hypothesis. Littermates lacking class II molecules (thereby CD4+CD25+ Treg cells) rarely developed type 1 diabetes in the absence of RIP-TNF, whereas they readily did so when TNF was expressed in the islets. In addition to this result, recent studies have shown that CD4+CD25+ T-cells also play a regulatory role (30–32). Thus, a low incidence of diabetes in class II–positive littermates could be due to several mutually nonexclusive mechanisms.

Even though neonatal expression of TNF-α in the islets of NOD mice can override the requirement for several of the NOD idd alleles, homozygosity for NOD alleles at the MHC locus (i.e., idd1) is mandatory for the development of accelerated diabetes in NOD.RIP-TNF mice (15,17). Interestingly, in the present study, diabetes was evident even in the F1 intercross of Aβ0.NOD.DQ8 and B6.RIP-TNF mice (both carrying the B6-derived disease-resistant idd1 allele because the inactivated H2-AB gene was also made originally on a diabetes-resistant background), indicating that the presence of NOD-derived idd1 is not mandatory. NOD idd1 consists of Kβ, Dβ, a nonexpressing H2-E, and H2-Aβ (18). In addition to the clear-cut involvement of NOD class II, class I molecules also seem to be important in the disease pathogenesis (4). Curiously, most, if not all, of the class I–restricted CD8+ T-cells/clones identified thus far from NOD mice seem to be restricted to Kβ (33–35). However, as evident from the present study, expression of Kβ is not always required, and diabetes can also occur in mice expressing Kβ and Dβ.

How TNF-α may cause/accelerate diabetes has been an area of active research. Green et al. (16,36) have shown that the local expression of TNF-α under the control of RIP resulted in early apoptosis of β-cells. The recent demonstration that TNF-receptor is indeed expressed in β-cells and is critical for the development of diabetes (37) supports the notion that TNF-mediated apoptosis of β-cells is a crucial event not only in the RIP-TNF model but also during the natural course of the disease, even in NOD mice (38). Apoptosis of β-cell recruits dendritic cells (DCs), which may then cross prime CD8+ T-cells independent of the CD40-CD40L pathway (15). A recent study based on a CD8+ T-cell–dependent diabetes model has also shown that in situ β-cell death can effectively prime CD8+ T-cells (39). In situ demonstration of DCs by immunochemical staining of affected islets underscores the importance of DCs in the pathogenesis (17).

Thus, in conclusion we have shown that accelerated diabetes in NOD.RIP-TNF mice is independent of the NOD allele at idd1 and is independent of the expression of functional class II molecules. Expression of class II is rather inhibitory to the development of diabetes. Expression of TNF in the pancreas thus seems to bypass the requirement of class II molecules for antigen presentation and might lead to a direct effector pathway in the context of class I and CD8 T-cells independent of CD4+ T-cells. This model can be useful to dissect roles of diabetogenic genes and class I/CD8 in the disease pathogenesis and to study therapeutic/preventive modalities.

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REFERENCES


