T Cell Receptor V Gene Usage of Islet β Cell–reactive T Cells Is Not Restricted in Non-Obese Diabetic Mice

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Summary

Five islet-reactive T cell clones were established from islet-infiltrating T cells of non-obese diabetic (NOD) mice. All clones expressed CD4, but not CD8, and responded to islet cells from various strains of mice in the context of I-A<sup>NOD</sup>. They could induce insulitis when transferred into disease-resistant I-E<sup>+</sup> transgenic NOD mice. The T cell receptor (TCR) sequences utilized by the clones were determined. Their usage of TCR V and J segments was not restricted but was rather diverse. One of the clones utilized V<sup>δ16</sup>. The expression of V<sup>δ16</sup> was significantly reduced in I-E<sup>+</sup> transgenic NOD, suggesting the possibility that the islet-reactive T cell clone expressing V<sup>δ16</sup> may be deleted or inactivated by I-E molecules. This clone might be one of the candidates that triggers insulitis.

The non-obese diabetic (NOD) mouse spontaneously develops insulin-dependent diabetes mellitus (IDDM) after T cell–mediated autoimmune insulitis (1–5). Several recessive genes are known to determine disease susceptibility (6, 7). One of these genes is closely linked to the MHC class II region characterized by the unique structure of I-A and the lack of I-E (6, 8). Recent work in I-A or I-E transgenic NOD mice (9–13) demonstrated that both the unique I-A<sup>NOD</sup> molecule and the lack of I-E expression are crucial in development of the autoimmune insulitis and diabetes. Therefore, the NOD mouse can be an ideal model to elucidate the role of MHC class II in generation of autoimmune T cells. Recently, a limited heterogeneity of TCR repertoire has been demonstrated in autoreactive T cells responsible for experimental autoimmune encephalomyelitis (EAE) (14, 15). However, it is still controversial whether the finding obtained from experimental autoimmune models is generalizable to spontaneous autoimmune diseases in mice and humans. In this study, CD4<sup>+</sup> islet-specific T cell clones were established from pancreatic islet-infiltrating cells of NOD mice, and the TCR sequences of these clones were determined. The usage of V and J gene segments was not restricted, but was diverse, which is in contrast to experimentally induced EAE. Some of T cell clones were distinguishable by bearing V<sup>β12</sup> and V<sup>δ16</sup> gene segments known to be deleted in I-E<sup>+</sup>-expressing mice. Their possible roles in the development of insulitis are discussed.

Materials and Methods

**Islet-reactive T Cell Clones.** Islet-reactive T cell clones were established from infiltrating T cells within islets isolated from 7–11-wk-old NOD mice. Isolated islets were cultured in RPMI 1640 containing 2% NOD serum for 1 wk in the presence of irradiated (3,000 rad) NOD spleen cells. During this period, most of the infiltrating cells died. Surviving cells were then cultured in RPMI 1640 containing 10% FCS and 20% culture supernatant of Con A-stimulated spleen cells, and the cells expanded were further stimulated with irradiated islet cells and NOD spleen cells without culture supernatant of spleen cells. These cell lines were cloned by limiting dilution and only the clones reactive against islet cells in the presence of NOD APC were selected. The reactivity of each T cell clone was assayed by stimulation of 10<sup>4</sup> rested T cells with 10<sup>4</sup> irradiated (3,000 rad) islet cells as antigens and 5 × 10<sup>5</sup> irradiated (3,000 rad) spleen cells as APC. A 72-h incubation, cells were pulsed with 0.5 μCi [H]Tdr for 12 h. [H]Tdr incorporation was measured by a liquid scintillation counter and is expressed as the mean of duplicate cultures.

**In Vivo Transfer of Islet-reactive T Cell Clones.** I-E<sup>+</sup> NOD mice (8–10 wk old, female) were sublethally irradiated (650 rad) and were transferred with 2–5 × 10<sup>6</sup> cells of islet-reactive T cell clones intravenously. After 2 wk, another injection with 2–5 × 10<sup>6</sup> cells of T cell clones was given, and recipient mice were killed 2 wk later after the second transfer for histological examination of pancreas.

**Isolation and Sequencing of TCR.** Total RNA was isolated from the cells of each clone using a standard (guanidine isothiocyanate/CsCl) method. Complementary DNA for each TCR α and β were synthesized from total RNA (0.3–1 μg) in a 25-μl reaction mixture containing 0.5 mM dNTPs, 200 U Mo-MLV reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD), 2 U human placenta ribonuclease inhibitor (Takara, Kyoto, Japan), and 100 pmol
Results and Discussion

We first analyzed TCR Vβ gene usage in peripheral T cells from NOD mice of various ages and in islet-infiltrating T cells by using available mAbs to Vβ gene products. The expression pattern of analyzed Vβ gene segments in peripheral T lymphocytes did not change before and after the onset of insulitis and diabetes (data not shown). The TCR Vβ gene usage in the locus of insulitis was almost comparable to that in peripheral T cells except that the proportion of Vβ3-expressing T cells was a little higher in infiltrating T cells than in peripheral T cells. For instance, in 15-wk-old NOD mice, mean percentages of Vβ3⁺, Vβ5⁺, Vβ6⁺, Vβ8⁺, Vβ9⁺, and Vβ11⁺ cells were 1.29, 2.89, 8.90, 28.9, 2.18, and 7.67% in islet infiltrating T cells, and 0.503, 3.17, 8.42, 29.3, 1.57, and 6.54% in inguinal and axillary lymph nodes, respectively. Although we do not know whether an increase of Vβ3⁺ cells in islet-infiltrating T cells is significant or not, the result indicates that islet-infiltrating T cells are rather heterogeneous in terms of Vβ gene usage.

However, the TCR repertoire of islet-infiltrating T cells may not necessarily reflect that of autoreactive T cells responsible for the development of insulitis. Islet-reactive T cell lines were therefore established by stimulation of infiltrating T cells with islet cells in the presence of irradiated NOD spleen cells as APC. All these T cell lines were found to express CD4 (data not shown) and were further cloned by limiting dilution. The specificities of these clones were then analyzed by using islet cells and APC from various strains of mice, including I-E expressing transgenic NOD mice that had previously been demonstrated not to develop insulitis (9, 10, 13). Fig. 1 shows the reactivity of a representative T cell clone, 4-1-L.6, to islet cells. This clone showed a strong reactivity to islet cells in the presence of splenocytes from NOD or I-E⁺ NOD as APC. Its inability to respond to islet antigens presented by APC from either BALB/c (H-2b) or C57BL/6 (H-2d) indicates that the clone is presumably restricted to class II I-A but not to class I, since the haplotypes of H-2K and -D of NOD are d and b, respectively. Furthermore, the α chain of I-A[NOD] is identical to that of BALB/c, indicating that the unique structure of AβNOD determines the I-A restriction of this clone. Islet cells from allogeneic mice such as BALB/c can also stimulate the clone in the presence of APC from NOD or I-E⁺ NOD. In addition, NOD insulinoma cells derived from a RIP-TAg-2 transgenic NOD mouse (16), which carries the SV40 large T antigen gene under the control of insulin promoter as a transgene, could also induce
Figure 2. Islet-reactive T cell clones can transfer insulitis into I-E-transgenic NOD mice. Pancreatic sections of an irradiated I-E" NOD mouse without transfer (a) or with transfer of an islet-reactive T cell clone, 4-1-G.4 (b), were shown. I-E" NOD mice (8-10 wk old, female) were sublethally irradiated (650 rad) and were transferred with $2-5 \times 10^6$ cells of the islet-reactive T cell clone intravenously. After 2 wk, another injection with $2-5 \times 10^6$ T cell clones was given, and recipient mice were killed 2 wk later after the second transfer.

proliferation of the clone (data not shown), indicating that it reacts to an antigenic determinant on \( \beta \) cells. The other T cell clones showed essentially the same reactive pattern (data not shown). The results indicate that all these T cell clones are reactive to \( \beta \) cell antigens in the context of NOD MHC class II (I\(^A\)\text{NOD}) but that \( \beta \) cell antigens stimulating the clones are not unique to the NOD mouse. It is also notable that APC from I-E" NOD mice can also present islet an-
tigens to these clones, suggesting that inhibition of insulitis in I-E\textsuperscript{+} NOD does not take place at the level of antigen presentation.

These T cell clones were tested for their ability to induce the insulitis. We used I-E\textsuperscript{+} NOD mice as the recipients since these mice never develop insulitis but have the identical genetic background to NOD except for the Eac\textsuperscript{d} transgene. These mice are also useful to determine whether any suppressive mechanisms in the effector phases are involved in I-E\textsuperscript{+} mediated prevention of insulitis. Each clone maintained under the stimulation of islet cells with NOD APC was expanded with IL-2 for a few days and then injected intravenously into sublethally irradiated I-E\textsuperscript{+} NOD recipients. The recipients received another injection of the same T cell clone 2 wk later and were tested for the development of insulitis 4 wk after the first injection. A typical pancreatic section is shown in Fig. 2. We could observe lymphocyte infiltration within islets in all the mice that received the T cell clones, although there still exist intact islets. This result demonstrates that all the clones could transfer insulitis in I-E transgenic NOD mice, and indicates that the islet-reactive T cells were not suppressed in I-E\textsuperscript{+} NOD mice.

TCR sequences of each T cell clone were analyzed. cDNA was synthesized from total RNA of each clone by using constant region–specific primers, and a poly(dA) tail sequence was introduced in the 3' terminal of each cDNA. The sequences containing V\textalpha or V\beta and joining regions were amplified by PCR, and the sequences of TCR of each clone were determined. The usage of TCR V and J gene segments of five individual T cell clones is summarized in Fig. 3. Four V\textalpha (V\alpha\textsubscript{1.1}, V\alpha\textsubscript{6.1}, V\alpha\textsubscript{2.2}, and V\alpha\textsubscript{1.1}) and four V\beta\textsubscript{3} (V\beta\textsubscript{15}, V\beta\textsubscript{16}, V\beta\textsubscript{12}, and V\beta\textsubscript{8.2}) were utilized by these five clones. V\alpha\textsubscript{1.1} was utilized by two clones, 4-1-E.2 and 7-10-D.3, and V\beta\textsubscript{15} by two clones, 4-1-L.6 and 7-10-D.3. Predominant usage of certain combinations of V\alpha, V\beta, J\alpha, and J\beta has been reported for T cells specific for cytochrome C (17, 18) and myelin basic protein (MBP) (14, 15). Amino acid sequences in the junctional regions equivalent to CDR3 of Ig are known to be selected in T cells with certain fine specificities (19, 20). However, in the present case, particular pairs of V\alpha and V\beta segments were not shared by more than two islet-reactive T cell clones. Junctional regions in TCR of these clones were also variable in each clone, as shown in Fig. 3. Thus, our results indicate that TCR usage of islet-reactive T cells is
not limited, but heterogeneous, and that multiple antigenic determinants on β cells may contribute to their generation or activation. Islet-specific T cell clones reported by Haskins et al. (21) also showed various specificities to islet cells of different sources, suggesting that multiple antigenic determinants may be recognized.

In an experimentally induced autoimmune model, EAE, the repertoire of the autoreactive T cells is known to be limited. The Vβ8.2 and Vα4 segments were dominantly utilized by MBP-specific T cells in H-2b mice such as PL/J and B10.PL (14, 15), while Vβ17a was frequently observed in H-2b mice such as SJL/J (22). It has recently been reported that MBP-specific T cell clones generated either from multiple sclerosis (MS) patients or control subjects predominantly expressed certain Vβ gene segments (23). Limited heterogeneity of TCR Vα gene expression was also reported in T cells from demyelinating brain plaques in MS patients (24). Our present finding is in contrast to these previously reported. There are two possibilities: (a) unlike EAE or MS, multiple antigenic determinants may be involved in the establishment of autoimmune insulitis in NOD mouse; or (b) although multiple antigenic determinants may be recognized by these clones, one or a few of them may be the diabetogenic targets that trigger autoimmune insulitis while the rest of the autoreactive T cells may be secondarily generated and responsible for progression of insulitis and diabetes mellitus. Previous experiments showing prevention of insulitis in I-E transgenic NOD mice (9, 10, 13) may support the latter possibility for the following reasons. All the T cell clones that we established reacted to islet cell antigens presented by APC from I-E+ NOD and transferred insulitis into I-E+ NOD mice. I-E-mediated prevention of insulitis is thus likely to be due to the clonal deletion or anergy of autoreactive T cells in I-E+ NOD mice rather than the functional suppression of autoreactive T cells in an effector phase or impaired presentation of autoantigens by I-E+ NOD APC. If this is the case, one or a few clones among heterogeneous autoreactive T cells may trigger autoimmune insulitis and be deleted or inactivated by I-E molecules.

T cells expressing certain Vβ segments such as Vβ17a, Vβ5, Vβ11, Vβ12, and Vβ16 have been known to be deleted by I-E molecules (25–28). Reich et al. (29) reported that the islet-reactive T cell clones expressing the Vβ5 gene segment possibly played a key role in the development of autoimmune insulitis in the NOD mouse. However, none of our T cell clones expressed Vβ5. Furthermore, in vivo depletion of Vβ5+ T cells by the mAb (MR9-4) did not affect the development of insulitis (data not shown), suggesting that Vβ5+ T cells may not be an essential requirement for the development of insulitis. Two of our T cell clones, 4-1-E.2 and 4-1-G.4, expressed Vβ12 or Vβ16, respectively. Northern blot analysis of splenic T cells revealed that Vβ16 but not Vβ12 expression was significantly decreased in the disease-resistant I-E transgenic NOD mouse, as shown in Fig. 4. Although our T cell clones including Vβ16+ 4-1-G.4 could not crossreact with I-E molecules (for example, 4-1-L.6 cells failed to proliferate in the presence of I-E+NOD splenocytes without islet antigens, as shown in Fig. 1), Vβ16+ T cells such as 4-1-G.4 might be similar to the case of Vβ11+ T cells, which were reported to be deleted by I-E within the thymus but not to be activated in vitro (27). It was recently reported that I-E expression on different subsets of immunocompetent cells by promoter-mutated Ea transgenics in NOD mice was not enough to prevent insulitis (30), suggesting that I-E-mediated clonal deletion might not necessarily correlate with prevention of insulitis. Therefore, depletion of Vβ16+ T cells may address the question whether 4-1-G.4 would trigger autoimmune insulitis when mAbs to the Vβ16 gene product are available.

![Figure 4. The Vβ gene expression in various strains of mice including NOD and I-E+ NOD.](image-url)
In this study, we have established islet-reactive T cell clones that could induce insulitis in disease-resistant I-E+ NOD mice, and determined their TCR sequences. These T cell clones will be useful for the identification of islet antigens presented by the unique I-ANOD molecule and for the understanding of the mechanisms of autoimmunity in the NOD mouse.

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