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^{NOD}. They could induce insulitis when transferred into disease-resistant I-E^+ 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 Vβ16. The expression of Vβ16 was significantly reduced in I-E^+ transgenic NOD, suggesting the possibility that the islet-reactive T cell clone expressing Vβ16 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)[1] 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^{NOD} 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, $CD4^+$ 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 Vβ12 and Vβ16 gene segments known to be deleted in I-E-expressing mice. Their possible roles in the development of insulitis are discussed.

[1] *Abbreviations used in this paper:* EAE, experimental autoimmune encephalomyelitis; IDDM, insulin-dependent diabetes mellitus; MBP, myelin basic protein; MS, multiple sclerosis; NOD, non-obese diabetic.

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 10^4 rested T cells with 10^4 irradiated (3,000 rad) islet cells as antigens and 5×10^5 irradiated (3,000 rad) spleen cells as APC. After a 72-h incubation, cells were pulsed with 0.5 μCi [^{3}H]TdR for 12 h. [^{3}H]TdR incorporation was measured by a liquid scintilation counter and is expressed as the mean of duplicate cultures.

In Vivo Transfer of Islet-reactive T Cell Clones. 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 islet-reactive T cell clones intravenously. After 2 wk, another injection with $2–5 \times 10^6$ 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

constant region specific primers, CA-2 AGAGGGTGCTGTCCTGAGAC (specific to Cα) and CB-2 TGGCCAAGCACACGAGGGTAGCC (specific to Cβ). A poly(dA) tail sequence was then introduced with terminal deoxynucleotidyl transferase (Bethesda Research Laboratories). Amplification was performed with thermus thermophilus HB8 DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). The first PCR was done with primers T7dT17, TAAGATCTAATACGACTCACTATAGGGAAGCTTTTTTTTTTTTTTTTT, and CA-2 or CB-2 with 30 cycles of denaturing at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 74°C for 2.5 min. 40 μl out of 100 μl PCR product was loaded onto 1% low-melting agarose gel; positions of 400–600 bp were cut out. The size-selected gel was melted, and 15 μl of the melted gel was subjected to the secondary PCR using the same primers and conditions as in the first one except for the higher annealing temperature (50°C). The product was size selected again and the final PCR was done with δT7 primer, TCTAAGTCGACTCACTATAGGGAAGC, and internal constant region specific primers containing restriction sites, CA-1*, CCGAGCTCCGAGGATCTTTTAACTGGTACAC (specific to Cα); or CB-1*, TCGAGCTCTGATGGCTCAAACAAGGAGAC (specific to Cβ). The annealing temperature for the final PCR was 55°C, and the rest of conditions were identical to the first PCR. After 40–45 cycles, a sharp band ~600 bp was observed. The final product was digested with SacI and SalI, and cloned into the PUC18 vector. In some cases, the products were digested only with SalI since the SacI site was found in the middle of the V regions. Transformed *Escherichia coli* colonies were screened with further internal constant region specific oligonucleotides; CA-0, AGGTTCTGGGTTCTGGATGTT (specific to Cα); or CB-0, GGTGGAGTCACATTTCTCAGAT (specific to Cβ). Plasmid DNAs were prepared from positive colonies, and alkali-denatured DNAs were sequenced by dideoxy chain termination method using a Sequenase™ Ver.2 kit (United States Biochemical Corp., Cleveland, OH) according to the manufacturer's instruction.

RNA Blot Analysis. Spleen cells from NOD, I-E$^+$ NOD, BALB/c, and C57BL/6 (B6) mice were cultured at 2 × 10^6/ml for 48 h with Con A (5 μg/ml). Poly(A)$^+$ RNA was prepared from total RNA with an oligo(dT) cellulose column. 5 μg of poly(A)$^+$ RNA obtained from each spleen cell culture was loaded onto an agarose gel and transferred to a nylon filter. RNA blots were hybridized with ^{32}P-labeled probes specific to Vβ8.2, Vβ12, Vβ15, Vβ16, and Cβ. Probes for these Vβ gene segments were synthesized by PCR using TCR β chain genes of the T cell clones as templates and Vβ gene–specific oligomers and δT7 as primers. Three individual filters were first hybridized with probes for Vβ12, Vβ15, and Vβ16. They were dehybridized and rehybridized with the constant region probe (Cβ). Then, the filter used for Vβ16 was also rehybridized with a Vβ8.2 probe.

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-2^d) or C57BL/6 (H-2^b) 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 $A\beta^{NOD}$ 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

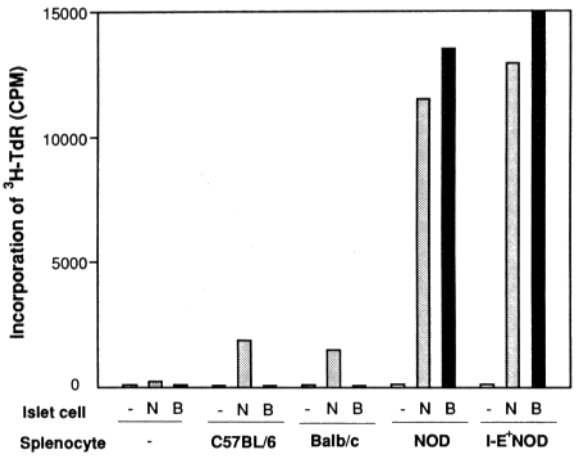

Figure 1. Islet reactivity and MHC specificity of a T cell clone (4-1-L.6) established from NOD pancreatic islet-infiltrating cells. Cloned T cells (4-1-L.6) were cultured without antigen (*open bars*), with NOD islet cells (*dotted bars*), or with BALB/c islet cells (*filled bars*) in the presence or absence of APC from C57BL/6, BALB/c, NOD, or I-E$^+$ NOD for 3 d. After a 72-h culture, cells were pulsed with [^{3}H]TdR for 12 h, and [^{3}H]TdR incorporation was measured.

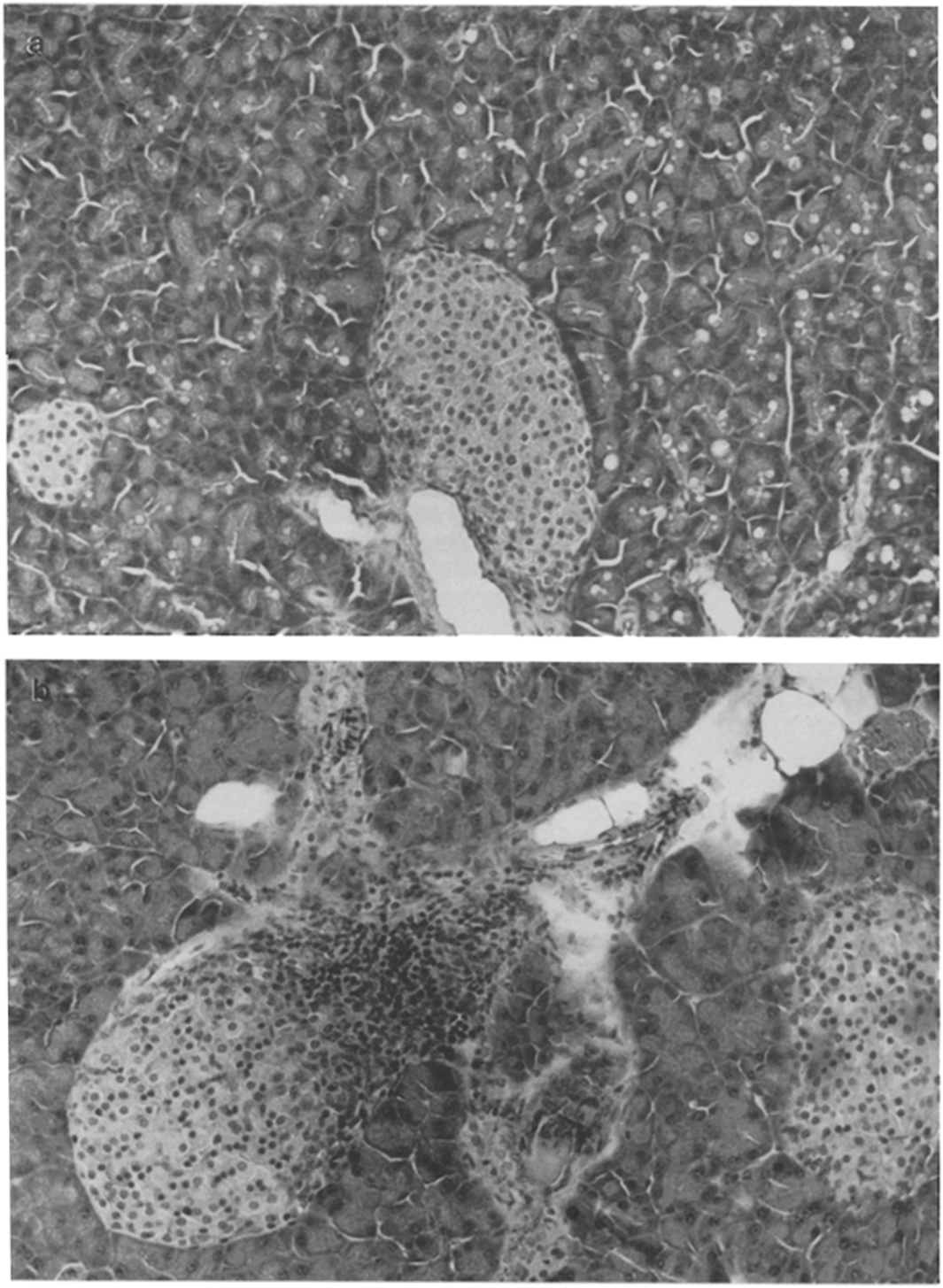

Figure 2. Islet-reactive T cell clones can transfer insulitis into I-E-transgenic NOD mice. Pancreatic sections of an irradiated I-E$^+$ NOD mouse without transfer (*a*) or with transfer of an islet-reactive T cell clone, 4-1-G.4 (*b*), were shown. I-E$^+$ NOD mice (8–10 wk old, female) were sublethally irradiated (650 rad) and were transferred with 2–5 × 10^6 cells of the islet-reactive T cell clone intravenously. After 2 wk, another injection with 2–5 × 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 β 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 β cell antigens in the context of NOD MHC class II (I-A^{NOD}) but that β 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$^+$ 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$^+$ NOD mice as the recipients since these mice never develop insulitis but have the identical genetic background to NOD except for the Eα^d transgene. These mice are also useful to determine whether any suppressive mechanisms in the effector phases are involved in I-E-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$^+$ 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$^+$ 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 Vα or Vβ 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 Vαs (Vα13, Vα6.1, Vα2.2, and Vα1.1) and four Vβs (Vβ15, Vβ16, Vβ12, and Vβ8.2) were utilized by these five clones. Vα1.1 was utilized by two clones, 4-1-E.2 and 7-10-D.3, and Vβ15 by two clones, 4-1-L.6 and 7-10-D.3. Predominant usage of certain combinations of Vα, Vβ, Jα, and Jβ 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 Vα and Vβ 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 indicates that TCR usage of islet-reactive T cells is

a

Clone	Vα	Junctional diversity	Jα
4-1-L.6	TGCGCACTGGAG C A L E Vα13	GGCCAT G H	TATGGGGGCAGTGGC Y G G S G TT11
4-1-K.1	TGTATCCTGAGA C I L R Vα6.1	GTAGCTACAAGACAA V A T R Q	GGAGGGTCTGCGAAG G G S A K TA27
4-1-G.4	TGTGCAGCACCC C A A P Vα2.2	TCGAAC S N	ACGGGTTACCAGAAC T G Y Q N E1
4-1-E.2	TGTGCAGTGGCA C A V A Vα1.1	GGA G	GGTGCAGATAGACTC G A D R L TA80
7-10-D.3	TGTGCAGTGAGC C A V S Vα1.1	CCG P	GGATACAACAAACTC G Y N K L D3

b

Clone	Vβ	D	Jβ
4-1-L.6	TGCGGTGCT C G A Vβ15	AGGGGCACT R G T	TATGAACAGTACTTC Y E Q Y F Jβ2.7
4-1-K.1	TGTGCCAGC C A S Vβ8.2	GGTGGG G G	TATGAACAGTACTTC Y E Q Y F Jβ2.7
4-1-G.4	TGTGCCAGCAGC C A S S Vβ16	TTAGGGACA L G T	ACAAACACCGGGCAG T N T G Q Jβ2.2
4-1-E.2	TGTGCCAGC C A S Vβ12	AGACTGGGA R L G	AACCAAGACACCCAG N Q D T Q Jβ2.5
7-10-D.3	TGTGGTGCT C G A Vβ15	GGACAGGGG G Q G	GGAAATACGCTCTAT G N T L Y Jβ1.3

Figure 3. Five independent islet-reactive T cell clones of the NOD mouse utilize distinct combinations of TCR Vα, Jα, Vβ, and Jβ. Sequences of junctional regions of TCR α chain (*a*) and β chain (*b*) of each clone were shown. TCR sequences containing Vα and Jα or Vβ and Jβ were amplified from all T cell clones by PCR (see Materials and Methods) and determined by a dideoxy termination method.

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-2^u mice such as PL/J and B10.PL (14, 15), while Vβ17a was frequently observed in H-2^s 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 autoimmunity 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 Eα transgenes 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.

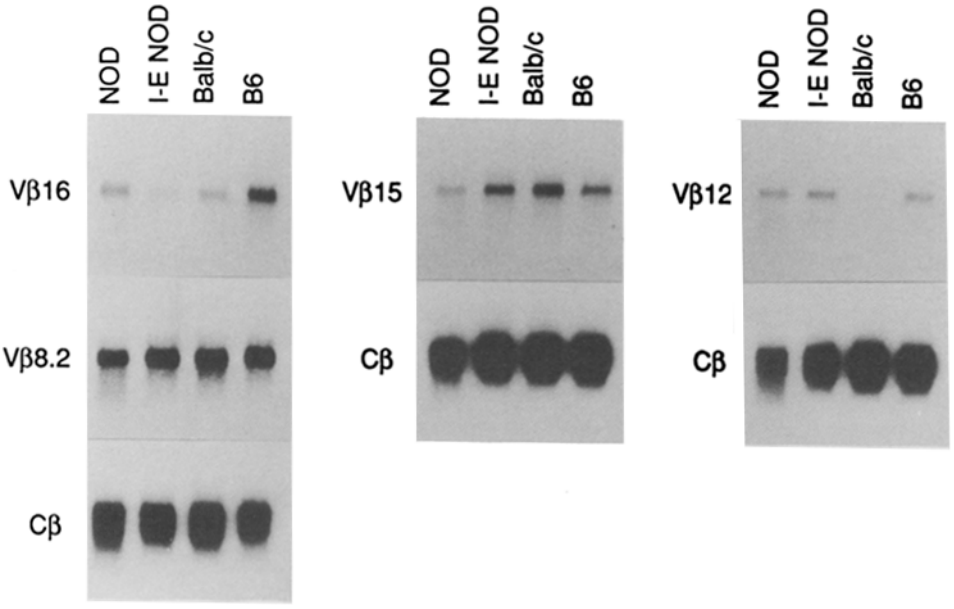

Figure 4. The Vβ gene expression in various strains of mice including NOD and I-E$^+$ NOD. Poly(A)$^+$ RNA was isolated from Con A–stimulated spleen cells of NOD, I-E$^+$ NOD, BALB/c, and C57BL/6 (B6). RNA blots were hybridized with ^{32}P-labeled probes specific to Vβ8.2, Vβ12, Vβ15, Vβ16, and Cβ.

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-A^{NOD} molecule and for the understanding of the mechanisms of autoimmunity in the NOD mouse.

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