

T-Cell Receptor Transgenic Response to an Endogenous Polymorphic Autoantigen Determines Susceptibility to Diabetes

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We have produced a T-cell receptor (TCR) transgenic NOD mouse, 6.9TCR/NOD, in which the expression of both diabetogenic T-cells and naturally occurring autoantigen were simultaneously controlled. The parent T-cell clone, BDC-6.9, and T-cells from 6.9TCR/NOD mice recognize a currently unidentified antigen present in NOD but not in BALB/c islet cells. A gene that codes for the antigen, or a protein that regulates the antigen, was previously mapped to a locus on chromosome 6. We have developed transgenic mice bearing the TCR α - and β -chains from the BDC-6.9 T-cell clone on a NOD congenic background in which the antigen locus on chromosome 6 of the NOD mouse is replaced by a segment from BALB/c. These NOD.C6 congenic mice lack the NOD islet cell antigen to which the BDC-6.9 T-cell clone responds. Diabetes in both male and female 6.9TCR/NOD mice is dramatically accelerated, but in 6.9TCR/NOD.C6 mice lacking the NOD islet cell autoantigen, we have not observed diabetes for up to 1 year of age. Thus, the generation of 6.9TCR transgenic mice provides a model of autoimmune diabetes whereby controlled expression of an endogenous polymorphic autoantigen effectively determines disease development. *Diabetes* 53:978–988, 2004

Type 1 diabetes results from pancreatic islet β -cell destruction, which leads to insulin insufficiency and hyperglycemia. Autoreactive T-cells play a critical role in β -cell death (1,2), and although it is clear that both CD4 and CD8 T-cells are involved (3,4), studies using T-cell clones (5) and T-cell receptor (TCR) transgenic mice (6–11) demonstrate that under some circumstances cells from only one lineage are sufficient to cause disease (5–11). Therefore, the presence of a domi-

nant islet-specific T-cell population of either lineage can disrupt an otherwise tolerant immune system.

Although the coordinate expression of autoreactive T-cells and autoantigen are prerequisites for autoimmunity, they are not sufficient. Autoreactive T-cells are present at low frequency in the periphery of individuals without detectable autoimmunity (12). Even when the repertoire is dominated by T-cells expressing an islet-specific TCR, as with 2.5TCR/NOD mice, diabetes may only rarely occur (13). In these instances, tolerance may be maintained at least in part by regulatory T-cells (13–16). Therefore, the mere presence of islet-reactive T-cells does not guarantee disease development, even when autoantigen is present.

Factors other than autoantigen identity and autoreactive T-cell specificity affect diabetes progression by influencing general immune function (17), and this is supported by the established polygenic nature of autoimmune diabetes (18–21). Although some susceptibility genes, such as the major histocompatibility and insulin genes, likely regulate autoreactive T-cell development and autoantigen expression, this may not hold true for others. For example, *Idd5* and *Idd13* appear to influence the transition from benign to destructive insulinitis, an event that occurs only after T-cells recognize islet antigen (22). Because the identity of most susceptibility genes is unknown, the mechanisms responsible for disease susceptibility remain largely unclear (23,24). In addition to identifying specific susceptibility genes, clarity might be gained by understanding how a susceptible genotype (such as the NOD mouse) influences basal immune function.

Due to the continuous presence of endogenous antigen and the lack of an antigen-negative reference mouse, characterizing the basal T-cell function of NOD mice is difficult. Even the very early stages of autoimmunity may result in baseline values for activation, apoptosis, cytokine production, and migration of T-cells that are different from those in a mouse that does not have any ongoing disease. In other words, in the autoimmune NOD mouse it is difficult to assay T-cell function in the absence of autoimmunity. This would require an intact NOD genome in mice that possess T-cells but lack disease (even early stages). One way to accomplish this is to generate NOD mice that express a transgenic TCR specific for a contrived antigen not present in NOD mice. Autoimmunity would not occur because the antigen is lacking. An alternative and perhaps more desirable approach includes the development of

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IFN, interferon; TCR, T-cell receptor.

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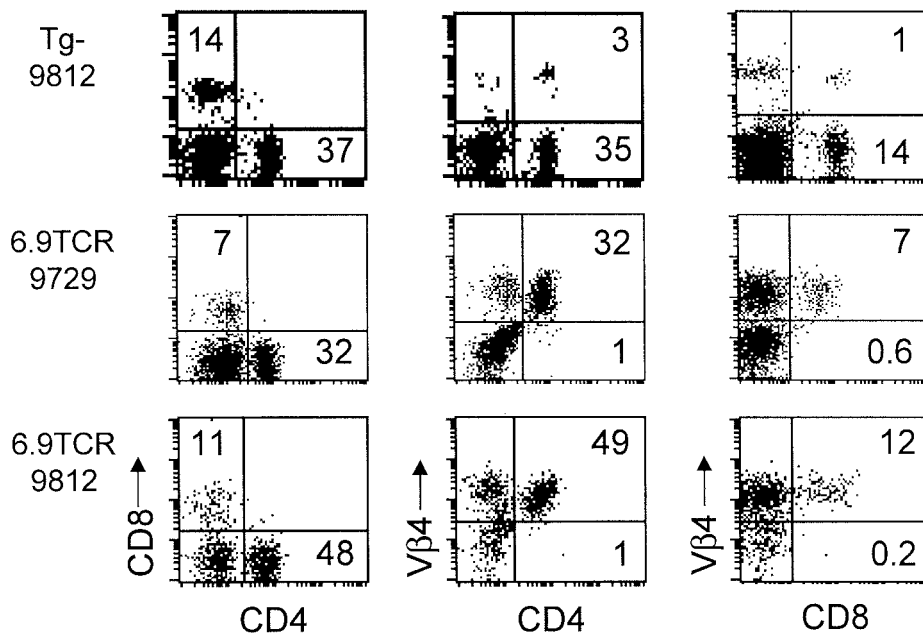


FIG. 1. T-cells from 6.9TCR mice express V β 4. Three-color flow cytometry of peripheral blood T-cells from 6.9TCR mice (9729 or 9812 line) showed that they belong primarily to the CD4 lineage. V β 4 was expressed on >95% of all T-cells from transgenic mice in contrast to 6–8% in non-transgenic littermates (Tg⁻). Percentages are indicated within quadrants of interest, and quadrant boundaries were based on parallel single-color control samples (data not shown).

NOD mice that possess T-cells capable of causing disease only under select circumstances. Here we document the development of a novel transgenic mouse model that may aid in uncovering the potentially subtle immune dysfunctions present in the diabetes-susceptible NOD mouse apart from disease development.

RESEARCH DESIGN AND METHODS

NOD and BALB/c mice. NOD and NOD.CB17-Prkdc^{scid}/J (NOD-scid) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) or the breeding colony at the Barbara Davis Center for Childhood Diabetes (Denver, CO). BALB/c mice were originally obtained from The Jackson Laboratory. Mice were bred and maintained under specific pathogen-free conditions at the University of Colorado Health Sciences Center's Center for Laboratory Animal Care (Denver, CO), the Scripps Rodent Breeding Colony (La Jolla, CA), and Southern Illinois University School of Medicine animal facility (Springfield, IL).

Generation of 6.9 transgenic and NOD.C6 mice. Rearranged TCR α - and β -chain genes were amplified from BDC-6.9 T-cell clone mRNA by RT-PCR using *Taq*DNA polymerase (Perkin-Elmer Life Sciences, Boston, MA) and the following primers: 5'-TGTGAGTCGACATGAAAACATACGCTCCTACATTAT T-3' (α -5'); 5'-AGTCAGTCGACGTCAGTCTTGCAGACCTC-3' (α -3'); 5'-GTCGACACTAGTCACATGGGCTCCATTTCCTCAGT-3' (β -5'); and 5'-AGGAGGC CGACTTGGCCGTTGAAGAACGGCTCA-3' (β -3'). PCR products were cloned directly into the pCRII vector (Invitrogen Life Technologies, Carlsbad, CA). Following sequence verification, the α - and β -genes were subcloned into separate mCD4e/*p*-sil expression vectors kindly provided by D. Littman (construct "i") (25). Briefly, this vector contains the murine CD4 minimal enhancer (339 bp), minimal promoter (489 bp), exon I, part of intron 1, and 32 bp of exon 2. A key intronic silencer element is absent, making this promoter capable of directing expression in both CD4 and CD8 T-cell lineages, as has been previously demonstrated (26). The CD4 exonic sequences encode 5' untranslated regions. Appropriate orientation of each gene was confirmed by sequencing 5' and 3' junctions. Linear *NotI* fragments containing each TCR transgene (α 5,740 bp and β 5,860 bp) were purified from unwanted vector sequences using the Qiagen Gel Extraction kit (Qiagen, Valencia, CA), mixed in equal molar ratios and injected into F2 (C57BL/6By \times BALB/cBy) embryos. Tail DNA from offspring was screened by PCR using the above primers. In the event that decreased fertility might result from early diabetes onset on the NOD background, transgenic founder mice were initially crossed to BALB/c mice and then subsequently backcrossed to either NOD or NOD.C6 mice (see description below) for three to five generations to generate 6.9TCR/NOD or 6.9TCR/NOD.C6 mice, respectively.

To create NOD.C6(N/B) mice, BALB/c mice were backcrossed to NOD mice for eight generations. Offspring were screened using PCR of tail DNA with microsatellite primers for D6Mit60 (ResGen; Invitrogen). Only mice that

maintained heterozygosity at D6Mit60 were selected to breed for the next generation. Intercrossing was initiated with the eighth backcross generation to create NOD.C6 mice (homozygous BALB/c or B/B at D6Mit60). Additional genotyping was performed to establish an interval of BALB/c genome between D6Mit52 and D6Mit57. Transgenic mice were backcrossed to NOD.C6(N/B) or NOD.C6(B/B) mice for at least five generations to create 6.9TCR/NOD.C6(N/N), 6.9TCR/NOD.C6(N/B), or 6.9TCR/NOD.C6(B/B) mice. To simplify nomenclature, NOD.C6(B/B) mice are referred to in some instances as NOD.C6.

Flow cytometry. Peripheral blood was collected or single cell suspensions of spleen, peripheral lymph nodes, and thymus were made and erythrocytes lysed using 0.85% ammonium chloride. Mononuclear cell suspensions were stained with a combination of anti-CD4-FITC (fluorescein isothiocyanate), anti-CD8-allophycocyanin, and anti-V β 4-biotin or anti-CD3-biotin. Anti-V β 4 and anti-CD3 binding were visualized with streptavidin-phycoerythrin. All

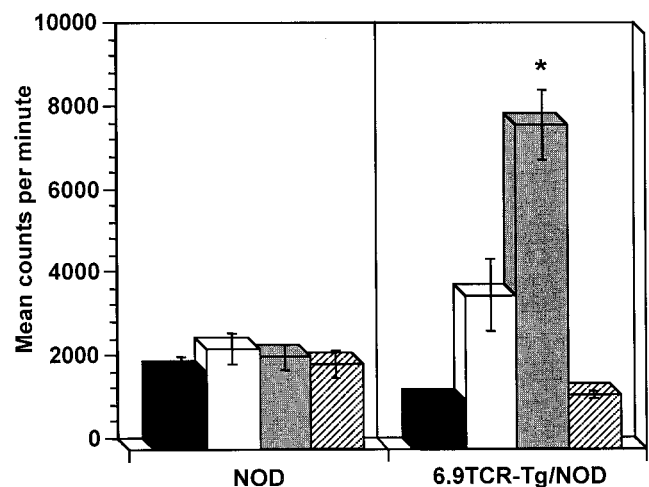


FIG. 2. 6.9TCR/NOD T-cells respond to NOD, but not BALB/c, islet cell antigen. Lymph node (mesenteric and pancreatic) mononuclear cells (5×10^5 /well) from 6.9TCR/NOD ($n = 6$) or age-matched nontransgenic littermate ($n = 3$) mice were cultured in the presence or absence of islet cells from NOD or BALB/c mice. Proliferative responses were determined by standard ^3H -thymidine incorporation. Data are the mean counts per minute, with SE indicated by error bars. 6.9TCR/NOD T-cells proliferated in response to 20,000 NOD islet cells (■) significantly (Student's *t* test) more than those of nontransgenic littermates (*). While this trend was also present in using 10,000 NOD islet cells (□), the results failed to reach statistical significance. Of note, there was no significant difference observed between 6.9TCR/NOD cells cultured alone or with BALB/c IC. ■, no islet cells; ▨, 15,000 BALB/c islet cells.

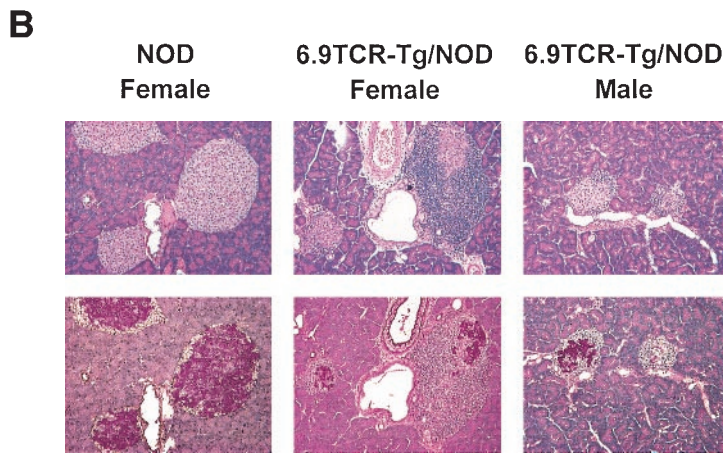
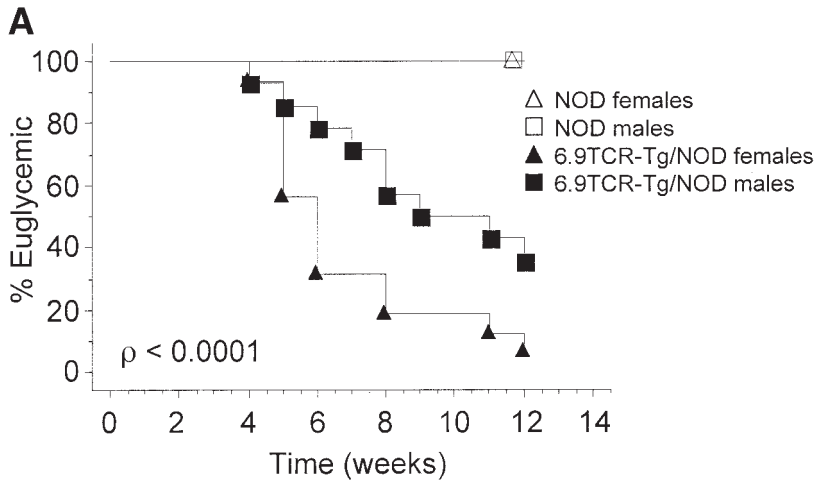


FIG. 3. Diabetes is severe and accelerated in 6.9TCR/NOD mice. **A:** The onset of diabetes was accelerated in 6.9TCR/NOD at backcross 4–5 ($n = 15$ females and 14 males) compared with sex-matched nontransgenic NOD ($n = 6$ females and 14 males) littermates. Urine glucose levels were monitored daily for the first 4 weeks of life and three times per week thereafter. Diabetes was confirmed in mice with glucosuria by measuring blood glucose levels. Mice were considered overtly diabetic when urine glucose was $>2\%$ and blood glucose was >15 mmol/l. The percentage of euglycemic mice is plotted on Kaplan-Meier curves, with statistical analysis performed using the Mantel-Cox log-rank test. **B:** Representative photomicrographs (original magnification, $\times 20$) of hematoxylin and eosin- (top) or aldehyde-fuchsin (bottom)-stained pancreas tissue sections from 3- to 5-week-old 6.9TCR/NOD or age-matched nontransgenic NOD mice. **C and D:** Increased islet inflammation and degranulation is consistent with an accelerated disease course in 6.9TCR/NOD compared with nontransgenic NOD littermates. Data were compiled from pancreas sections taken from 3- to 5-week-old 6.9TCR/NOD mice and age-matched nontransgenic littermates (6–9 mice/group) as described in RESEARCH DESIGN AND METHODS. **C:** \square , no infiltrate; \square , polar infiltrate; \square , peri-islet infiltrate; \blacksquare , intraislet infiltrate. **D:** \square , fully granulated; \square , 50–75% granulated; \square , 20–50% granulated; \blacksquare , fully degranulated.

antibodies were purchased from BD Pharmingen (San Diego, CA). Immunofluorescence was detected using a FACS (fluorescence-activated cell sorter) Calibur, and analysis was performed on cells with lymphoid light-scatter properties using CellQuest version 3.2 software (Becton Dickinson). **Proliferation and cytokine assays.** Islet cells were used as a source of antigen to stimulate T-cell proliferation and cytokine production. Islets were harvested from whole pancreas by collagenase digestion as previously described (27,28).

To measure proliferative responses, mesenteric and pancreatic lymph node cells were harvested and pooled from 9- to 10-week-old 6.9TCR/NOD or nontransgenic age-matched NOD mice. Lymph node cells (5×10^5 cells/well) were cultured in duplicate in the presence or absence of islet cells from NOD ($1-2 \times 10^4$ cells/well) or BALB/c (1.5×10^4 cells/well) mice. All cultures were maintained in complete medium, which was prepared by supplementing Dulbecco's modified Eagle's culture medium (Life Technologies, Gaithersburg, MD) with 44 mmol/l sodium bicarbonate, 0.55 mmol/l L-arginine, 0.27 mmol/l L-asparagine, 1.5 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 mg/l gentamicin sulfate, 50 μ mol/l 2-ME, 10 mmol/l HEPES, and 10% FCS. Cultures were incubated at 37°C with 10% CO₂. After 60 h, cultures were pulsed with [³H]thymidine (1 μ Ci/well), and 12 h later incorporation was de-

termined using a Tomtec Harvester 96 Mach III (Tomtec, Hamden, CT) and a Wallac 1450 MicroBeta liquid scintillation counter (Perkin-Elmer Life Sciences).

To measure interferon (IFN)- γ production, spleen and lymph node mononuclear cells were harvested and pooled from a 12-week-old 2.5TCR/NOD mouse or from 8-week-old 6.9TCR/NOD or nontransgenic NOD littermate mice. Cells (5×10^5 /well) were cultured in the presence or absence of islet cells (10,000/well) from NOD.C6 or BALB/c mice. Triplicate samples were cultured in complete medium for 72 h, and then cell-free supernatants were assayed for IFN- γ by enzyme-linked immunosorbent assay using purified and biotinylated antibody pairs according to an established BD Pharmingen protocol.

Adoptive transfers. Splenocytes were harvested from 6.9TCR/NOD, 6.9TCR/NOD.C6, or appropriate nontransgenic littermate mice. Mononuclear cells were prepared using gradient centrifugation with Lympholyte M (Cedarlane Laboratories Limited, Hornby, ON, Canada). Flow cytometry was used to determine the percentage of CD4 cells present. Cell concentrations were normalized based on CD4 cell number before injection of adult (intravenous; 2×10^6 CD4 equivalents) or neonatal (intraperitoneal; 2.4×10^6 CD4 equivalents) NOD-*scid* recipients. Alternatively, before injection of neonatal NOD-*scid* recipients, CD4 cells were purified from the spleens of 6.9TCR/NOD.C6

TABLE 1
Summary of peripheral blood phenotypes

	n	Mean percentage of positive cells and 4 to 8 ratios in peripheral blood				
		% CD4	% CD8	4-to-8 ratio	% V β 4*	% V β 4†
9812 Tg-	6	32.3 \pm 8.5	8.7 \pm 3.4‡	3.9 \pm 0.7	9.9 \pm 1.8‡	4.4 \pm 0.7‡
9812 Tg+	16	28.1 \pm 5.8	6.2 \pm 1.8	4.7 \pm 1.0	88.1 \pm 7.1	96.4 \pm 3.8
9729 Tg-	4	27.5 \pm 14.7	7.1 \pm 4.8	4.6 \pm 1.8	8.5 \pm 2.2‡	6.0 \pm 2.8‡
9729 Tg+	22	24.2 \pm 8.4	7.0 \pm 4.5	4.1 \pm 2.1	94.4 \pm 6.0	98.5 \pm 1.8

Data are means \pm SD. *Gated on CD4-positive cells; †gated on CD8-positive cells; ‡statistically significant ($P < 0.05$) using Student's *t* test when compared with transgenic mice.

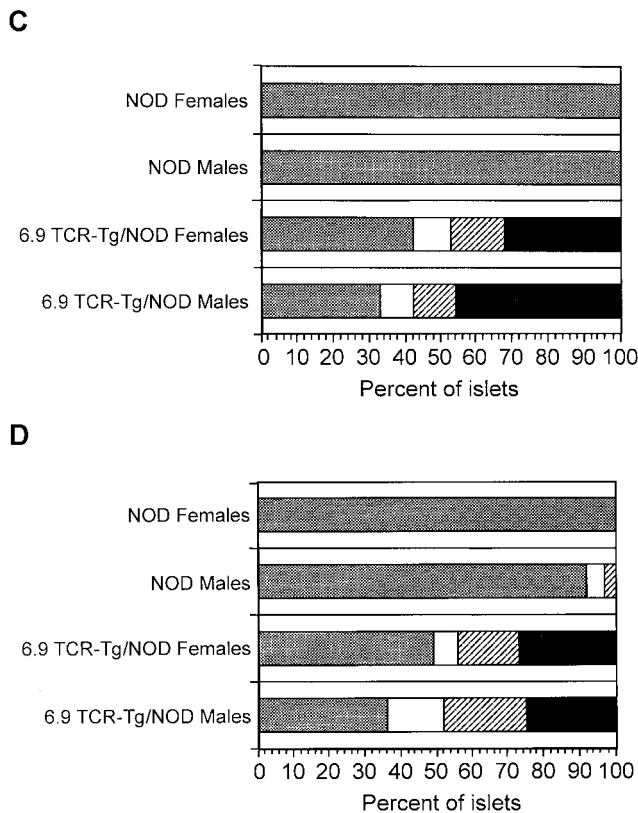


FIG. 3. Continued

mice using mouse CD4 (L3T4) MicroBeads, MS+ Separation Columns, and the MiniMACS magnet system from Miltenyl Biotec (Auburn, CA). Purity of the CD4+ population was always >95%, as determined by flow cytometric analysis. Diabetes onset was monitored as described below.

Diabetes onset. Blood glucose measurements were made using OneTouch Ultra (Life Scan, Milpitas, CA) or Precision QID (MediSense, Waltham, MA) glucose meters. Mice were considered diabetic when blood glucose levels were >15 mmol/l (270 mg/dl). As a less-invasive screening method, urine glucose was measured using Diastix in some cases (Miles Laboratories, Elkhart, IN). Hyperglycemia was always verified for mice with >2% glucosuria. Urine and/or blood glucose levels were measured daily, weekly, or every 2 weeks as appropriate for the expected disease time course. Diabetes onset was graphed using Kaplan-Meier nonparametric survival curves, and statistical significance was determined by the Mantel-Cox log-rank test with StatView software.

Histology, insulinitis, and granulation. Pancreas tissue from 6.9TCR/NOD, 6.9TCR/NOD.C6, or appropriate nontransgenic littermate mice was paraffin embedded, sectioned, and stained as previously described (29,30) with hematoxylin and eosin to determine mononuclear cell infiltration and/or with aldehyde-fuchsin to determine the extent of β -cell granulation. Sections from each mouse (containing on average 12 islets per section) were microscopically examined, and each islet was scored for the level of infiltration or β -cell granulation. Photographs were taken at $\times 200$ (original magnification).

RESULTS

Generation of 6.9TCR/NOD transgenic mice. In designing a new diabetes model we took advantage of the previously well-characterized T-cell clone, BDC-6.9, which was derived from a newly diabetic NOD mouse. The BDC-6.9 clone is I-A^{g7} restricted, specific for an unknown islet β -cell antigen, and diabetogenic upon injection into NOD or NOD-*scid* mice (29–31). Unlike the specificities of other NOD-derived T-cell clones, the BDC-6.9 clone is selectively responsive to islet antigen from NOD and NOD-related mouse strains (32). For example, BDC-6.9 cells do not proliferate or secrete cytokines when presented with islet cell antigens from BALB/c mice. Because

we sought to exploit this discrimination, we first generated transgenic mice (6.9TCR) using rearranged TCR genes from the BDC-6.9 T-cell clone. Using PCR to screen for transgene integration, 7 founder mice were identified from 38 offspring: 1 integrated only 6.9 α -, 1 integrated only 6.9 β -, and 7 integrated both α - and β -transgenes (data not shown). Only founders that integrated genes for both chains were bred for further analysis.

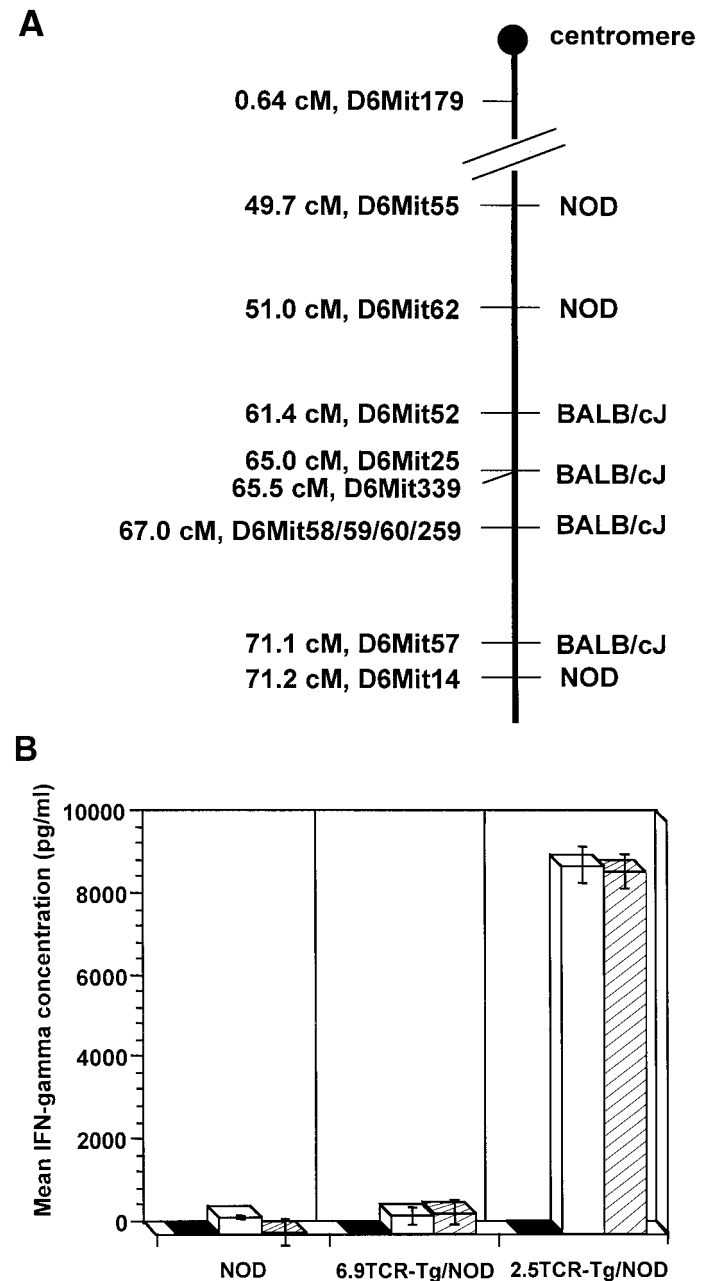


FIG. 4. Neither NOD.C6 nor BALB/c islet cells can stimulate 6.9TCR/NOD T-cells. **A:** As diagrammed here, microsatellite analysis indicates that NOD.C6 mice contained BALB/c genome in a region between D6Mit52 and D6Mit57. **B:** Islet cells from NOD.C6 mice were indistinguishable from BALB/c islet cells as an antigen source for T-cells from 6.9TCR/NOD. Little or no IFN- γ was secreted in response to either islet cell (IC) preparation. However, both preparations contained sufficient and equivalent levels of islet cell antigen to stimulate significant IFN- γ production by 2.5TCR/NOD T-cells. As expected from the low precursor frequency of islet-reactive T-cells in young NOD mice, IFN- γ secretion by these cells remained almost undetectable. IFN- γ was measured by enzyme-linked immunosorbent assay. Data are mean values of triplicate cultures. ■, no IC; □, BALB/c IC; ▨, NOD.C6IC.

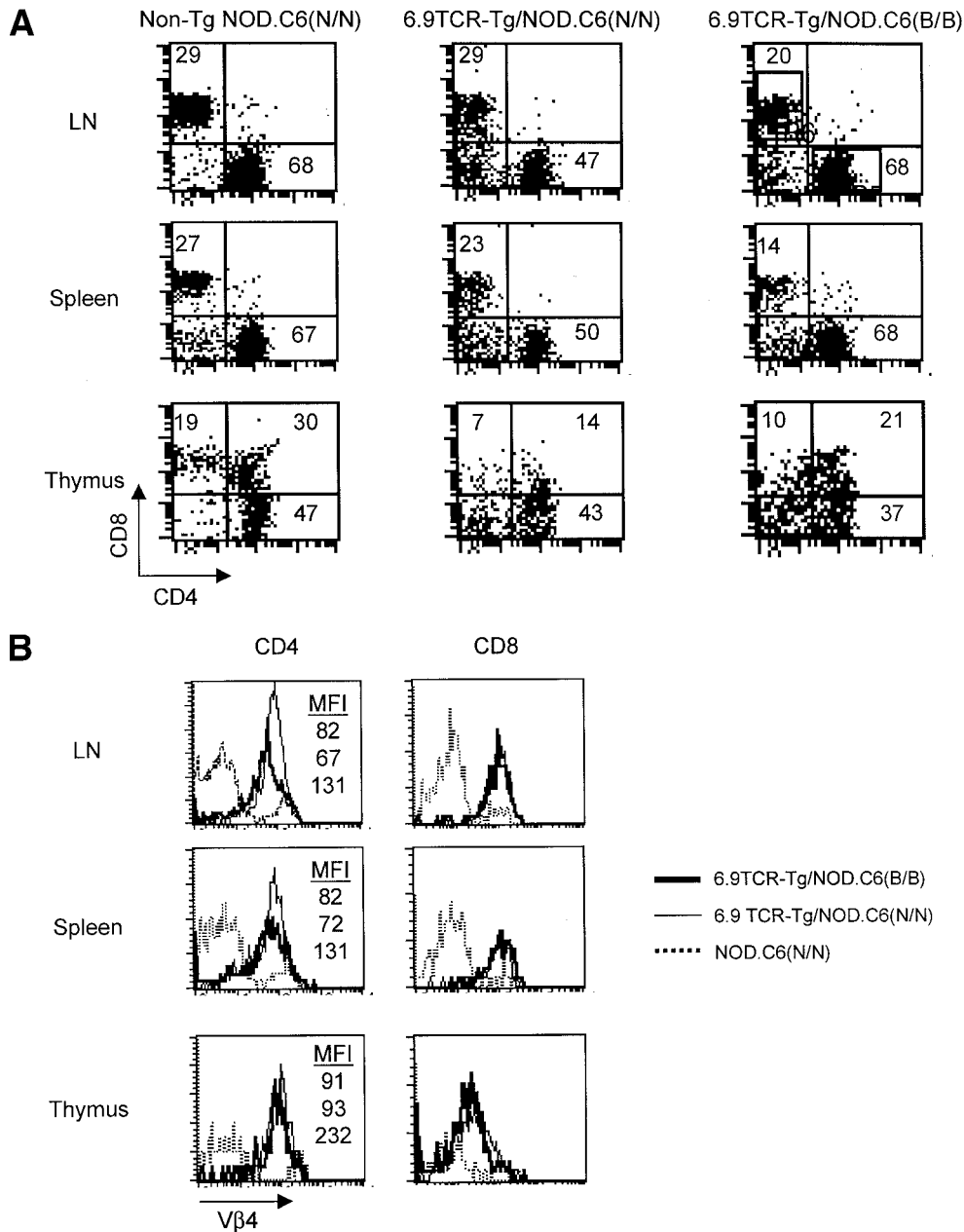


FIG. 5. Inheritance of NOD alleles on chromosome 6 (61.4–71.1 cM) does not affect V β 4 expression on T-cells but does affect diabetes incidence. **A and B:** Three-color flow cytometry of splenic, lymph node, and thymic T-cells from nontransgenic NOD.C6(N/N), 6.9TCR/NOD.C6(N/N), and 6.9TCR/NOD.C6(B/B) mice (average age, 7.5 \pm 1.9 weeks old) showed that V β 4 was expressed on >95% of all T-cells from transgenic mice. Both the dot plots and the histograms were gated on CD3-positive cells. Percentages indicated within quadrants are from individual representative mice. At least three separate experiments were performed ($n = 3$ –6 mice per genotype). Data for each set of tissues shown are from a single mouse per genotype. Mean channel fluorescence (MFI) is indicated for V β 4-expressing CD4 cells within the relevant histograms in the following order (top to bottom): 6.9TCR/NOD.C6(B/B), 6.9TCR/NOD.C6(N/N), and nontransgenic NOD.C6(N/N). **C:** Diabetes onset was determined by monitoring blood glucose levels among female 6.9TCR/NOD.C6(N/N) ($n = 12$), 6.9TCR/NOD.C6(N/B) ($n = 19$), 6.9TCR/NOD.C6(B/B) ($n = 14$), and nontransgenic NOD.C6(N/N) ($n = 18$) littermates. Kaplan-Meier plots show the percentage of mice remaining euglycemic over time. Statistically significant differences were observed using the Mantel-Cox log-rank test for comparisons made between nontransgenic NOD.C6(N/N) mice and 6.9TCR/NOD.C6(B/B) or 6.9TCR/NOD.C6(N/B) mice. **D:** Similar analyses were performed with male 6.9TCR/NOD.C6(N/N) ($n = 7$), 6.9TCR/NOD.C6(N/B) ($n = 33$), 6.9TCR/NOD.C6(B/B) ($n = 19$), and nontransgenic NOD.C6(N/N) ($n = 14$) littermate mice. **E and F:** All transgene-negative mice exhibited disease incidence profiles similar to those observed with the nontransgenic NOD.C6(N/N) mice shown in **C** and **D**. Additional mouse genotypes shown here include NOD.C6(N/B) ($n = 40$ females and 20 males) and NOD.C6(B/B) ($n = 14$ females and 18 males).

Transgene β -chain expression was detected by flow cytometry and was used in addition to PCR to identify transgenic mice (Fig. 1). Intact expression of the BDC-6.9 TCR expression could not be demonstrated by flow cytometry since neither a clonotypic antibody nor an α -chain-specific antibody (V α 13) exists. However, offspring from two lines (9729 and 9812) demonstrated consistent germline transmission of α - and β -TCR chains (data not shown)

and possessed peripheral T-cells exhibiting a high degree of expression of the transgene (Fig. 1) (Table 1). The CD4-to-CD8 ratios in peripheral blood lymphocytes were typically more than 4, and >85% of CD4 and 95% of CD8 T-cells expressed the transgene V β chain (Fig. 1) (Table 1). As studies progressed, no difference was observed between these lines, and therefore data from the 9729 line are shown unless otherwise indicated. A sample of V α

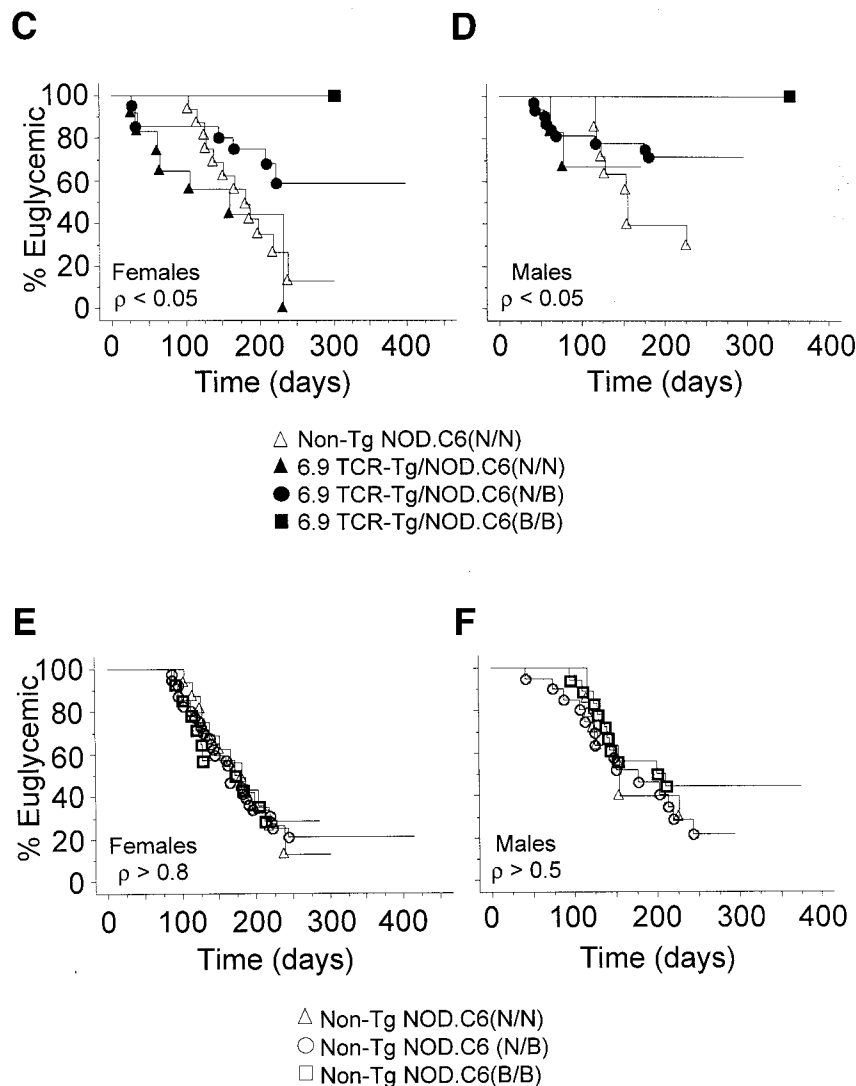


FIG. 5. Continued

expression in the transgenic repertoire was obtained by examining peripheral CD4⁺ T-cells by flow cytometry with two commercially available V α antibodies (V α 2 and 8.3). Significantly fewer V α 2 (2.4%)- and V α 8.3 (0.7%)-positive cells were found in transgenic compared with nontransgenic mice (V α 2 [9.8%] and V α 8.3 [4.8%]).

6.9TCR/NOD T-cells recognize NOD β -cell antigen specifically. In the absence of BDC-6.9 TCR α -chain expression data, we sought to confirm the functional expression of the BDC-6.9 TCR by taking advantage of the established selective specificity of the BDC-6.9 T-cell clone (32). T-cells from 6.9TCR/NOD transgenic mice proliferated when provided with islet cell antigen from NOD, but not BALB/c mice (Fig. 2). In contrast, T-cells from age-matched nontransgenic littermates did not proliferate significantly to islet antigens from either source, reflecting the diverse repertoire of young NOD mice. Furthermore, 6.9TCR/NOD splenocytes (as few as 5×10^5) transferred disease when injected into adult NOD-*scid* recipients (data not shown). Both in vitro and in vivo peripheral 6.9TCR/NOD T-cell responses were consistent with a repertoire composed primarily of T-cells expressing the TCR of BDC-6.9.

Diabetes onset is markedly accelerated in 6.9TCR/NOD transgenic mice. To determine whether disease incidence was affected by expression of the BDC-6.9 TCR, blood glucose levels of a cohort of 6.9TCR/NOD mice (from backcross generations 3–5) were compared with nontransgenic littermates. Disease onset was significantly accelerated not only among female 6.9TCR/NOD mice but also among transgenic males (Fig. 3A). More than 60% of

TABLE 2

Lymphoid organ mean cell yields and T-cell phenotypes: mean total cell yields

	Mean cell number ($\times 10^6$)		
	Non-Tg NOD.C6(N/N)	6.9TCR/ NOD.C6(N/N)	6.9TCR/ NOD.C6(B/B)
<i>n</i>	3–5	4–5	4–6
Lymph node	6.7 ± 2.7	8.6 ± 4.0	$12.3 \pm 4.7^*$
Spleen	97.9 ± 50.6	60.5 ± 13.4	64.2 ± 24.3
Thymus	89.3 ± 5.3	151.5 ± 55.0	$215.3 \pm 62.8^*$

Data are means \pm SD. *Statistically significant ($P < 0.05$) using Student's *t* test when compared with nontransgenic NOD.C6(N/N) mice. Non-Tg, nontransgenic.

TABLE 3
Lymphoid organ mean cell yields and T-cell phenotypes: T-cell phenotypes

	<i>n</i>	Mean percentage of positive cells and 4-to-8 ratios							
		DP	CD4	Non-Tg NOD.C6(N/N)				6.9TCR/NOD.C6(N/N)	
				% Vβ4*	CD8	% Vβ4†	4 to 8	DP	CD4
Lymph node	5–6	—	69.2 ± 2.6	10.6 ± 2.4	29.0 ± 2.3	8.7 ± 8.2	2.4 ± 0.3	—	50.1 ± 4.4‡
Spleen	5–6	—	66.9 ± 1.6	10.0 ± 1.2	27.5 ± 2.0	7.3 ± 4.3	2.4 ± 0.2	—	57.5 ± 6.5‡
Thymus	3–4	29.6 ± 6.1	48.0 ± 3.5	—	19.4 ± 2.7	—	—	40.6 ± 19.0	26.3 ± 11.7‡

Data are means ± SD. All data are gated on CD3-positive cells. *Gated on CD4-positive cells; †gated on CD8-positive cells; ‡statistically significant ($P < 0.05$) using Student's *t* test when compared with Non-Tg NOD.C6(N/N) mice. DP, double positive; non-Tg, nontransgenic.

all transgenic mice from backcrosses 3–5 were hyperglycemic by 12 weeks of age, whereas nontransgenic littermates remained euglycemic. The kinetics of disease onset were extreme, with hyperglycemia being detected as early as 4 weeks of age. At 3–5 weeks of age, NOD mice typically show few if any signs of impending disease, whereas at this time point, islet tissue from 6.9TCR/NOD mice showed profound inflammation (Fig. 3B). Islets were extensively infiltrated, and islet β-cell degranulation was significant (Fig. 3C and D). These results indicate that autoimmune diabetes was well established in young 6.9TCR/NOD mice. Disease was clearly accelerated, and the sex bias typically observed among NOD mice was almost nonexistent.

Inheritance of BALB/c genome on chromosome 6 (61.4–71.1 cM) protects islet tissue from destruction by 6.9 T-cells. Given that both the BDC-6.9 T-cell clone and 6.9TCR/NOD T-cells exhibited strain-specific antigen responsiveness, we sought to control autoantigen expression *in vivo*. Previous mapping studies localized the gene encoding (or regulating) the β-cell autoantigen to a region on chromosome 6 (Fig. 4A). In these studies, microsatellite markers D6Mit58 and D6Mit60 showed 100% correlation with inheritance of the strain-specific phenotype (32). That is, the BDC-6.9 clone proliferated in response to islet antigen from mice with at least one NOD allele (defined at D6Mit58/60), but not if both alleles were of BALB/c origin.

In the current study, BALB/c mice were backcrossed for eight generations with NOD/LtJ mice and then intercrossed to generate NOD.C6 congenic mice. Microsatellite analysis of chromosome 6 allowed development of NOD mice that possessed NOD [NOD.C6(N/N)], BALB/c [NOD.C6(B/B)], or both [NOD.C6(N/B)] loci as defined by D6Mit60. Additional microsatellite analysis of the eighth backcross generation established that the BALB/c loci extended from D6Mit52 telomeric to D6Mit57 (data not shown).

Because type 1 diabetes is a complex polygenic disorder and both susceptibility and resistance loci have been identified (17,18,21,23), it was possible that BALB/c susceptibility and/or resistance genes might be located within or adjacent to the D6Mit52–57 interval. The presence of such elements could affect diabetes onset in our new model independent of TCR expression. To test this, we followed the onset of diabetes among male and female nontransgenic NOD.C6 mice. Onset of hyperglycemia was nearly identical among NOD.C6 mice of all three genotypes [NOD.C6(BB), (N/B), or (N/N)] (Fig. 5E and F). Onset of hyperglycemia among NOD.C6 females followed the typical time course for NOD mice, where ~80% become diabetic at 3–7 months (33). Together, these data

suggested that even if BALB/c disease-resistance genes remained within the NOD.C6 genome, they had little influence on diabetes onset among nontransgenic mice.

To verify the deficiency in autoantigen, islet tissue from NOD.C6 mice was used to stimulate T-cells from either 6.9TCR/NOD or 2.5TCR/NOD mice. For comparison, islet tissue from BALB/c mice was also tested. Islets obtained from both BALB/c and NOD.C6 mice elicited robust IFN-γ secretion from 2.5TCR/NOD cells (Fig. 4B). This is consistent with the high frequency of BDC-2.5 TCR-expressing T-cells in 2.5TCR/NOD mice and the known reactivity of the parental BDC-2.5 T-cell clone, neither of which can discriminate between NOD and BALB/c islet antigen (32). In contrast, 6.9TCR/NOD cells produced little or no IFN-γ in response to either islet source. Even though islet tissue from NOD.C6 mice maintained expression of at least one naturally occurring islet autoantigen (recognized by 2.5TCR/NOD cells), expression of the BDC-6.9-specific antigen was not detectable.

In the absence of NOD β-cell autoantigen, 6.9TCR transgenic mice do not become hyperglycemic. Once established, eighth-generation NOD.C6 or NOD.C6(N/B) mice were bred to 6.9TCR/NOD mice to generate transgenic mice that possessed NOD [6.9TCR/NOD.C6(N/N)], BALB/c (6.9TCR/NOD.C6), or both [6.9TCR/NOD.C6(N/B)] loci at D6Mit60. As with the 6.9TCR/NOD transgenics, nearly all peripheral T-cells from the 6.9TCR/NOD.C6 mice expressed Vβ4 (Fig. 5A and B). Expression was independent of chromosome 6 genotype, demonstrating that 6.9 T-cells successfully completed thymic positive selection even in the absence of endogenous autoantigen (Fig. 5A and B) (Tables 2 and 3). However, the level of Vβ4 expression was lower in peripheral CD4 cells from transgenic mice compared with that of controls (Fig. 5B). Reduction in TCR levels does not appear to result from peripheral downregulation of receptor expression since similar levels are found on single positive CD4 thymocytes of transgenic mice. Rather, this may reflect the inherent level of expression required for 6.9TCR transgenic CD4 cells to proceed successfully through thymic education, avoiding negative selection. Given the major histocompatibility complex class II restricted nature of the 6.9TCR, it was not surprising that thymic profiles of transgenic mice exhibited strong skewing to the CD4 lineage. However, the presence of double-negative CD3-positive thymocytes was unexpectedly increased in all 6.9TCR transgenic mice [Fig. 5A: percentage double negative (mean ± SD) for nontransgenic NOD.C6(N/N), 3.0 ± 0.3; for 6.9TCR/NOD.C6(N/N), 28.0 ± 11.0; and for 6.9TCR/NOD.C6(B/B), 28 ± 5.1%]. This likely results from early TCR expression during

TABLE 3
Continued

Mean percentage of positive cells and 4-to-8 ratios									
6.9TCR/NOD.C6(N/N)				6.9TCR/NOD.C6(B/B)					
% V β 4*	CD8	% V β 4†	4 to 8	DP	CD4	% V β 4*	CD8	% V β 4†	4 to 8
92.8 \pm 3.9‡	29.0 \pm 3.1	99.2 \pm 0.4‡	1.7 \pm 0.1	—	54.7 \pm 6.9‡	93.8 \pm 3.4‡	23.7 \pm 5.2	99.3 \pm 0.6‡	2.4 \pm 0.7
88.2 \pm 5.5‡	22.2 \pm 4.6‡	98.2 \pm 1.1‡	2.7 \pm 0.6	—	60.5 \pm 5.1‡	78.8 \pm 24.0‡	19.7 \pm 4.9‡	90.4 \pm 7.4‡	3.3 \pm 0.9
—	10.1 \pm 3.1‡	—	—	26.0 \pm 8.0	32.7 \pm 14.0	—	13.7 \pm 4.1	—	—

the double-negative stage of thymocyte development (data not shown), as has been previously described (26) with other transgenes using the same promoter. In some, but not all, 6.9TCR-Tg/NOD.C6(N/N) mice, a reduction in the percentage of double-positive thymocytes was observed compared with that of nontransgenic or 6.9TCR-Tg/NOD.C6(B/B) mice (Fig. 5A). This may be secondary to diabetes-induced elevations in glucocorticoids (34,35) because loss of double-positive thymocytes occurred in mice that were hyperglycemic at the time of analysis. Analysis of other 6.9TCR-Tg/NOD.C6(N/N) mice that were not yet hyperglycemic yielded profiles similar to those of 6.9TCR-Tg/NOD.C6(B/B) mice (Table 3). Since increased glucocorticoid levels are associated with hyperglycemia and can result in increased thymocyte apoptosis (36,37), the loss of double-positive thymocytes in some 6.9TCR-Tg/NOD.C6(N/N) mice may be glucocorticoid induced.

As with 6.9TCR/NOD mice, we observed an acceleration of disease onset among both male and female 6.9TCR/NOD.C6(N/N) mice (from backcross generations 5–8) compared with that of nontransgenic littermates (Fig. 5C and D). Hyperglycemia was detected as early as 21 days of life. Disease onset was also significantly accelerated among transgenic mice possessing only one NOD allele [6.9TCR/NOD.C6(N/B)]. However, disease penetrance before 12 weeks appeared to be reduced compared with that of 6.9TCR/NOD mice (compare Fig. 3A and Fig. 5C and D). This difference notwithstanding, diabetes still progressed with an overall accelerated time course for 6.9TCR/NOD.C6(N/N) and 6.9TCR/NOD.C6(N/B) mice.

Most importantly, and in contrast to transgenic NOD.C6 mice containing NOD alleles within the C6 locus, it was found that with 6.9TCR/NOD.C6 mice, in which the C6 locus is homozygous for BALB/c, no animals became hyperglycemic during the course of this study. This was true for both males and females over a period of up to 349 days. Despite the complete lack of hyperglycemia among 6.9TCR/NOD.C6 mice, islet infiltration was still evident (Fig. 6A). In young (8- to 13-week-old) mice, the extent of infiltration (Fig. 6B) and degranulation among 6.9TCR/NOD.C6 mice was relatively low compared with their 6.9TCR/NOD counterparts, but was still detectable. Whether this islet inflammation is due to a small population of nontransgenic islet-reactive T-cells or to an unrelated defect in immune function is currently unclear and awaits analysis of 6.9TCR/NOD.C6 mice on a *scid* or *Rag*^{null} background.

Because peri-insulitis without frank diabetes occurs in 6.9TCR/NOD.C6 mice, it was possible that an abnormality in the transgenic T-cell pool existed, rendering them no longer diabetogenic. However, either purified CD4 lymph node T-cells or unseparated splenocytes from nondiabetic

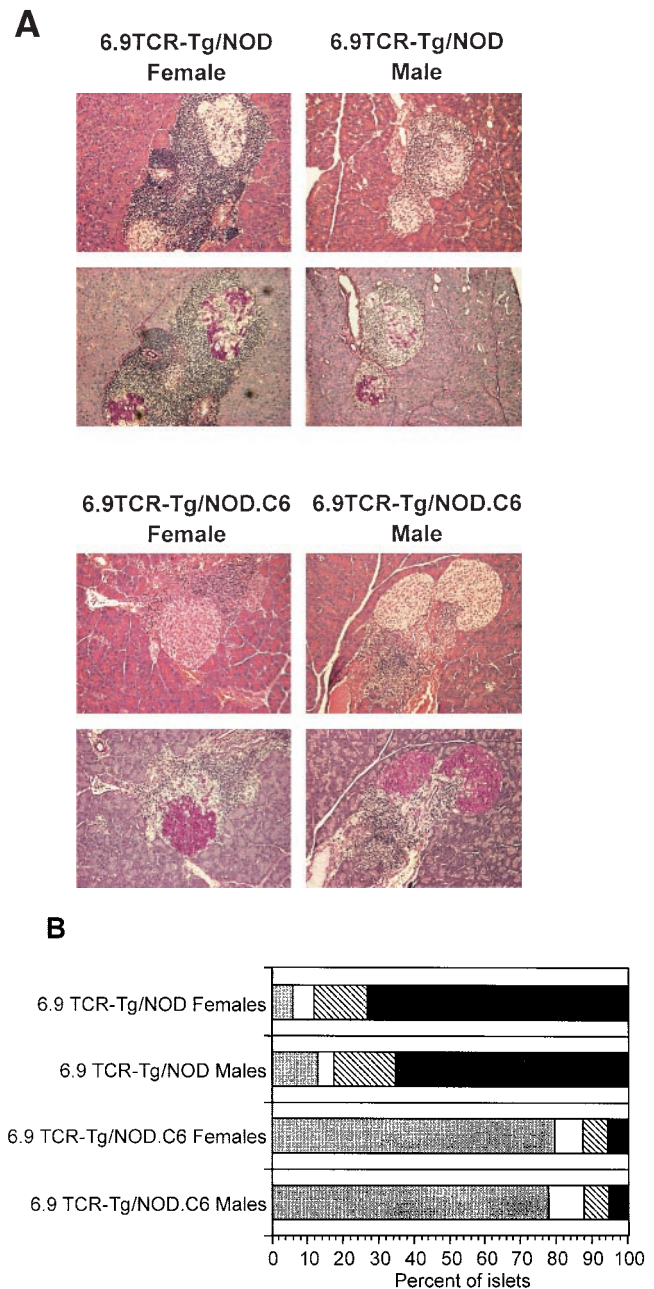


FIG. 6. Mild islet infiltration is evident in 6.9TCR/NOD.C6 mice. A: Representative photomicrographs (original magnification, $\times 20$) of hematoxylin and eosin (*top*)– or aldehyde-fuchsin (*bottom*)–stained pancreatic tissue sections from 8- to 13-week-old 6.9TCR/NOD mice or age-matched 6.9TCR/NOD.C6 mice. B: The extent of islet inflammation in 6.9TCR/NOD.C6 mice was low compared with that of 6.9TCR Tg/NOD mice. Data are compiled from pancreas sections taken from 8- to 13-week-old 6.9TCR/NOD and age-matched 6.9TCR/NOD.C6 mice (3–9 mice/group) as described in RESEARCH DESIGN AND METHODS. □, no infiltrate; ▨, polar infiltrate; ▩, peri-islet infiltrate; ■, intra-islet infiltrate.

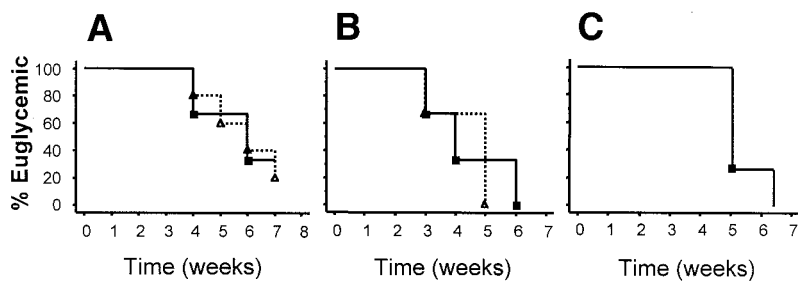


FIG. 7. Splenocytes from diabetes-resistant 6.9TCR/NOD.C6 mice retain their diabetogenic potential upon transfer into NOD-*scid* recipients. Splenocytes harvested from nondiabetic 6.9TCR-Tg/NOD.C6 (■) or diabetic nontransgenic (△) littermates were injected into NOD-*scid* adult (A) or neonatal (B) mice. CD4 cells (10×10^6 cells per injection, two injections per recipient, 1 week apart) were purified from the lymph nodes of nondiabetic 6.9TCR/NOD.C6 and were injected into 9-day-old NOD-*scid* mice (C). Blood glucose levels were measured weekly, following cell transfer. Data are represented in Kaplan-Meier plots. No significant difference was observed between groups.

6.9TCR/NOD.C6 mice were capable of transferring disease to NOD-*scid* recipients (Fig. 7A–C). The transfers with purified CD4 T-cell populations are especially compelling since these experiments indicate that transfer of disease by the transgenic T-cells does not require the presence of CD8 T-cells, recapitulating the behavior in vivo of the diabetogenic parent BDC-6.9 clone. The kinetics of disease transfer were similar to those observed with the transfer of splenocytes from diabetic nontransgenic littermates. Thus, despite the lack of diabetes in 6.9TCR/NOD.C6 mice, CD4 transgenic T-cells retained their diabetogenic function when placed in an antigen-competent host.

DISCUSSION

Generation of 6.9TCR transgenic mice has allowed the development of a flexible model of autoimmune diabetes whereby controlled expression of a naturally occurring autoantigen effectively determines disease development. As is observed with the BDC-6.9 T-cell clone, T-cells from the 6.9TCR transgenic mouse respond only to islet cells from NOD mice and not to BALB/c islet cells. This feature of restricted islet cell antigen specificity of the BDC-6.9 clone allowed for the expression of the TCR in a NOD mouse lacking the autoantigen for these T-cells. The NOD.C6 congenic mouse is BALB/c in the chromosome 6 interval containing the BDC-6.9 antigen locus, and the expression of the BDC-6.9 TCR in these mice leads to a transgenic animal that is protected against developing diabetes. In the 6.9TCR mouse on the NOD.C6 background, we did not observe diabetes in animals for up to 1 year. Whereas transgenic mice with one or two NOD alleles on chromosome 6 (~67 cM) develop diabetes with accelerated kinetics, hyperglycemia does not occur if both alleles are of BALB/c origin. Importantly, splenocytes from 6.9TCR/NOD.C6 mice remain functionally capable of responding to cognate antigen as demonstrated by disease development following transfer into NOD-*scid* recipients. Therefore, absence of hyperglycemia in 6.9TCR/NOD.C6 mice most likely reflects a change in islet cell autoantigen expression.

The 6.9TCR/NOD mouse expresses TCR genes from BDC-6.9, a highly diabetogenic clone from our panel of Th1 cell clones (29). Unlike the situation with the TCR transgenic mouse made from the BDC-2.5 clone from our panel, in which diabetes incidence is quite low (13), the expression of the BDC-6.9 TCR on the NOD background results in a mouse exhibiting a markedly accelerated disease phenotype, with 60% of male and female transgenic animals developing hyperglycemia before 12 weeks of age. In this respect, the 6.9TCR/NOD mouse more closely resembles the 4.1-NOD TCR transgenic mouse described by Schmidt et al. (38), in which the T-cell

repertoire was also derived from a pathogenic CD4+ T-cell clone.

Although autoreactive T-cells appear in the periphery of normal mice (and humans), their activation is controlled and autoimmunity is prevented by peripheral tolerance mechanisms that include regulatory cells (15,39,40). In some cases, regulatory T-cells are effective even in the presence of a nearly monospecific autoreactive T-cell population (13,41,42), and, for example, DX5⁺ cells limit the diabetogenic effect of T-cells in 2.5TCR/NOD mice (13). Our results would suggest that regulatory T-cells may not be effective at preventing disease in 6.9TCR/NOD mice, or, alternatively, these cells may be absent or severely reduced in number. The reason(s) for the striking difference in disease outcome between 6.9TCR/NOD (or 4.1-NOD) and 2.5TCR/NOD is not clear. One possibility is that autoantigen-specific T-cells may differ in affinity for antigen, trafficking capability, or other unknown characteristics, making them more or less affected by regulatory T-cells. Alternatively, differences in transgene expression and TCR α allelic exclusion may result in thymic development, which allows production of regulatory cells in the 2.5TCR mouse, but not the 6.9TCR mouse. Studies aimed at discriminating between these possibilities await analysis of transgenic NOD-*scid* or NOD-*Rag*^{null} mice.

Both susceptibility (*Idd6* and *Idd20*) and resistance (*Idd19*) loci have been identified on chromosome 6 in crosses with *Mus spretus*, feral PWK, and the more closely related C3H mouse strains (43–45). However, recent mapping studies (32,45) show that none of these loci overlap with the locus controlling the BDC-6.9 autoantigen. Inheritance of BALB/c-derived susceptibility or resistance genes would be expected to change the incidence and/or kinetics of hyperglycemia. Although we cannot formally rule out simultaneous inheritance of susceptibility and resistance genes in the NOD.C6 mice, disease onset among nontransgenic NOD.C6 mice was nearly identical to that of NOD mice, strongly suggesting that NOD.C6 mice did not inherit BALB/c disease-modifying genes on chromosome 6.

In current NOD mouse model systems of autoimmune diabetes, determining whether an immune phenotype associated with disease development, such as increased activation-induced expression of type 1 cytokines (46), is caused by a genetic predisposition to disease or results from the disease process itself can be difficult. For example, these two possibilities cannot adequately be deciphered with current model systems where subclinical disease may be present (e.g., male NOD or young female NOD) or T-cells are absent (e.g., NOD-*scid*). The 6.9TCR/NOD.C6 congenic mouse, which possesses the genetic predisposition of the NOD strain but does not become hyperglycemic, may provide a new approach to separating

disease development and genetic susceptibility. In other TCR transgenic mice produced with TCR from autoreactive T-cell clones, such as the 2.5TCR mouse, it cannot be determined at what point the T-cells are "naïve" for their self-antigen (47). However, in the 6.9TCR/NOD.C6 mouse in which the islet autoantigen is absent, T-cells arising in this mouse are truly naïve. Unchallenged mice will provide an opportunity to evaluate naïve T-cell function both in vitro and following in vivo immunization with peptide or cellular antigen. Therefore, 6.9TCR/NOD.C6 mice may be helpful in future studies aimed at defining complex susceptibility traits that might influence basal immune function in NOD mice.

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