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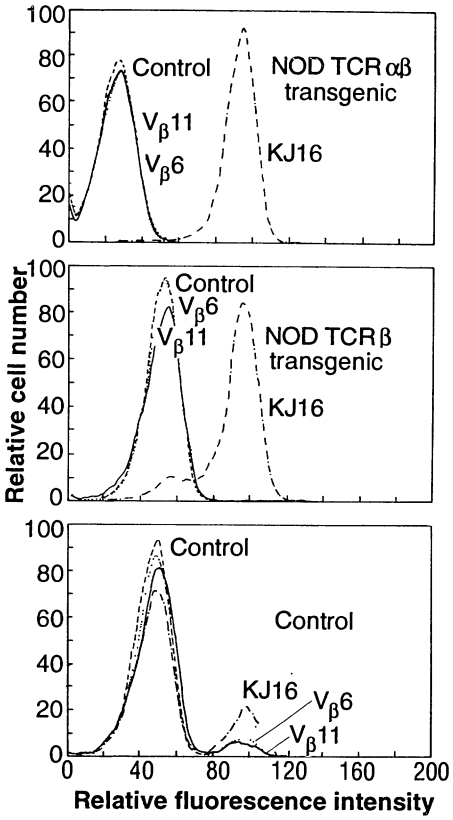

Fig. 1. Phenotype of peripheral T lymphocytes of transgenic αβ (upper), β (middle), and control (nontransgenic littermate) (lower) NOD mice by FACS analysis. Cell suspensions from pooled mesenteric, axillary, and inguinal lymph nodes were prepared by gentle teasing through a wire screen and T cells enriched by passage over an anti–immunoglobulin M immunoaffinity column (Biotex). T cells were stained as described (*3*) and analyzed on an Ortho Diagnostics Inc. Cytofluorograf IIs analyzer, gating for size by forward light scatter. The following MAb and antisera were used for staining: KJ16 [(*6*), reactive to $V_β$8.1 and $V_β$8.2], RR-4.7 [anti-$V_β$6; (*8*)], RR3.15 [anti-$V_β$11; (*9*)]; FITC-conjugated goat antibody to rat immunoglobulins (KPL) was used as a secondary antibody. Equivalent numbers of cells were counted for the control and the transgenic mice. Flow cytometry on other transgenic β and αβ NOD mice confirmed the data shown.

Progression to Diabetes in Nonobese Diabetic (NOD) Mice with Transgenic T Cell Receptors

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The T cell receptor (TCR) requirements in the pathogenesis of insulin-dependent diabetes were examined with transgenic NOD mice bearing nondisease-related TCR α and β chains. In both TCRβ and TCRαβ transgenic NOD mice the β chain transgene was expressed by >98% of peripheral T cells. The α chain transgene was also highly expressed. Insulitis developed in both sets of transgenic animals with most of the lymphocytes in the lesion expressing the transgenic β chain and with depletion of the endogenous TCR $V_β$ genes. Nonetheless, NOD animals transgenic for TCRβ and TCRαβ developed diabetes similar to controls. Thus, skewing the TCR repertoire did not diminish autoimmune susceptibility in NOD mice.

The NOD mouse develops spontaneous insulin-dependent diabetes mellitus (IDDM), characterized by the invasion of lymphocytes into pancreatic islets (insulitis) and the selective destruction of the insulin-producing pancreatic beta cells, similar to human type I diabetes. IDDM in both NOD mice and humans is probably T cell–mediated (*1*), but the autoantigens that trigger the autoimmune response are unknown. One approach to examining the role of T cells in IDDM and the antigens that drive them has been the derivation of islet-specific T cell clones. Characterization of the TCR gene usage from these clones has shown that T cells with receptors of diverse sequences can transfer insulitis and diabetes (*2*). Nonetheless, the issue of preferential TCR gene usage in islet targeting remains unsettled.

To investigate the importance of the TCR repertoire in IDDM we created transgenic NOD mice. These mice predominantly expressed single TCRβ or TCRαβ sequences that were not related to diabetes; their endogenous repertoire was suppressed by allelic exclusion. We reasoned that if the pathogenic T cells in diabetes were focused on a very limited set of peptide epitopes, autoantigen recognition would be dependent on a limited number of specific TCR and depleting these sequences would pro-

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tect against disease. To our surprise, dramatically skewing the TCR repertoire neither prevented the development of insulitis nor slowed the kinetics of diabetogenesis in NOD mice.

The β chain transgene consisted of the rearranged allele (V_β8.2, D_β1, J_β1.1, and C_β2) from the T cell hybridoma DO11.10 and the murine immunoglobulin heavy chain enhancer element (3). Two different lines of transgenic NOD mice expressing this β chain were established. NOD mice transgenic only for the β chain were created by mating NOD mice to a β chain transgenic founder that was the product of mating between (C57BL/6 × SJL)F_1 hybrid mice. The progeny that carried the transgene were backcrossed to NOD mice. The first backcross generation mice was doubly selected for the presence of the transgene and for *H-2*^{g7} homozygosity by Southern blot analysis of tail DNA. These animals were then progressively backcrossed to NOD mice, selecting for the transgene, to the ninth backcross generation.

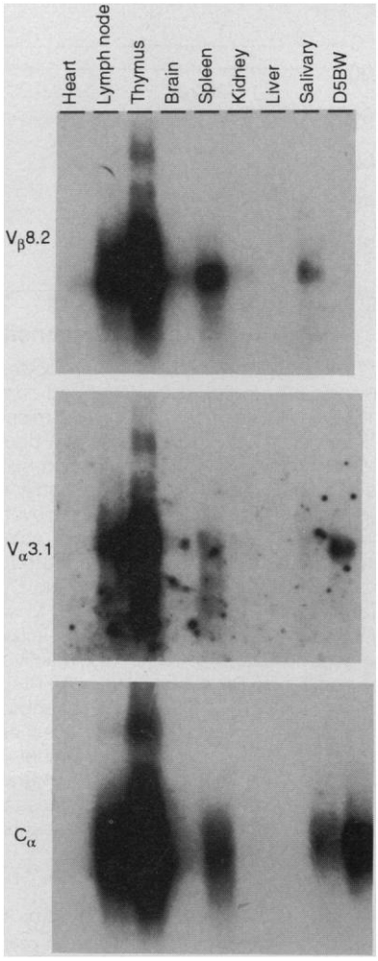

Fig. 2. Tissue specificity of transgene transcription in TCR αβ chain transgenic NOD mice. Total cellular RNA (10 μg) was isolated from various organs of a transgenic mouse, separated on a 1% agarose formaldehyde gel, transferred to nitrocellulose, and sequentially hybridized to ^{32}P-labeled V_α3.1 and V_β8.2 and C_α probes, and exposed to Kodak XAR films for 18 to 72 hours (*11*). Expression of V_β8.2 (1.3 kb), V_α3.1 (1.7 kb), and C_α (1.7 kb) was as indicated. The same blot was sequentially hybridized with each probe and stripped between hybridizations. Total RNA (10 μg) derived from the V_α3.1-expressing D5BW hybridoma was run as a positive control.

Double αβ transgenic NOD mice were generated by coinjecting the same TCRβ transgene and a functionally rearranged TCRα gene directly into NOD pronuclei. The α transgene was derived from the cytotoxic T cell clone 2C, which has specificity for the L^d class I major histocompatibility complex (MHC) antigen (*4*) and contained the immunoglobulin heavy chain enhancer element between the leader and the V region segments. One transgenic male founder mouse had integrated several copies of both the α and β transgenes as determined by hybridizing V_α3.1 and V_β8.2 probes to a Southern blot of tail DNA. All transgenic offspring from this founder possessed both α and β transgenes, indicating that the transgenes had integrated together into a single chromosomal site (*5*).

To characterize cell surface expression of the TCRβ transgene and other V_β families in the transgenic β and transgenic αβ mice, we analyzed the T cells by flow cytometric analysis with a panel of monoclonal antibodies (MAbs) (Fig. 1). About 94% and 98% of the T cells from the β and αβ transgenic mice, respectively, reacted with the MAb KJ16 (anti-V_β8.1 and V_β8.2) (6). Because 85.5% and 94.8% of this T cell population from β and αβ mice, respectively, were $CD3^+$ (*7*), most peripheral T cells expressed the transgene on their cell surface. In contrast, when expression of V_β6 (*8*) and V_β11 (9) was examined, staining of T cells from both the β and αβ transgenic mice was negligible (<0.6%), whereas expression in control (transgene-negative littermates) was 8.3% and 6.5%, respectively (Fig. 1). In addition, V_β5 (*10*) expression was undetectable in β and αβ transgenic mice, compared to 2% in control mice. Thus, the presence of the β transgene in both β and αβ TCR transgenic mice resulted in its surface expression on most T cells with few endogenous NOD TCRβ genes expressed. This observation is consistent with the process of allelic exclusion. These V_β expression patterns were preserved in diabetic β and αβ TCR transgenic mice, indicating that "escape" from allelic exclusion did not account for the progression to disease.

The transgenic V_α3.1 gene segment was not recognized by the clonotypic MAb IB2

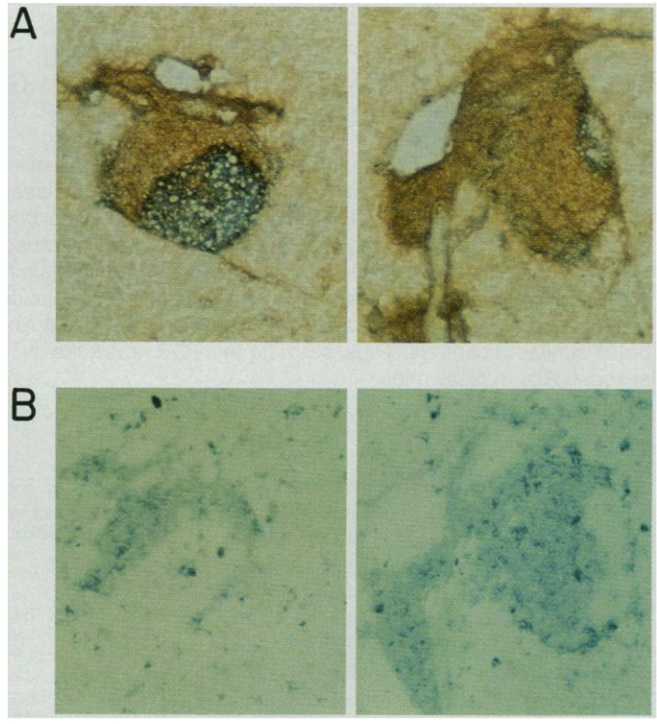

Fig. 3. Serial sections of pancreas from a 3-month-old β chain transgenic NOD mouse demonstrating that the majority of the T cells in the lesion express the transgene-encoded gene product. Analysis of the insulitis lesions of TCRαβ transgenic mice showed similar findings. (**A**) Double-staining of pancreatic islets with the MAb to T cell antigen Thy1.2 (stained brown) and the anti-islet MAb A2B5 (stained blue). Pancreata were snap-frozen in liquid nitrogen, 4-μm-thick sections were fixed with acetone, and the sections incubated for 15 min with biotin-conjugated Thy1.2 (Becton Dickinson). MAb A2B5 ascites (*15*) were then added and after washing were incubated with peroxidase-conjugated Streptavidin (Sigma). Alkaline phosphatase conjugated to antibodies to mouse immunoglobulin M was added and after washing incubated with DAB and hydrogen peroxide, then developed in a staining solution consisting of levamisole (Sigma) and Fast Blue (Sigma). (**B**) Staining with MAb KJ16 (anti-V_β8) (stained blue). Sections were prepared as described above, incubated for 45 min with KJ16 supernatant (straight), washed, incubated with alkaline phosphatase conjugated to antibodies to rat immunoglobulin (Sigma), washed, and stained as above.

(4), which recognizes an epitope whose conformation is dependent on both the V_α3.1 and V_β8.2 chains of the 2C TCR. Northern (RNA) blot analysis (*11*), however, with a V_α3.1-specific probe revealed an abundant full-length transcript of 1.7 kb in the thymus, lymph nodes, and spleen (Fig. 2). V_α3.1 expression was not detected in lymph node or spleen cells of nontransgenic NOD mice under the same hybridization conditions. Faint transcripts of V_α3.1 (and V_β8.2) transgenes were present in the salivary glands of αβ transgenic mice, consistent with the histological evidence of severe infiltation of V_β8-bearing T cells in the salivary glands. The V_α3.1 and V_β8.2 transcripts could not be detected in other nonlymphoid tissues (liver, kidney, heart, and brain).

To quantitate the relative abundance of the V_α3.1 transgene mRNA in the lymph nodes of the TCRαβ transgenic mice, we cloned the expressed TCRα mRNA, by anchored polymerase chain reaction (PCR) (*12*). We observed that 83% (20/24) of the C_α-positive cDNA plasmid clones derived from an αβ transgenic mouse were V_α3-positive. In contrast, <5% (0/20) of the C_α-positive clones derived from the nontransgenic littermate were V_α3-positive (*13*).

To confirm the frequencies of the TCRα transgene, we generated T cell hybridomas by fusing nylon wool–purified splenic T cells from an αβ transgenic and nontransgenic littermates with the TCRαβ negative cell line BW5147 (*14*). Slot blot hybridization of mRNA prepared from each clone with radioactive V_α3.1 and C_α DNA probes showed that at least 12/14 (86%) of T cell clones expressed the V_α3.1 transgene. The remaining two hybridomas had a weak C_α signal and thus the expression of V_α3.1 could not be determined with certainty. All the V_α3.1-positive clones were V_β8.2-positive. The TCRα and TCRβ transgenes were thus coexpressed by the majority of T cells. This high expression may have been aided by the tandem integration of the coinjected α and β transgenes and by the inclusion of the immunoglobulin H enhancer in these constructs.

Both the β and αβ transgenic mice developed insulitis similar to nontransgenic littermates. To determine whether T cells in the lesion expressed the β transgene, we double-stained cryostat sections of pancreas with a MAb to the T cell marker Thy1.2 and a MAb to islet ganglioside, A2B5 (*15*), and then examined adjacent sections stained with the MAb KJ16 (anti-V_β8) (Fig. 3). Most of the T lymphocytes in the lesions (Fig. 3A) expressed the transgene-encoded β chain (Fig. 3B).

We amplified the rearranged TCR genes from cells in the lesion by PCR (*16*) (Fig. 4). Cell lysates were prepared from frozen tissue sections (*17*). Oligonucleotide primers corresponding to all 17 of the sequenced murine V_β subfamilies and a J_β2 primer from the J_β2.6 region were used (*18*); only the rearranged genes were efficiently amplified (Fig. 4A). Specificity of the amplified products was assessed by hybridization under high stringency conditions with an oligonucleotide probe internal to the primers. This system will detect the native V_β-J_β2 rearrangements but will not detect the transgene, which uses J_β1.1 (and thus belongs to the other J family). The intensity of the signal from the different V_β gene segments reflects both the prevalence of the individual V_β regions and the efficiency of amplification of the particular V_β-J_β2.6 primer combination.

The lesions of the nontransgenic NOD littermates showed many V_β-chain gene families with a complex pattern of amplifi-

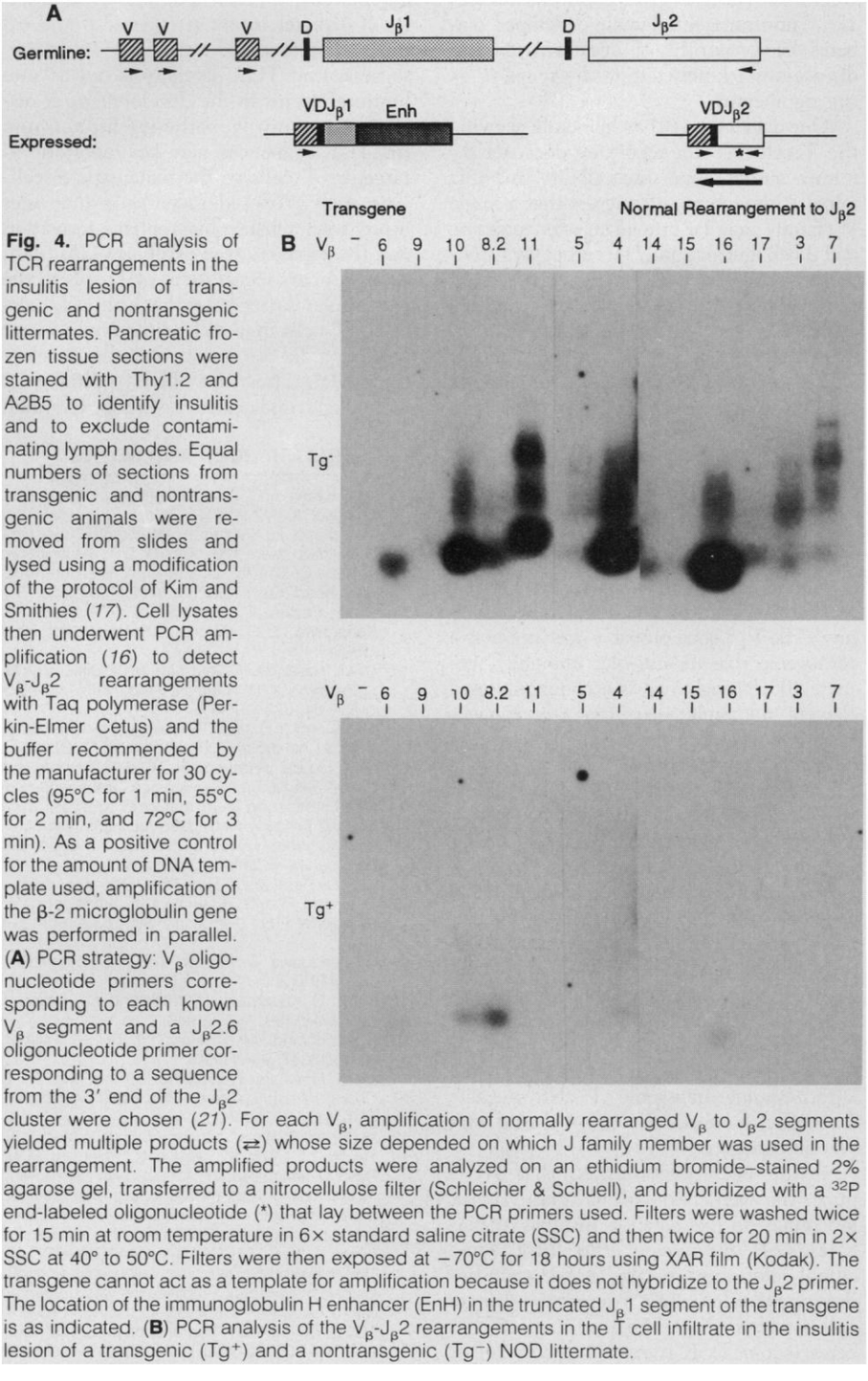

Fig. 4. PCR analysis of TCR rearrangements in the insulitis lesion of transgenic and nontransgenic littermates. Pancreatic frozen tissue sections were stained with Thy1.2 and A2B5 to identify insulitis and to exclude contaminating lymph nodes. Equal numbers of sections from transgenic and nontransgenic animals were removed from slides and lysed using a modification of the protocol of Kim and Smithies (*17*). Cell lysates then underwent PCR amplification (*16*) to detect V_β-J_β2 rearrangements with Taq polymerase (Perkin-Elmer Cetus) and the buffer recommended by the manufacturer for 30 cycles (95°C for 1 min, 55°C for 2 min, and 72°C for 3 min). As a positive control for the amount of DNA template used, amplification of the β-2 microglobulin gene was performed in parallel. (**A**) PCR strategy: V_β oligonucleotide primers corresponding to each known V_β segment and a J_β2.6 oligonucleotide primer corresponding to a sequence from the 3′ end of the J_β2 cluster were chosen (*21*). For each V_β, amplification of normally rearranged V_β to J_β2 segments yielded multiple products (⇄) whose size depended on which J family member was used in the rearrangement. The amplified products were analyzed on an ethidium bromide–stained 2% agarose gel, transferred to a nitrocellulose filter (Schleicher & Schuell), and hybridized with a ^{32}P end-labeled oligonucleotide (*) that lay between the PCR primers used. Filters were washed twice for 15 min at room temperature in 6× standard saline citrate (SSC) and then twice for 20 min in 2× SSC at 40° to 50°C. Filters were then exposed at −70°C for 18 hours using XAR film (Kodak). The transgene cannot act as a template for amplification because it does not hybridize to the J_β2 primer. The location of the immunoglobulin H enhancer (EnH) in the truncated J_β1 segment of the transgene is as indicated. (**B**) PCR analysis of the V_β-J_β2 rearrangements in the T cell infiltrate in the insulitis lesion of a transgenic (Tg⁺) and a nontransgenic (Tg⁻) NOD littermate.

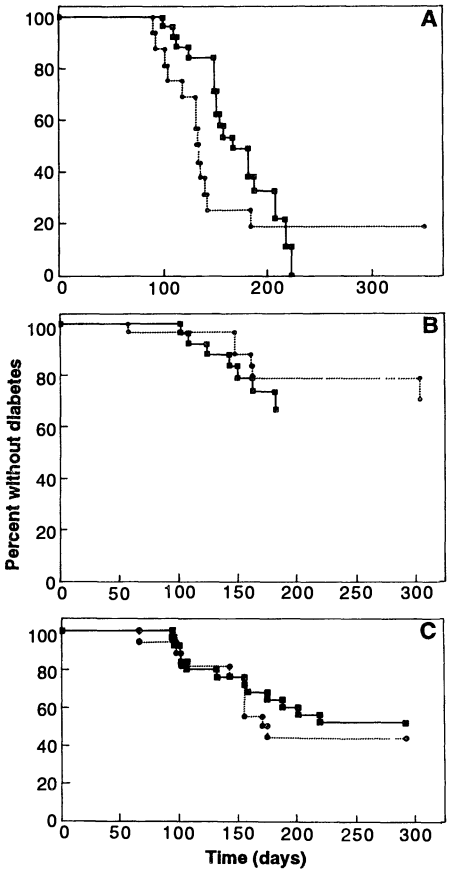

Fig. 5. Lifetable analysis of the progression to diabetes in NOD transgenic (●---●) and nontransgenic (■—■) littermates. (**A**) Comparison of female αβ transgenic and nontransgenic littermates. (**B**) Comparison of male αβ transgenic and nontransgenic littermates. (**C**) Comparison of female TCRβ transgenic chain and nontransgenic littermates.

cation, consistent with a polyclonal distribution of V_β-chain gene usage (Fig. 4B). In contrast, the signals amplified in the lesion of the transgenic animals were diminished, with rearrangement of most V_β-chain families undetectable. Therefore, the TCR repertoire was altered not only in the periphery but also in the lesion of the transgenic NOD mice. The few faint signals detected in the lesion of the pancreas of the transgenic NOD mice were also amplified to a similar degree in the thymus of these animals, suggesting that these signals do not represent clonal outgrowths of TCR subpopulations in the lesion. These rearranged genes, however, may not be expressed as functional receptors (*19*).

In spite of this perturbation of the TCR repertoire, both the transgenic β and αβ NOD mice developed diabetes similar to control nontransgenic littermates (*20*). Life-table analysis of the risk of progression to diabetes revealed that the time of onset of disease in the female αβ transgenic NOD mice was slightly accelerated compared with nontransgenic female littermates (Wilcoxon rank-sum test, $P = 0.01$), with 14/18 transgenic versus 18/25 nontransgenic females developing diabetes by 6 months of age (Fig. 5A). No significant difference in the disease kinetics, however, was noted in the male αβ mice (Wilcoxon rank sum test, $P = 0.41$) with 6/29 transgenic versus 7/25 nontransgenic littermates developing diabetes by 6 months of age (Fig. 5B). In the seventh and eighth backcross generation of TCRβ transgenic mice (Fig. 5C) 9/17 transgenic littermates versus 12/25 nontransgenic female developed diabetes by 6 months of age, with similar disease-onset kinetics in both groups (P = not significant).

Our data suggest that markedly skewing the T cell receptor repertoire does not diminish autoimmune susceptibility in NOD mice. Although reports suggest that a single V_β family may be critical in islet targeting and β cell destruction (*21*), subsequent studies show considerable heterogeneity in TCR gene usage in islet-specific, diabetogenic T cell clones (*2*), perhaps suggesting that diabetes results from a response to a complex antigen or multiple antigens. Our findings are consistent with the hypothesis that there may be sufficient antigen complexity in the NOD mouse to give rise to a multitude of self-epitopes such that T cells with a wide variety of specificities and receptor sequences (including the transgene-bearing T cells) can mediate disease.

Could the β-chain transgene be playing a specific role in the induction of autoimmunity? The $V_\beta 8$ gene probably does not play a pathogenic role in anti-islet immunity, because (i) although $V_\beta 8$ is a commonly used element in immune recognition, the frequency of $V_\beta 8$ in insulitis lesions of NOD mice is proportional to the peripheral blood (*2, 22*); (ii) when a deletion that included $V_\beta 8$ was bred into NOD mice, the resultant $V_\beta 8$-deficient mice developed diabetes at similar rates to controls, demonstrating that $V_\beta 8$ (specifically $V_\beta 8.2$) is not required for diabetogenesis (*23*); (iii) when the submandibular salivary glands (which are consistently affected by cellular infiltration in NOD mice) of the TCRαβ transgenic mice were examined histologically, severe infiltration of transgene-bearing T cells was detected, similar to the insulitis lesions. It is thus unlikely that the Vβ8.2-bearing transgenic T cells mediate their effect by selectively cross-reacting with islet autoantigens.

It has been hypothesized that NOD mice transgenic for MHC class II I-E molecules do not develop insulitis or diabetes (*24*) because of the intrathymic clonal deletion of certain TCR V_β families that might otherwise participate in disease pathogenesis (*21*). The insulitis and diabetes in our TCR transgenic NOD mice, despite elimination of virtually the entire endogenous NOD V_β repertoire, provides further evidence that the protective effect of I-E in NOD mice is not mediated by the clonal deletion of particular TCR V_β families (*24*).

Our studies suggest that an intact TCR repertoire is not required for the generation of pancreatic β-cell destruction in the NOD mouse. Could a small number of specific T cells escaping allelic exclusion in our transgenic animals be initiating the autoimmune process? The preserved kinetics of diabetes in our transgenic β and αβ mice, despite a reduced TCR repertoire, suggests that TCR diversity is not a "rate limiting" factor in the development of disease. Alternatively, pathways distinct from αβ TCR sequences may be important in targeting T cells to the pancreatic β cell. One such proposed scenario is that islet injury results first in macrophage activation and the production of cytokines (*25*), with the secondary recruitment of T cells to the lesion. Our data suggest that although functional T cells may still be required for amplification in the lesion and the development of insulitis, the TCR specificity of individual lymphocytes may not be essential.

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12. Anchored PCR was performed as described by E. Y. Loh, J. F Elliott, S. Cwirla, L. L Lanier, and M. M Davis [*Science* **243**, 217 (1989)], with the following modifications. Total mRNA (5 μg) was reversed transcribed with the ribonuclease H$^-$ MMLV reverse transcriptase (BRL) using the C_α-specific primer (5'-GATTCCAAGAGCAATGGGGCC-3'). The first-strand cDNA was tailed with poly(dC) instead of poly(dG). Amplification was performed with a nested C_α primer (5'-CGGCACATTGATTTGGGAGTC-3') and the anchor primer (5'-GATAGTCGACGGGGGGGGGGG-3'). One-hundredth

of the amplified product was subjected to a second round of amplification using a third internal C_α primer (5′-GTGAACAGGCAGAGGGTGCTG-3′) and the same anchor primer. The amplified products were ligated to the Hph I–digested pCR1000 plasmid vector (Invitrogen) and transformed into the INVαF′ bacteria. The resultant kanamycin colonies were screened successively with C_α and V_α3.1 probes (same probes as described for Northern analysis).

13. TCRα surface expression and heterogeneity were assessed by subjecting immunoprecipitates from surface ^{125}I-labeled lymphocytes to two-dimensional electrophoresis employing pH-dependent separation in the horizontal dimension and then SDS–polyacrylamide gel electrophoresis in the vertical dimension [P. A. O'Farrell, H. M. Goodman, P. H. O'Farrell, *Cell* **12**, 1133 (1977)]. Although the basic β chain components of control NOD mice exhibited considerable microheterogeneity in terms of both charge and molecular size, there was little structural heterogeneity detectable in the acidic α chain components. These findings, which are similar to the reports of others [B. W. McIntyre and J. P. Allison, *Cell* **34**, 739 (1983); E. L. Reinherz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4104 (1983)], precluded analysis of the α chain repertoire by this approach.
14. We generated T cell hybridomas by fusing, according to the fusion procedure of L. Glimcher and E. M. Shevach [*J. Exp. Med.* **156**, 640 (1982)], nylon-wool–purified splenic T cells (1×10^7 to 2×10^7) from a 6-month-old transgenic and a nontransgenic littermate with the TCRαβ$^-$ thymoma [J. White *et al.*, *J. Immunol.* **143**, 1822 (1989)]. T cell hybridomas were cloned by limiting dilution. Total mRNA from 1×10^6 to 2×10^6 hybridoma T cells were prepared from each clone, and were transferred onto nitrocellulose by the slot-blot method (Schleicher & Schuell, Keene, NH). The same blots were sequentially hybridized with ^{32}P-labeled V_α3.1, V_β8.2, and C_α probes and stripped between hybridizations.
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A Functional Role for GTP-Binding Proteins in Synaptic Vesicle Cycling

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The squid giant synapse was used to test the hypothesis that guanosine-5′-triphosphate (GTP)–binding proteins regulate the local distribution of synaptic vesicles within nerve terminals. Presynaptic injection of the nonhydrolyzable GTP analog GTPγS irreversibly inhibited neurotransmitter release without changing either the size of the calcium signals produced by presynaptic action potentials or the number of synaptic vesicles docked at presynaptic active zones. Neurotransmitter release was also inhibited by injection of the nonhydrolyzable guanosine diphosphate (GDP) analog GDPβS but not by injection of AlF_4^-. These results suggest that a small molecular weight GTP-binding protein directs the docking of synaptic vesicles that occurs before calcium-dependent neurotransmitter release. Depletion of undocked synaptic vesicles by GTPγS indicates that additional GTP-binding proteins function in the terminal at other steps responsible for synaptic vesicle replenishment.

Rapid transmission of signals between neurons is achieved by secretion of neurotransmitters at synapses. Neurotransmitters are stored in synaptic terminals in membrane-bound organelles, the synaptic vesicles, and are secreted by the process of exocytosis. During repeated bouts of synaptic transmission, neurotransmitters are newly synthesized while synaptic vesicles are retrieved and refilled through a local cycling pathway (*1*, *2*). The molecular mechanisms that mediate the cycling of synaptic vesicles are not known. Because the trafficking of other intracellular organelles involves proteins that bind GTP (*3*) and such proteins are also associated with synaptic vesicles (*4*–*6*), GTP-binding proteins may regulate synaptic vesicle traffic (*6*). To test this proposal, we used the squid giant synapse to examine how the activation of GTP-binding proteins affects two consequences of vesicle trafficking: the release of neurotransmitters and the distribution of synaptic vesicles within the terminal.

Transmission across the giant synapse was assayed by measurement of postsynaptic currents (PSCs) or potentials (PSPs) that were evoked by presynaptic action potentials (*7*). Both iontophoretic and pressure microinjections of guanosine-5′-O-(3-thio-triphosphate) (GTPγS), a nonhydrolyzable analog of GTP, into the giant presynaptic terminal produced a slow and irreversible depression of synaptic transmission (Fig. 1A). Because the nucleotide was injected only into the presynaptic terminal, this inhibition reflects a decrease in neurotransmitter release from the terminal. This effect contrasts with the case of mast cells, where GTPγS stimulates secretion (*8*). In this experiment (Fig. 1A), release diminished to ~15% of its preinjection value when transmission was assayed 30 min after the start of the injection (at $t = 54$ min), and there was no reversal of the effect. Similarly, irreversible depression was ob-

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