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Immunology

Widespread expression of an autoantigen-GAD65 transgene does not tolerize non-obese diabetic mice and can exacerbate disease

(autoimmunity/tolerance/glutamic acid decarboxylase/diabetes/transgenic mice)

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ABSTRACT **Glutamic acid decarboxylase (GAD)65 is a pancreatic β cell autoantigen implicated as a target of T cells that initiate and sustain insulin-dependent diabetes mellitus (IDDM) in humans and in non-obese diabetic (NOD) mice. In an attempt to establish immunological tolerance toward GAD65 in NOD mice, and thereby to test the importance of GAD in IDDM, we generated three lines transgenic for murine GAD65 driven by a major histocompatibility complex class I promoter. However, despite widespread transgene expression in both newborn and adult mice, T cell tolerance was not induced. Mononuclear cell infiltration of the islets (insulitis) and diabetes were at least as bad in transgenic mice as in nontransgenic NOD mice, and in mice with the highest level of GAD65 expression, disease was exacerbated. In contrast, the same transgene introduced into mouse strain, FvB, induced neither insulitis nor diabetes, and T cells were tolerant to GAD. Thus, the failure of NOD mice to develop tolerance toward GAD65 reflects at minimum a basic defect in central tolerance, not seen in animals not predisposed to IDDM. Hence, it may not be possible experimentally to induce full tolerance toward GAD65 in prediabetic individuals. Additionally, the fact that autoimmune infiltration in GAD65 transgenic NOD mice remained largely restricted to the pancreas, indicates that the organ-specificity of autoimmune disease is dictated by tissue-specific factors in addition to those directing autoantigen expression.**

Non-obese diabetic (NOD) mice are widely used as a model for insulin-dependent diabetes mellitus (IDDM) in humans. In both, a gradual progression to diabetes is marked by insulitis, a prediabetic lymphocytic infiltration of the islets of Langerhans that is particularly strong in the NOD mouse (1, 2). Intensive effort is focused on elucidating polygenic (3, 4) and environmental factors (5, 6) responsible for insulitis and disease. Among the genetic components, the strongest is a polymorphism (identical in humans and NOD mice) at position 57 of the major histocompatibility complex (MHC) class II A-β chain (3, 4). This component emphasizes the fundamental role in disease played by MHC class II-reactive T cells. Additionally, mice lacking either $CD4^+$ or $CD8^+$ T cells fail to develop insulitis and disease (7–9).

Much research has focused on the targets of the T cell attack on the pancreas. Among putative autoantigens (10), glutamic acid decarboxylase (GAD) appears important in the prediabetic and the diabetic stages. In rodents and in humans, two isomers of GAD, defined by their molecular weights (GAD65 and GAD67) are respectively encoded by each of two genes (11–13). Autoantibodies against GAD65 first were detected in patients, sometimes years before clinical onset of disease (14, 15). Although autoantibodies to GAD65 also have been reported in young NOD mice (16), the more striking observation is that GAD65 is a target of autoreactive T cells during early insulitis (17, 18).

To assess the significance of such GAD-reactive T cells, two basic approaches have been taken. One has been to show that a GAD65-reactive T cell line can induce diabetes after adoptive transfer to irradiated NOD-severe combined immunodeficient (SCID) mice (R.S.S., unpublished results). The second has been to show that disease is affected by altering immunity to GAD. Thus, young NOD mice injected intrathymically or intravenously with GAD65 showed reduced proliferative T cell responses both to GAD65 and to other autoantigens, and insulitis and diabetes were reduced. Strikingly, inoculation with heat shock protein or peripherin reduced neither GAD immunoreactivity nor disease (17, 18). Interestingly, GAD65-inoculated mice maintained autoantibodies to GAD65, indicating that disease reduction was not dependent on complete immunological tolerance to GAD65. Likewise, diversion of the GAD-reactive inflammatory Th1 response toward a largely humoral, Th2 response inhibited disease progression in prediabetic mice and prolonged survival of syngeneic islets grafted into diabetic NOD mice (19). Diabetes also was reduced in NOD mice fed GAD67-transgenic plants (20).

T cell tolerance toward antigens can be achieved in the thymus (during T cell development) or in the periphery. T cells bearing T cell antigen receptors (TCRs) with strong affinity for complexes of MHC and self-peptide expressed on thymic antigen-presenting cells (APCs) undergo intrathymic (central) deletion during negative selection (21–24). In contrast, T cells with high affinity for self-peptides that are not obviously expressed in the thymus may be positively selected in the thymus (through weak interactions with other peptides), but functionally inactivated in the periphery (anergized) by engagement of the high affinity target on nonprofessional APCs (25). Rodent GAD65 expression has been reported for neurons, pancreatic islets, testis, and fallopian tubes (27, 28), none of which normally may tolerize lymphocytes. Therefore, because they are ignorant of their cognate antigen, GAD-reactive T cells in the periphery may remain inactive until congenital and/or environmental events induce presentation of GAD by professional APCs in the context of tissue damage and inflammation (24, 26). A prediction of this hypothesis is that congenitally induced, widespread expression of GAD should ameliorate GAD T cell reactivity, and hence the development of insulitis and diabetes. This prediction has been tested by the establishment of GAD65 transgenic NOD mice.

MATERIALS AND METHODS

Mice. NOD and NOD-SCID mice from Jackson Laboratory and FvB mice from Taconic Farms were bred locally, specific pathogen free. Diabetes was monitored regularly.

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Abbreviations: GAD, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes mellitus; NOD, non-obese diabetic; MHC, major histocompatibility complex; TCR, T cell antigen receptor; HGH, human growth hormone; SCID, severe combined immunodeficient.

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Generation of Transgenic (tg+) Mice. The transgenic construct (Fig. 1*A*) was made by inserting a 4-kb genomic *Eco*RI–*Nru*I DNA fragment containing the enhancer and promoter of mouse MHC class I H2-K^b into the *Xho*I site of pBluescript SK(+). Mouse GAD65 cDNA (gift of R. Tisch, Stanford University, CA) was inserted between the *Xho*I and *Hin*dIII sites. Human growth hormone gene sequences (2.1 kb) (gift of R. Perlmutter, University of Washington) extending from a *Bam*HI site in the first exon to the *Eco*RI site after the poly(A) addition signal were removed from vector p1017 (29) by *Bam*HI and *Not*I digestion and placed between *Eco*RV and *Not*I sites downstream of the GAD65 cDNA. The GAD65 cDNA was mutated by using PCR (30) by substituting leucine and alanine for the histidine-395 and lysine-396 that are critical to pyridoxal phosphate coenzyme binding (31). The MHC class I-GAD-human growth hormone (HGH) fragment was released by *Bss*HII digestion and microinjected into fertilized NOD or FvB eggs that then were transferred to pseudopregnant NOD or FvB mice. Transgene positive founders were identified by Southern blot of *Bgl*II-digested tail DNA by using a 700-bp GAD65 cDNA fragment as a probe (Fig. 1 *A* and *B*).

Analysis of mRNA Expression. Five micrograms of tissue RNA extracted with guanidium isothiocyanate/phenol (RNAzol, Cinna/Biotecx, Houston, TX) were primed with random hexamers, reverse-transcribed with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), and equal amounts (quantified by spectrophotometry) amplified by PCR. In the case of islets, cDNA was quantified by PCR titration, using hypoxanthine-guanine phosphoribosyltransferase (HPRT) primers. Transgene-specific primers were: 5′-ACT CTG TGA CAT GGA ATC CT-3′ and 5′-TTG GGA TAT AGG CTT CAA-3′; mouse HPRT-specific primers were: 5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′ and 5′-GAG GGT AGG CTG GCC TAT GGC T-3′. PCR conditions were 94°, 60°, and 72°C for 1′ each for 35 cycles.

Analysis of Transgenic Protein Expression. Tissues were homogenized in 1% Nonidet P-40 lysis buffer (plus protease inhibitors) in a dounce homogenizer at 4°C, and the 100,000 *g* supernatants quantified with a Bio-Rad ^{D}C Protein Assay Kit II (Bio-Rad). Equal amounts of lysates were immunoprecipitated

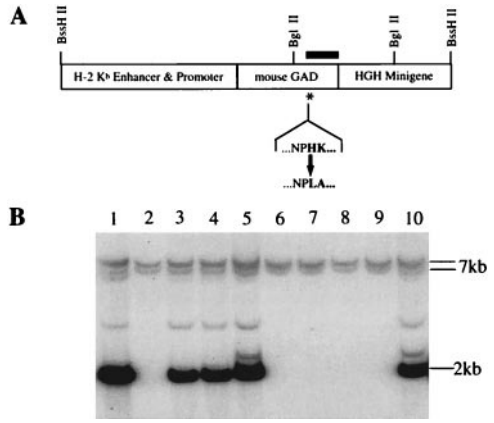

FIG. 1. GAD65 transgenic construct and Southern blot of tail DNA. (*A*) Map of the transgene, comprising the enhancer and promoter regions of the MHC class I K^b gene, ligated 5′ of the murine GAD65 coding region, ligated 5′ of a series of noncoding exons from the HGH gene. The introduced mutations replaced the pyridoxal phosphate binding motif NPHK with NPLA. The black bar represents the 700-bp probe used on Southern blots to detect a predicted 2-kb *Bgl*II fragment. (*B*) Southern analysis of *Bgl*II-digested genomic DNA extracted from mouse tails: lanes 1, 3, 4, 5, and 10 are tg(+) line 14; lanes 2, 6, 7, 8, and 9 are non-tg(+) littermates. The 7-kb doublet and 2-kb band correspond to endogenous and transgenic bands, respectively. Tg(+) mice of lines 12 and 13 gave essentially the same results as those shown for line 14.

with Stiff Man Syndrome sera (32) and Protein G Sepharose CL-4B beads (Pharmacia LKB). Proteins were transferred from SDS/8% polyacrylamide gels to poly(vinylidene difluoride) membrane (Bio-Rad), probed with mN65 mAb (33), and detected by enhanced chemiluminescence (DuPont). The membrane was stripped and reprobed with 7673 antisera (34).

Histochemistry and Assessment of Insulitis. Pancreata were fixed in 10% formalin, embedded in paraffin (35), sectioned, and stained with hematoxylin and eosin. By light microscopy, insulitis was scored as follows: −, islets free of insulitis; +, islets with peri-insulitis; ++, islets with lymphocyte infiltration in less than 50% of the area; +++, islets with lymphocyte infiltration in more than 50% of the area; ++++, islets completely filled with lymphocytes. Other organs were examined by the same method, and statistical differences was assessed by Mann–Whitney *U* test.

Immunocytochemistry on Frozen Sections. Tissue was fixed in periodate/lysine/paraformaldehyde (36), kept on ice for a minimum of 4 hr, processed through three consecutive sucrose solutions [10%, 20%, and 30% (wt/vol)], and snap-frozen in Tissue-Tek OCT compound (Miles) by submersion into 2-methylbutane (Aldrich). Six-millimeter sections were transferred onto lysine-treated glass slides and stained (37). Endogenous peroxidase activity was quenched by using 1.25% H_2O_2 in PBS (30 min). Slides then were preblocked with 3% BSA in PBS, Tween 20 (0.05%; 30 min). After washing, they were stained with biotin-labeled antibodies, as follows: anti-CD4 (clone RM4–5); anti-CD8 (clone 53–6.7); and anti-B220 (clone RA3–6B2) (all PharMingen, CA), for 1 hr at room temperature. Bound antibody was detected by using horseradish peroxidase-conjugated Streptavidin (30 min at room temperature), and developed with 3,3′-diaminobenzidine substrate solution. Hematoxylin was used lightly as a counterstain.

Diabetes Measurements. Weekly measurements of urine glucose by using Diastix (Miles) were confirmed by blood glucose measurements with One Touch Basic glucose meter (Lifescan, Mountain View, CA). Mice were scored diabetic if two consecutive measurements of blood glucose were greater than 250 g/dl. Statistical differences were assessed by Mann–Whitney *U* test and χ^2 test.

T Cell Proliferation Assays. Spontaneous reactivity to GAD65 was analyzed by culture of freshly isolated splenocytes from 7- to 10-week-old mice at 4×10^5 cells per flat-bottom well, in HL-1 serum-free medium supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, and 100 mg/ml of steptomycin. The cells, cultured in 96-well flat-bottom plates for 3 days, were pulsed with 1 μCi [^{3}H]thymidine for 18 hr, and assayed by scintillation counting. Statistical differences were assessed by Mann–Whitney *U* test. Previous immunization was by injection into hind footpads of either 25 μg of GAD65 (derived from rat brain and exhaustively purified as described in ref. 34) or ovalbumin, each dissolved in water at 1 mg/ml and thoroughly emulsified (1:1) with complete Freund's adjuvant (Sigma-Aldrich).

Adoptive Transfer of Diabetes. Spleen cells (10^7) from diabetic GAD65 transgenic mouse were adoptively transferred i.v. to 7-week-old female NOD-SCID mice. Recipients were monitored for diabetes biweekly thereafter.

RESULTS

Generation of Transgenic Mice and Demonstration of Transgenic mRNA and Protein Expression. Our previous failure to generate transgenic mice expressing GAD65 from an MHC class I promoter (unpublished work) suggested that ubiquitous expression of functional GAD65 might be toxic because of the widespread generation by GAD of γ-butyric acid, a major inhibitory neurotransmitter. To avoid this toxicity, histidine-395 and the adjacent lysine-396, which are critical to pyridoxal phosphate coenzyme binding, and hence to decarboxylase activity (30) (Fig. 1*A*), were mutated. The mutations are neither part of, nor close to, any known immunogenic epitopes of GAD65 (17, 38), and thus should not influence GAD65 processing or presentation.

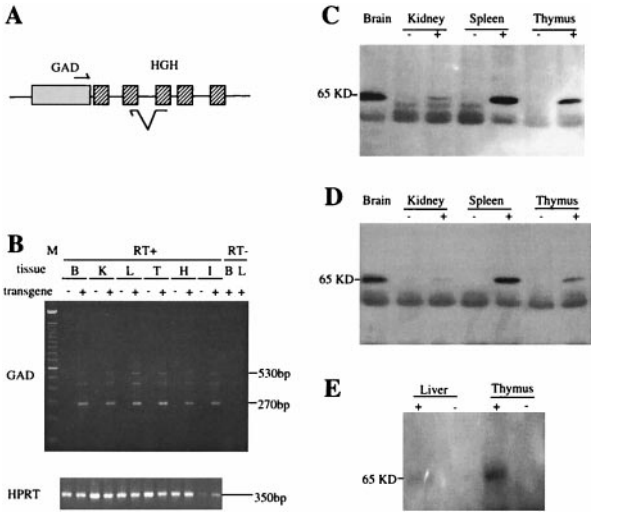

FIG. 2. Transgenic mRNA and protein expression. (*A*) Design of primers to detect spliced hybrid GAD-HGH RNA (see text). (*B*) Reverse transcription–PCR analysis of tissues from tg(+) mice (+) and non-tg(+) mice (−) mice, in the presence (RT+) or absence (RT−) of avian myeloblastosis virus reverse transcriptase (AMV-RT) by using transgene-specific primers (*Upper*) and hypoxanthine-guanine phosphoribosyltransferase-specific primers (*Lower*). M, 100-bp molecular marker (GIBCO/BRL); B, brain; K, kidney; L, liver; T, thymus; H, heart; I, islets. The origin of the different bands is discussed in the text. Tissue lysates from adult (*C* and *D*) or day 2 (*E*) tg(+) mice (+) and non-tg(+) mice (−) were immunoprecipitated with Stiff Man Syndrome patient serum and Western blotted with mAb mN65 against GAD65 (*C* and *E*). The membrane in *C* was stripped and reprobed with GAD65-specific rabbit polyclonal antisera, 7673 (*D*). Sizes of proteins were estimated from coelectrophoresed, prestained markers (not shown).

The mutant GAD65 cDNA was placed 3′ of the H2-K^b enhancer-promoter to promote widespread expression (Fig. 1*A*), which was enhanced by use of a HGH minigene (39) (see *Materials and Methods*). Three founder lines (lines 12, 13, and 14) were bred with non-tg(+) NOD mice and maintained as heterozygotes. To genotype (Fig. 1*B*), *Bgl*II-digested tail DNAs were blotted and hybridized to a 700-bp GAD65 specific probe (Fig. 1*A*). In addition to two endogenous GAD65 bands around 7 kb, a supramolar 2-kb band [predicted from the map (Fig. 1*A*)] was present in about 50% of the samples. The copy numbers of integrated 2-kb bands were determined by densitometry (ImageQuant, Molecular Dynamics) to be 3, 14, and 7 for lines 12, 13, and 14, respectively. Additional faint bands most likely represent flanking fragments.

Transgene-specific mRNA was detected by reverse transcription–PCR by using a forward primer for GAD65 cDNA and a 20-nt reverse primer that comprised two nucleotides complimentary to the end of HGH exon two and 18 nucleotides complimentary to the beginning of HGH exon 3 (Fig. 2*A*). A product of expected size (270 bp) for spliced GAD-HGH mRNA was detected in all tissues (brain, kidney, liver, thymus, heart, and pancreatic islets) of tg(+) mice (Fig. 2*B*). Other, fainter PCR products (shown in Fig. 2*B*) proved to be partially spliced transcripts and heteroduplex hybrids of partially spliced and fully spliced forms. No signals were detected in non-tg(+) littermates (Fig. 2*B*), whereas amplification of hypoxanthine-guanine phosphoribosyltransferase transcripts was equivalent in tg(+) and non-tg(+) samples (Fig. 2*B*). Islet expression of GAD65 was further analyzed by direct sequencing of PCR products derived by the use of primers that amplify endogenous and transgenic GAD cDNA equally well. Endogenous GAD65 sequences were readily recovered from all islets, whereas an excess of mutant GAD65 transgene sequences were recovered from tg(+) samples (data not shown).

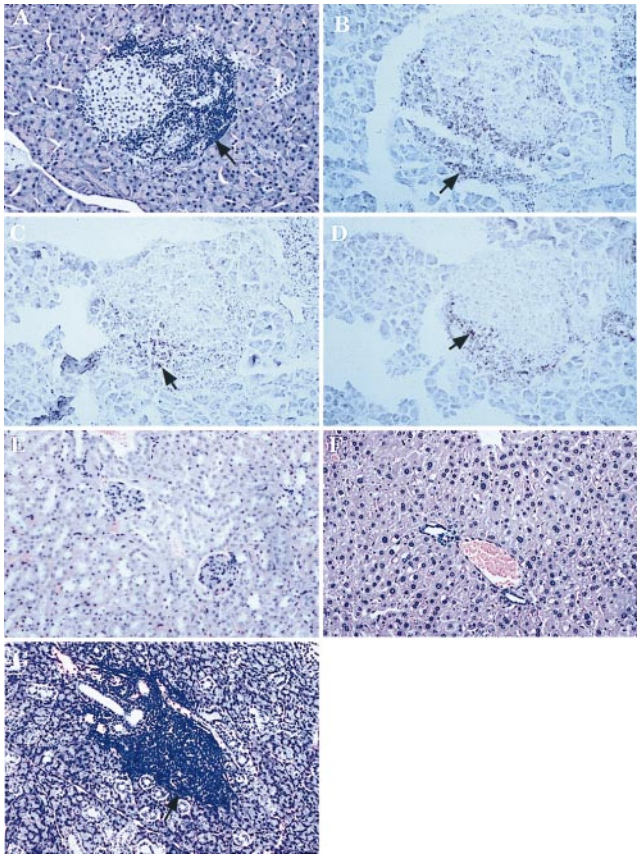

FIG. 3. Histological analysis of tissues from transgenic mice. Paraffin-embedded tissue sections stained with hematoxylin/eosin revealed the presence of infiltrating leukocytes in the pancreas (*A*) and occasionally salivary gland (*G*), but not in kidney (*E*) or liver (*F*). (*B-D*) Consecutive frozen sections of the pancreas showing the same islet stained with antibody against CD4 (*B*), CD8 (*C*), and B220 (*D*). Arrows in *A-D* and *G* indicate infiltrates.

To assess protein expression, organ lysates of 4-week-old mice were immunoprecipitated with serum from a Stiff Man Syndrome patient, with high anti-GAD65 reactivity, and then Western blotted. A band of 65 kDa was detected at equivalent strength in brain extracts of tg(+) and non-tg(+) mice, after probing with mAb mN65 raised against the GAD65 N terminus (Fig. 2*C*). Endogenous GAD65 is highly expressed in brain. A comigrating 65-kDa band was detected in kidney, spleen, and thymus of tg(+) mice, but not non-tg(+) mice. The identity of this band as GAD was confirmed by stripping the membrane and reprobing with rabbit polyclonal antibody 7673, specific for the GAD65/67 C terminus (Fig. 2*D*). Other bands reflect the Ig heavy chain captured in the immunoprecipitate (55 kDa) and some minor cross-reactive species that may not be GAD derived, because they were not detected with antibody 7673 (Fig. 2*D*). By densitometry, GAD65 expression in the thymus of line 14 was ≈150% of that in line 13, whereas the excess in the liver and spleen was close to 3-fold. At 2 days postpartum, there already was a clear GAD65 signal in tg(+) thymus and a weak signal in tg(+) liver, but no detectable expression in non-tg(+) thymus or liver (Fig. 2*E*). The immunoprecipitation and Western blot detection of transgene-encoded GAD65 by all of several anti-GAD reagents tested indicated that the mutations introduced into GAD65 did not significantly change its overall structure.

Effect of Transgenic GAD65 on Insulitis. Insulitis was commonly observed in hematoxylin and eosin-stained sections of pancreata of tg(+) mice (indicated by arrows in Fig. 3*A*). To compare this finding with insulitis in non-tg(+) NOD mice, frozen sections were stained with antibodies against CD4 (Fig. 3*B*), CD8 (Fig. 3*C*), and the B cell CD45 epitope, B220 (Fig. 3*D*),

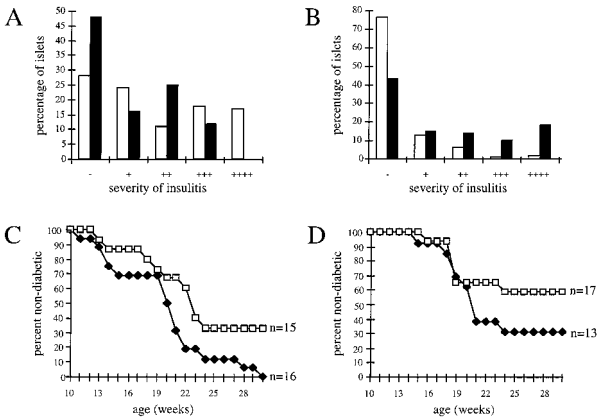

FIG. 4. Frequency and severity of insulitis and diabetes. Insulitis in 7-week-old female (*A*) and 10-week-old male (*B*) tg(+) (filled bar) and non-tg(+) (empty bar) mice. (*A*) tg(+), $n = 6$; non-tg(+), $n = 7$. (*B*) Non-tg(+), $n = 10$; tg(+), $n = 10$. Severity of insulitis on a scale of − to ++++ was assessed as described in the text. Incidence of spontaneous diabetes in female (*C*) and male (*D*) tg(+) and non-tg(+) mice. Blood glucose levels were measured weekly starting from 10 weeks of age. ◆ and □ represent weekly assessments of tg(+) mice and non-tg(+) littermates, respectively.

respectively. The patterns of the infiltrates (indicated by arrows in Fig. 3), and in particular the excess of $CD4^+$ cells, were typical of NOD insulitis. Additionally, there was occasional florid infiltration of the salivary gland (Fig. 3*G*), as is commonly the case for non-tg(+) NOD mice (data not shown). In contrast, there was no infiltration of kidney or liver (Fig. 3 *E* and *F*).

The insulitis in line 14 tg(+) and non-tg(+) NOD littermates was graded blind by an independent, experienced observer (Fig. 4 *A* and *B*): − reflects no infiltration; +, peri-insulitis; ++, lymphocyte infiltration in <50% of the islet area; +++, lymphocyte infiltration in >50% of the islet area; and ++++, islets completely filled with lymphocytes and overtly damaged. Although insulitis was statistically comparable among tg(+) and non-tg(+) females (Fig. 4*A*), male tg(+) mice displayed more severe insulitis (Mann–Whitney *U* test, $P = 0.005$) than non-tg(+) littermates [Fig. 4*B*, >40% of tg(+) scores were between ++ and ++++, whereas this was true in only 9% of non-tg(+) scores].

Effect of Transgenic GAD65 on the Incidence of Diabetes. GAD65 tg(+) and non-tg(+) littermates housed under the same specific pathogen-free conditions were scored diabetic when two consecutive measurements of blood glucose were >250 g/dl. Some females of tg(+) line 14 developed diabetes by 11–12 weeks, and by 15 weeks, one-third of tg(+) females were diabetic [◆ in Fig. 4*C* represent weekly scores of diabetes in tg(+) mice]. This onset of disease was significantly earlier than that seen in non-tg(+) littermates (□ in Fig. 4*C*) (Mann–Whitney *U* test, $P < 0.05$). Cumulative disease incidence also was significantly greater in tg(+) mice (x^2 test, $P < 0.05$), all of which had succumbed by 30 weeks, whereas >30% of non-tg(+) mice remained disease free. A disease incidence of 100% in females of line 14 is significantly greater than both the range of ≈67–70% seen in our control group, and the range of ≤ 80–85% commonly reported for other NOD colonies (2).

Consistent with the increased insulitis in tg(+) males, ≈70% of line 14 tg(+) males developed diabetes by 30 weeks, compared with only ≈40% of non-tg(+) mice (consistent with other reports for NOD males) (Fig. 4*D*). Diabetes in cohorts of tg(+) mice of lines 12 and 13 was likewise at least as severe as that in non-tg(+) NOD littermates (data not shown).

Diabetes in NOD Transgenic Mice Is an Immunological Disease and Not Caused by β Cell Toxicity Induced by the Transgene. β cell transgenes can elicit nonautoimmune diabetes, because of interference with islet cell function (40, 41). To determine whether islet toxicity was induced by the mutant GAD65, the same construct was used to generate transgenics in the nondiabetic FvB strain. Tg(+) animals again were detected in Southern blots by a band of ≈2 kb (Fig. 5*A*) [in FvB, the restriction fragment encompassing the endogenous GAD65 gene is ≈14 kb (Fig. 5*A*)]. Two lines (9 and 27), each with ≈7 copies of the transgene, were established as breeding colonies. In both, the transgene was expressed in thymus, spleen, liver, and pancreatic islets (Fig. 5 *B–D*). Densitometric comparison of the levels of endogenous GAD65 protein in FvB and NOD brains, respectively, with GAD65 transgene expression in different tissues showed that expression levels were roughly comparable in transgenic FvB and NOD mice. For example, transgene expression in the thymus and the liver of the FvB lines was greater than that in NOD tg(+) line 13, and in the thymus, was 75% of the high-expressing NOD line 14 [the transgene copy number (seven copies) was also similar between NOD line 14 and the FvB lines]. And yet, none of 25 tg(+) FvB mice of either line, housed under specific pathogen-free conditions, developed diabetes by 35 weeks of age. Moreover, there was no obvious damage to the islets examined at either 12 or 35 weeks. This segregation of the transgene from disease indicates that disease in NOD transgenics is not simply attributable to transgene-induced islet toxicity.

A further indication that disease in NOD tg(+) mice was immunologic was provided by adoptive transfers. Each of two NOD-SCID mice inoculated with 10^7 spleen cells from a recently diabetic line 14 tg(+) NOD mouse developed diabetes within 4–5 weeks posttransfer, consistent with results obtained by using splenocytes from recently diabetic, unmanipulated NOD mice (e.g., ref. 35).

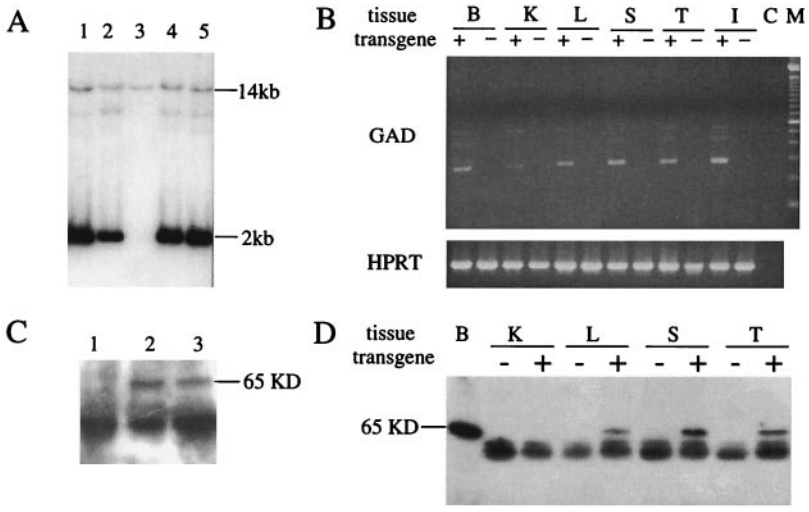

FIG. 5. Establishment of MHC class I-GAD65 FvB transgenic mice. (*A*) Southern blot of tail genomic DNA. Lanes 1, 2, 4, and 5 were tg(+); lane 3 was non-tg(+). Lanes 1 and 2 are from line 27; lanes 4 and 5 from line 9. (*B*) Reverse transcription–PCR analysis of tg(+) (+) and non-tg(+) (−) tissues for transgene-specific RNA expression. M, 100-bp marker (GIBCO/BRL); B, brain; K, kidney; L, liver; S, spleen; T, thymus; I, pancreatic islets of Langerhans; C, water. (*C*) Immunoprecipitation/Western blot analysis of thymus lysates of 2-day-old non-tg(+) (lane 1), line 9 tg(+) (lane 2), and line 27 tg(+) (lane 3) mice. Immunoprecipitation was by mN65, Western by 7673 (see text). (*D*) GAD65 immunoprecipitation/Western blot analysis of lysates from adult tg(+) (+) or non-tg(+) (−) mice. Immunoprecipitation was by Stiff Man Syndrome patient serum, Western by mN65 (see text). B, brain; K, kidney; L, liver; S, spleen; T, thymus.

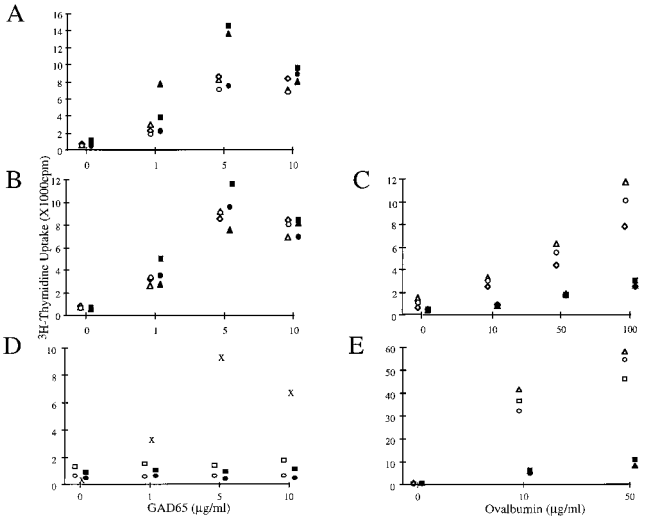

FIG. 6. Proliferative responses to antigen of splenocytes from individual NOD or FvB mice immunized with GAD65 or ovalbumin (*ova*). (*A* and *B*) 4×10^5 splenocytes per well from three tg(+) NOD mice (closed symbols) and three non-tg(+) NOD mice (open symbols) were stimulated *in vitro* with GAD65 9 days after immunization with GAD65 (*A*) or *ova* (*B*). (*C*) 4×10^5 splenocytes per well from two sets of three tg(+) NOD mice were stimulated *in vitro* with *ova*, 9 days after immunization with either GAD65 (closed symbols) or *ova* (open symbols). (*D*) 4×10^5 cells per well from spleen (squares) or draining lymph nodes (circles) of FvB tg(+) (closed symbols) and FvB non-tg(+) mice (open symbols), and from the spleen of non-tg(+) NOD mouse (x) were stimulated *in vitro* with GAD65, 9 days after immunization with GAD65. FvB symbols represent data from three mice. (*E*) 4×10^5 splenocytes per well from two sets of three FvB mice [two tg(+), 1 non-tg(+)] were stimulated *in vitro* with *ova*, 9 days after immunization with GAD65 (closed symbols) or *ova* (open symbols). Proliferation assessed as uptake of [^{3}H]thymidine, added during the last 18 hr of a 90-hr culture. Data are mean cpm from triplicate wells. SDs were less than 10%.

Expression of GAD65 in NOD Mice Did Not Elicit T Cell Tolerance Toward GAD65. The disease in tg(+) NOD mice suggested that widespread expression of the transgene had not elicited tolerance toward GAD65. To examine this, a series of tg(+) and non-tg(+) NOD littermates were immunized, either with GAD65 or with ovalbumin (*ova*), and then tested for responses to either antigen. The results in Fig. 6*A* indicate a dose-dependent response to GAD65 of splenocytes from either immunized tg(+) mice (solid symbols) or immunized non-tg(+) mice (open symbols), confirming that the GAD65 transgene did not elicit T cell tolerance. Indeed, tg(+) responses were usually higher than non-tg(+) responses. Furthermore, the average response to GAD65 was equally strong among mice immunized with either GAD65 or *ova* (Fig. 6 *A* and *B*), indicating that T cells in *ova*-immunized NOD mice had been spontaneously primed to GAD. In contrast, tg(+) NOD mice immunized with *ova* (Fig. 6*C*, open symbols) had a significantly heightened response to *ova* compared with mice immunized with GAD65 (Fig. 6*C,* closed symbols) (Mann–Whitney *U* test, $P < 0.001$), indicating that the mice had not been spontaneously primed to *ova*.

When the same experiment used FvB mice, neither tg(+) (Fig. 6*D*, closed symbols) nor non-tg(+) FvB mice (Fig. 6D, open symbols) primed with GAD65 showed a proliferative response to GAD (the response marked by x is from an NOD mouse). Conversely, both types of mice immunized with *ova* showed strong secondary responses to *ova* (Fig. 6*E* and data not shown). Hence, the lack of tolerance to GAD65 depends on the NOD genetic background, and on this background, engineered widespread expression of GAD65, including in the perinatal thymus (Fig. 2) failed to tolerize T cells.

DISCUSSION

Transgenes expressed in the thymus [e.g. simian virus 40 T-antigen (42) and mutant mouse type II collagen (43)], and blood-borne self-antigens that gained access to the thymus from the circulation (44) both induced intrathymic deletion of developing autoreactive thymocytes. Such central tolerance is easily reconciled with clonal deletion induced by cognate antigen in TCR transgenic mice (22, 45–48). In contrast, thymic expression of S100β (implicated in experimental autoimmune pancephalitis) failed to delete S100β-reactive T cells (49), and T cells specific for myelin basic protein and for acetylcholine receptor (both expressed in the thymus) are normal components of peripheral blood (50–52). Additionally, peripheral blood samples of IDDM patients, healthy individuals, and NOD mice all have yielded insulin-specific T cell clones, despite thymic expression of insulin RNA (42, 53, 54), and in TCR transgenic NODxC57BL/6 mice, T cells expressing reduced levels of TCR cross-reactive with NOD-derived class II escaped from the thymus and caused joint-specific rheumatoid arthritis (55). Thus, the degree to which central tolerance is mounted varies with regard both to antigen and genetic background. Here we show that NOD mice, but not FvB mice, fail to develop tolerance to ubiquitous and perinatal expression of GAD65. A lack of thymic deletion also was reported for NOD mice that were transgenic either for antigens implicated in IDDM (proinsulin or Hsp60) expressed from a promoter expressed in thymic MHC class II(+) cells, or for a TCR from islet cell antigen-specific CD4$^+$ T cells (56–59).

In contrast, negative selection to islet cell antigens could be achieved when antidiabetogenic MHC class II molecules were introduced onto the NOD genetic background (60). Thus the failure of transgenic GAD65 to induce tolerance in NOD mice may be because of the inability of NOD-MHC (e.g. Ia^{g7}) to present GAD peptides in a way that can negatively select, despite a capacity to elicit strong negative selection toward other peptides and to super antigens such as mtv-3 (61). Other thymic abnormalities in NOD mice may compound the difficulty in establishing tolerance to GAD65 (62–65). Indeed, thymic expression of GAD65 may actually positively select autoreactive T cells, leading to exacerbated insulitis and diaetes, as is seen in tg(+) line 14 and in mice injected intrathymically with GAD65 peptides (66).

And yet, anti-GAD T cell reactivity was reduced in NOD mice inoculated intrathymically with GAD65 (18), suggesting *a priori* that central deletion might be achieved. Alternatively, intrathymic injection of concentrated antigen may have induced high-dose antigen tolerance of circulating, GAD-reactive T cells, as an indirect consequence of which cortical thymocytes may have been killed by cytokine and steroid toxicity. Such has been proposed to account for the deletion of cortical thymocytes in TCR-transgenic mice inoculated with cognate peptide. It is also possible that intrathymic injection of GAD induced a Th2 response. This would reduce the inflammatory attack on the pancreas and the subsequent activation of other auto-reactive T cells. Deliberate attempts at Th2 immunodiversion successfully have regulated IDDM in NOD mice (19) and might likewise account for reduced disease in young NOD mice or diabetes-prone BB rats receiving islet transplants into the thymus (65, 68). Consistent with Th2 induction, GAD-reactive antibodies were maintained in GAD-inoculated mice (18). Interestingly, unafflicted relatives of diabetics also can show high titers of anti-GAD-reactive antibodies (69). However, the linkage of high titers of anti-GAD-reactive antibodies with Stiff Man Syndrome (SMS), a serious human disease (27), might caution against treatments that enhance anti-GAD antibodies. While there is no mouse model for SMS, severe complications of immunodiversion were demonstrated in marmosets, in which multiple scelerosis was converted to a lethal demyelinating disorder after experimental skewing toward Th2 (70).

Despite essentially ubiquitous expression of GAD65 in tg(+) NOD mice, inflammation was limited to islets and salivary glands. This finding demonstrates that the expression pattern of a particular autoantigen is important but insufficient to dictate the organs affected by autoimmune disease, a finding that can readily explain how broadly-expressed antigens such as heat shock proteins can be implicated in organ-specific disease (71).

Finally, disease is more severe in female rather than male NOD mice (2). Castration or administration of sex hormones can regulate disease incidence (72), suggesting an interaction of sex hormones and immune function (73). In our study, transgenic GAD65 expression induced a disease incidence of approximately 70% in line 14 males, comparable to that in non-tg(+) NOD females. Disease exacerbation in male NOD mice previously was reported in tg(+) mice expressing IL-10 in islet α cells (74), and in mice transgenic for a TCR from a $CD4^+$ diabetogenic clone (60). Clearly, the immunological and endocrine effects that combine to skew disease incidence in males and females are important areas for further study.

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