Differential Protection in Two Transgenic Lines of NOD/Lt Mice Hyperexpressing the Autoantigen GAD65 in Pancreatic β-Cells

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Although expressed at very low levels in islets of NOD mice, GAD65 is a candidate islet autoantigen. Two transgenic lines of NOD/Lt mice expressing high levels of human GAD65 from a rat insulin promoter were generated. Transgenes were integrated on proximal chromosome 15 of the A line and on the Y chromosome of the Y line. Transgenic A-line mice were obligate hemizygotes, since homozygous expression resulted in developmental lethality. A twofold higher level of hGAD65 transcripts in A-line islets from young donors was associated with higher GAD protein and enzyme activity levels. Y-line males developed diabetes at a similar rate and incidence as standard NOD/Lt males. In contrast, A-line mice of both sexes exhibited a markedly lowered incidence of diabetes. Insulitis, present in both transgenic lines, developed more slowly in A-line mice and correlated with a reduction in the ratio of γ-interferon to interleukin-10 transcripts. Splenic leukocytes from young A-line donors transferred diabetes into NOD-*scid* recipients at a retarded rate compared with those from nontransgenic donors. Further, nontransgenic NOD T-cells transferred diabetes more slowly in NOD-*scid* recipients that were congenic for A-line transgenes as compared with standard NOD-*scid* recipients. Primed T-cell responses and spontaneous humoral reactivity to GAD65 failed to distinguish transgenic from nontransgenic mice. Quantitative differences in expression level or insertional mutagenesis are possible mechanisms of protection in the A line. *Diabetes* 47:1848–1856, 1998

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DAPI, 4′,6′-diamidino-2-phenylindole; ECL, enhanced chemiluminescence system; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IFN-γ, γ-interferon; IL, interleukin; LAK, lymphokine-activated killer; MHC, major histocompatibility complex; NFDM, nonfat dry milk; NTP, nucleotide triphosphate; OD, optical density; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLP, pyridoxal phosphate; RT, reverse transcription; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing 0.1% Tween-20; VNTR, variable number tandem repeat.

A variety of β-cell autoantigens have been identified as targets of T-cell–mediated autoimmune attack in humans and in the NOD mouse (1). Experimental manipulations entailing potentially tolerogenic exposure of prediabetic NOD mice to candidate autoantigens, including transgenic expression of proinsulin (2) or heat shock protein-60 (3), parenteral injections of insulin (4,5), or GAD (6–12) are capable of deviating the immune attack directed against the islets. Among these candidate autoantigens, GAD has received considerable attention because of a possible molecular mimicry between a GAD peptide and the P2-C region of Coxsackievirus, a human pathogenic virus speculated to be a trigger for IDDM (13). A GAD-peptide–specific $CD4^+$ T-cell line has recently been reported that can transfer IDDM into NOD-*scid*/*scid* recipients (14). Two independently encoded isoforms of the GAD enzyme (GAD65 and GAD67) catalyze the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). Human β-cells contain only the GAD65 isoform, which is also the major isoform in rat islets (15,16). In contrast, mouse islets predominantly express the GAD67 isoform, albeit at low levels (16–18). Although GAD67 transcripts are readily detected in total mouse pancreatic RNA preparations (19,20), GAD65 transcripts are either very low or below the limit of detection by reverse transcription–polymerase chain reaction (RT-PCR) assay (18). Some pancreatic transcripts may derive from GABA-ergic neurons (21). Because of the negligible level of constitutive GAD65 expression in mouse β-cells, the autoimmune IDDM-prone NOD mouse is ideal for assessing the consequences of high levels of β-cell expression of GAD65 from a transgene (16); in this study, we compared the effects on IDDM development in two independent lines of transgenic NOD/Lt mice expressing high levels of human GAD65 in β-cells. Assuming that β-cell–expressed GAD is a primary target of the earliest T-cells infiltrating islets, a major increase in β-cell–restricted GAD in NOD/Lt mice to levels more consistent with the higher levels demonstrable in rat and human β-cells was expected to modulate IDDM development, either by accelerating initial rates of β-cell destruction or by producing anergy in the earliest effector cells.

RESEARCH DESIGN AND METHODS

Mice. NOD/Lt mice were produced in a specific pathogen-free research vivarium at The Jackson Laboratory. Mice were allowed free access to diet (autoclaved diet 96WA; Emory Morse, Guilford, CT) and acidified water. When these studies were done, diabetes incidence by age 1 year in the NOD/Lt colony was 100% in females and 50% in males. Husbandry conditions for the NOD/LtSz-*Prkdc*^{scid} stock

(designated NOD-*scid*) used in adoptive transfer studies has been described previously (22). Females of the NOD/Lt A-line transgenic stock (see below) were intercrossed with a NOD/LtSz-*scid* homozygous male. Transgene-positive F1 mice were intercrossed with NOD-*scid* males to produce an F2 generation in which two transgene-positive NOD/LtSz-*scid* homozygous males were identified. These males were mated to homozygous NOD/LtSz-*scid* females to establish the *scid* congenic line, designated NOD/LtSz-*Prkdc*^{scid}.TgN(RIP7hGAD65)Lt4 (A line). For brevity, this stock is designated as the NOD/LtSz-*scid*.A-line.

Assessment of diabetes and insulitis in mice. Mice in diabetes incidence studies were tested every 2 weeks, starting at age 12 weeks, for urinary glucose using Diastix (gift of Bayer Diagnostics, Elkhart, IN). After initial detection of glycosuria, diabetes was confirmed when mice were consistently glycosuric for 3 consecutive weeks. Insulitis was scored histologically on three nonoverlapping sections per specimen, as described previously (23), after Bouin's fixation and aldehyde fuchsin staining to detect granulated β-cells. Diagnosis of diabetes was confirmed by histological demonstration of end-stage insulitis and the virtual absence of aldehyde fuchsin–positive β-cells.

Production of NOD/Lt-TgN(RIP7hGAD65)Lt4 transgenic mice. The RIP7 promoter represents a fusion of a 9.5-kb *Eco*RIfragment from the rat insulin II RIP3 and a 1.0-kb *Eco*RI-*Hind*III fragment from the rat insulin II RIP5 promoter. The fusion construct was inserted at the *Eco*RI-*Hind*III sites of Bluescript-KS (Stratagene, La Jolla, CA). Thus the RIP7 plasmid contained the 9.5-kb promoter sequence upstream from the transcription initiation site and the first intron of the rat insulin II gene and a poly A terminator from a class II *Ea* gene. A transgenic construct (RIP7hGAD65) was engineered by insertion of 1,757-bp cDNA encoding the human GAD65 enzyme into a *Cla*I site downstream of the RIP7 promoter (made by Y.S.). *Not*I and *Xho*I restriction enzymes were used to excise the 12-kb RIP7hGAD65 transgene. This transgene construct was injected directly into naturally ovulated NOD/Lt zygotes by the Microinjection Service of The Jackson Laboratory and implanted without prior culture into pseudopregnant (B6 × SJL)F1 recipients. Of 68 progeny, two males were transgene positive and used as founders for two NOD/Lt-TgN(RIP7hGAD65)Lt4 transgenic lines: the autosomal (A) line and the Y line. These lines are referred to as NOD-transgenic A and Y line, respectively. PCR was used to identify hGAD65 transgene-positive mice. Tail snip preparations of genomic DNA were tested using the following primer pairs: RIP-2′, (5′ -AGCACTTTCTGCAGACCTAGCAC-3′) and hGAD-216, (5′-GGCTTCTCGGCGTCTCCGTAGA-3′). A 25-µl reaction mixture contained 2 µl of genomic DNA, 1 × PCR buffer (Perkin Elmer, Norwalk, CT), 1.5 mmol/l $MgCl_2$, 0.25 mmol/l deoxynucleotide triphosphates, 1 U *Taq* polymerase (AmpliTaq; Perkin Elmer), and 10 pmol each of the above primers. Reactions were run in a thermal cycler (Perkin Elmer) for 31 cycles of 1 min at 94°C, 1.5 min at 60°C, and 2 min at 72°C. The product was 550 bp.

Transgene copy number. Southern blot analysis was used to determine the copy number of each of the transgenes. Then 10 µg of *Msp*I-digested DNA from individual NOD A- and Y-line and standard NOD/Lt mice were Southern blotted and probed with a 1.7-kb *Msp*I fragment of the RIP7hGAD65 transgene construct. Transgene copy number was estimated using a phosphoimager (Fuji Photo Film, Tokyo, Japan) to compare the intensity of the transgenic insert bands to the endogenous germ-line band on the Southern blot.

Transgene integration site. Fluorescent in-situ hybridization was used to map the chromosomal site into which the A-line transgenes had been inserted. Metaphase chromosome spreads were prepared from lymphokine-activated killer (LAK) cell cultures from A-line males. Spleen leukocytes were depleted of macrophages by incubation for 45 min on fetal calf serum (FCS)–coated flasks in RPMI 1640. Macrophage-depleted cells were seeded at a density of 2 × 10^6/ml in RPMI 1640 supplemented with 2 mmol/l glutamine, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mmol/l nonessential amino acids, 1 mmol/l sodium pyruvate, 50 mmol/l 2-mercaptoethanol, and 1,000 U/ml human recombinant interleukin-2 (IL-2) (Genzyme, Cambridge, MA). Cultures were maintained at 37°C in a humidified 5% CO_2 incubator and subcultured every 48 h into fresh IL-2–supplemented medium at a density of 1 × 10^6 cells/ml. For preparation of metaphase chromosomes, LAK cultures 4–14 days old were subcultured and allowed to grow for 24 h. Then 10 ml of cells were incubated at 37°C for 40 min in 25 µmol/l ethidium bromide in complete medium. Colcemid (Sigma, St. Louis, MO) was added at 0.01 µg/ml for 20 min at 37°C. Cells were centrifuged at 200*g* for 10 min; the supernatant was removed, and 5 ml 75 mmol/l KCl, previously warmed to 37°C, were added to the pellet. Cells were resuspended and incubated at 37°C for 20 min. Next 1 ml of freshly prepared fixative (3:1 methanol:acetic acid) was gently added to the cell suspension and the mixture was incubated for 20 min at room temperature. Cells were centrifuged at 200*g* for 8 min, and all except 500 µl of the fixative was removed. The cell pellet was tapped to resuspend the cells, and 5 ml of fresh fixative were slowly added while mixing. This step was repeated twice. Cells were then resuspended in 500 µl fixative. Using a Pasteur pipette, fixed cells were dropped onto clean, warm, and double-distilled H_2O–wetted glass slides. Slides were air dried and baked at 65°C for at least 4 h, and then probed with the biotin-labeled 13-kb *Not*I-*Xho* RIP7hGAD65 fragment. The Oncor Chromosome In Situ Hybridization and Detection Kit (S1333-BF) (Oncor, Gaithersburg, MD) was used for fluorescent detection and amplification on preparations counterstained with propidium iodide or 4′,6′-diamidino-2-phenylindole (DAPI). Analysis was performed by computerized image analysis (MetaSystems ISIS software, Belmont, CA), which allows DAPI-stained chromosome images to be displayed in black and white (pseudo-Giemsa banding).

Western blotting of mouse islets. Islets were isolated from five mouse pancreases from each line by a modification of the bile duct infusion method of Gotoh et al. (24). Purified islets were boiled in SDS-sample buffer (80 mmol/l Tris, 2% SDS, 15% sucrose, 5% β-ME, 0.006% bromphenol blue) for 5 min at a concentration of 5 islets/µl. The protein concentration in extracts was measured by the Coomassie Plus Protein Kit (Pierce, Rockford, IL). An extract from 300 islets was subjected to SDS-PAGE using a 12% polyacrylamide gel and the buffer system of Laemmli (25). Proteins were transferred to a Protran Pure Nitrocellulose Transfer and Immobilization Membrane (Schleicher & Schuell, Keene, NH) using a semidry electroblotting apparatus (E & K Scientific, Campbell, CA) according to the manufacturer's instructions. The nitrocellulose membrane was washed in 50 mmol/l Tris/HCl (pH 7.6) and 150 mmol/l NaCl (Tris-buffered saline [TBS]), containing 0.1% Tween-20 (TBST) and 10% nonfat dry milk (NFDM), followed by three 5-min washes in TBST, incubation with a primary antibody in TBST containing 5% NFDM for 1 h, three 5-min washes in TBS containing 0.3% Tween-20, incubation with a secondary antibody in TBST for 30 min, and three 5-min washes in TBS containing 0.3% Tween-20. The primary antibodies used were GAD6 (dilution 1:250), a mouse monoclonal antibody specific for GAD65, and 1701 (dilution 1:5,000), a rabbit polyclonal antibody that recognizes both GAD65 and GAD67. Both antibodies react equally well with mouse and human GAD (16). The secondary antibodies were a peroxidase-coupled sheep anti-mouse Ig (dilution 1:2,000) and a donkey anti-rabbit Ig (dilution 1:8,000), respectively (Amersham, Buckinghamshire, U.K.). Immunostaining was visualized using the enhanced chemiluminescence system (ECL) (Amersham).

Measurement of GAD enzymatic activity. Glutamic acid decarboxylation in mouse islets was measured using a modification of the method of Wu et al. (26). Collagenase-isolated islets from standard NOD/Lt males aged 5–6 weeks and age-matched males from both transgenic lines (350–530 islets/group) were washed twice in ice-cold phosphate-buffered saline (PBS). The islet pellets were then homogenized in a microhomogenizer with a minimal volume of homogenization buffer (100 mmol/l potassium phosphate [pH 6.8], 0.25% Triton X-100, 0.2 mmol/l EDTA, and freshly added 0.02 mmol/l pyridoxal phosphate [PLP]). Homogenates were removed to 1.5-ml Eppendorf tubes and centrifuged at 8,000*g* for 10 min. Supernatants were removed and kept at 4°C. An aliquot was reserved for measurement of protein by the Bradford protein assay (Bio-Rad, Hercules, CA). Reaction vessels were 15-ml polystyrene conical tubes (Corning, Medfield, PA) with the top one-third removed to reduce dead air space. The tubes were sealed with serum stoppers to which were affixed a plastic cup (Kontes Glassware #882320-0000, Vineland, NJ) containing 100 µl hyamine hydroxide (Du Pont-NEN, Boston, MA). Reactions were run under both limiting and saturation substrate conditions. The reaction mixture contained 50 µl islet homogenate supernatant, 70 µl diluting buffer (50 mmol/l potassium phosphate [pH 7.2], 1 mmol/l aminoethylisothiuronium bromide, 0.2 mmol/l PLP, 9 µl cold glutamic acid at 4 or 40 mmol/l, and 2 µl glutamic acid L-[1-^{14}C] or [U-^{14}C] [Du Pont-NEN] lyophilized and resuspended in 1 mmol/l HCl at the original concentration). The reaction was incubated at 37°C for 90 min, stopped by the injection of 50 µl 0.5N H_2SO_4 into the reaction mixture, and then incubated for an additional 90 min at 37°C to ensure complete release of CO_2. Radioactivity trapped in the plastic cup was counted in scintillation vials containing 5 ml scintillation fluid in an LKB 1218 RackBeta liquid scintillation counter (Wallac, Gaithersburg, MD). Samples in which PLP or the islet homogenate was omitted served as background controls.

Lymph node cell proliferation assay. Mice were injected in the hind footpad with a 1:1 emulsion of complete Freund's adjuvant (Sigma) and 25 µg/50 µl recombinant hGAD65 (gift of Dr. Mark Atkinson, University of Florida, Gainesville, FL). Ten days after priming, the popliteal lymph node cells were set up in an in vitro proliferation assay, as described previously (27). The hGAD65 antigen was added at various concentrations in 10 µl per well in RPMI 1640 supplemented as described above, but with 1% Nutridoma SP (Boehringer Mannheim, Indianapolis, IN) replacing FCS. After 3 days in culture at 37°C in a humidified 5% CO_2 atmosphere, the cells were pulsed with 1 µCi/well [^{3}H]thymidine for an additional 18 h. Cultures were harvested and incorporation of [^{3}H]thymidine was measured with an LKB Betaplate 1205 spectrometer.

RT-PCR semiquantification of GAD and cytokine mRNA. Total RNA was extracted from islets of NOD/Lt, A-line, and Y-line male mice aged 6 weeks or from neonatal thymuses using a single-step guanidinium-isothiocyanate method (28). At least 500 islets collected from five mice were pooled to make each RNA sample. RNA samples were treated with DNase (DNase I, amplification grade; Life Technologies, Grand Island, NY); ~0.5 µg of DNase-treated RNA from each sample was reverse transcribed to cDNA (60 min at 42°C) in a 30-µl reaction con-

taining 50 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 10 mmol/l $MgCl_2$, 0.5 mmol/l spermidine, 10 mmol/l dithiothreitol, 1 mmol/l of each dNTP, 40 U RNasin (Promega, Madison, WI), 600 ng random hexamers (Pharmacia, Uppsala, Sweden), and 30 U avian myeloblastosis virus–reverse transcriptase (Promega) The resulting cDNA from islets was amplified by radiolabeled PCR using oligonucleotide primers for β-actin (sense, TGGAATCCTGTGGCATCCATGAAA; antisense, TAAAACGCAGCTCAGTAACAGTCC); γ-interferon (IFN-γ) (sense, AGCGGCTGACTGAACTCAGATTGT; antisense, GTCACAGTTTTCAGCTGTATAGGG), provided by Dr. Robert Evans (The Jackson Laboratory, Bar Harbor, ME.); IL-10 (sense, GCAGGGGCCAGTACAGCCGGGAA; antisense GCTTTTCATTTTGATCATCATGT) (29); murine GAD65 (sense, CTCTGGCGATGGAATCTTTTCTCCTGGTGG; antisense, CTTGTGAGGATTCCATGTCACAGAGTTGGC); murine GAD67 (sense, CCTCCCAGTCTGACATCGATTTCC; antisense, ACATGCTATGGTCTAGGACTAAGC) (30); and human GAD65 (sense, GGGAAAATGATTCCATCTG; antisense, TAAATCTTGTCCAAGGCGTTC). The β-actin, IFN-γ, IL-10, mGAD67, mGAD65, and hGAD65 PCR products were 348, 220, 479, 354, 490, and 833 bp, respectively. PCR reactions contained 5 µl cDNA, 50 mmol/l KCl , 10 mmol/l Tris-HCl (pH 9.0), 0.1% Triton-X 100, 2.5 mmol/l $MgCl_2$, 0.1 mmol/l of each dNTP, 50 pmol of the appropriate sense and antisense primers, 2.5 U *Taq* DNA polymerase (Promega), and 0.3 µCi [α-^{32}P]dCTP. Reactions were loaded in a Thermal Cycler 9600 (Perkin-Elmer), heat treated at 94°C for 3 min, and then subjected to 23 (β-actin), 35 (IL-10, IFN-γ), or 38 (hGAD65, mGAD65, and mGAD67) cycles of amplification. These cycle numbers were empirically established to be in the linear phase of the reactions. Primer annealing temperatures were 55°C (β-actin and IFN-γ) or 60°C (IL-10, hGAD65, mGAD65, and mGAD67). The cDNAs from neonatal thymus were tested for GAD65 expression using the hGAD65 primers, with cDNA from adult NOD mouse brain used as a positive control. After amplification, 5 µl of the PCR products were electrophoresed in a 1% polyacrylamide gel for 45 min at 300 V. Gels were dried and the radiolabeled PCR products were quantified using the Fuji phosphorimaging system. To correct for variations in the amount of RNA used and variability in RT efficiency, the radioactive signal for each PCR product was normalized to the β-actin signal produced by the same sample. The results of the PCR experiments are representative of two independent PCR runs, using the same RNA samples.

Adoptive transfers. Adoptive transfers into homozygous NOD/LtSz-*scid* females of splenic leukocytes from prediabetic (age 8 weeks) and diabetic NOD/Lt donors, as well as from nondiabetic NOD A-line transgenic mice aged 8 and 21 weeks, were performed as described previously (22). Next 2×10^7 donor cells were injected intravenously into the tail vein of the recipient mice in a volume of 200 µl PBS. Percentages of T- and B-cells in spleen were determined by flow cytometry after staining with monoclonal antibodies, as described elsewhere (31). In another experiment, a common pool of splenic leukocytes from standard NOD/Lt female donors aged 5 weeks was also transferred into groups of homozygous NOD-*scid* females and NOD/LtSz-*scid*.A-line females aged 6–8 weeks.

Measurement of serum hormones and antibodies. Insulin and glucagon were measured using commercially available radioimmunoassay kits. (Linco, St. Louis, MO). Mouse serum antibodies binding to hGAD65 were identified by enzyme-linked immunosorbent assay (ELISA), as described previously (27), for five antibody isotypes: IgG1, IgG2a, IgG2b, IgG3, and IgM. Measurements reported in OD405 units were all performed in the same assay.

Statistical analysis. Significance of differences in IDDM-free survival rates was tested using survival analysis (log-rank Mantel-Cox test) (StatView; Abacus Concepts, Berkeley, CA). Significance was assumed at $P \leq 0.05$.

RESULTS

Transgene copy number and integration site. Two founder males were obtained from a total of 68 viable offspring recovered from direct injections into NOD/Lt zygotes. These males were mated with transgene-negative sibs. One founder male transmitted transgenes exclusively to his male progeny, establishing transgene integration onto the Y chromosome (Y line). This was evident after screening five litters of his progeny, in which all 18 males were transgene positive and all 23 females were transgene negative. The second male transmitted transgenes to progeny of both sexes, confirming transgene integration into an autosome (A line). FISH analysis using the 13-kb RIP7hGAD construct as a hybridization probe, followed by analysis of the pseudo Giemsa-banded metaphase spread, established that the transgene was inserted on proximal chromosome 15 adjacent to the centromere. Despite many generations of mating hemizygous A-line parents, no live-born transgene homozygotes were ever observed. The presence of 6 of 19 resorbing embryos in placenta of gravid A-line females killed at 12.5 and 15 days after coitus confirmed a developmental lethality. Hence all A-line transgene-positive mice of either sex and Y-line males were obligate hemizygotes. Densitometric comparison of the intensity of transgenic insert bands to endogenous germline bands on a Southern blot showed that 4–5 copies of the RIP7hGAD transgene are present in A-line hemizygotes, and approximately 17 copies of the transgene are present in Y-line hemizygotes.

Transgene expression. RT-PCR analysis confirmed that the transgenes were expressed in islets of A- and Y-line mice. Data in Fig. 1 provide semiquantitative analysis of GAD transcript levels relative to β-actin transcript levels in the same samples, and show the highest expression level in the A line. Primers based on mouse GAD65 failed to amplify any product from 300 handpicked islets from nontransgenic NOD males aged 6 weeks (Fig. 1*A*), whereas primers based on the mouse GAD67-specific sequence detected very low transcript levels in the same cDNA sample (Fig. 1*C*). In contrast, mouse GAD65 and GAD67 primers both amplified high levels of product relative to β-actin in islet cDNAs from age-matched A- and Y-line male donors, respectively. This represented cross-reactivity to the human GAD65 product pro-

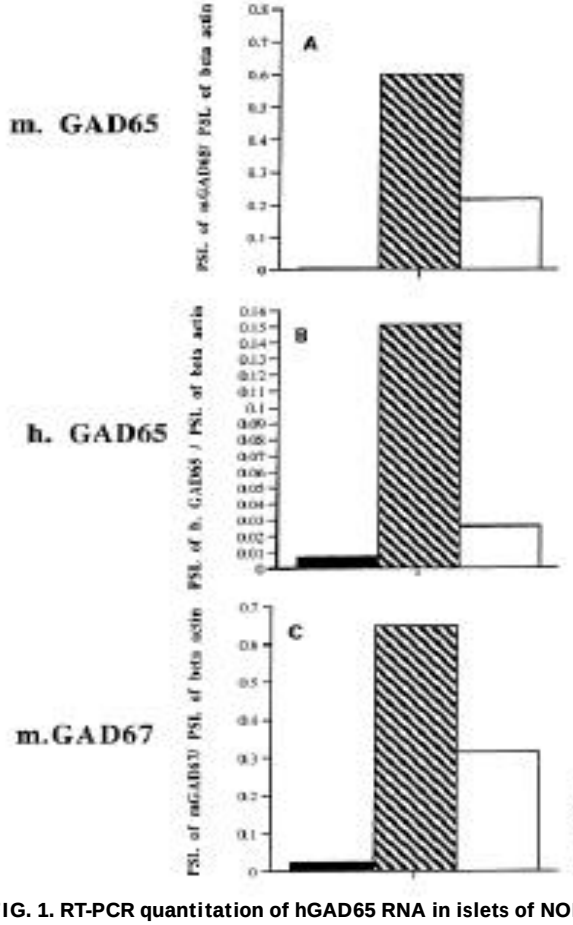

FIG. 1. RT-PCR quantitation of hGAD65 RNA in islets of NOD, A-line, and Y-line mice aged 6 weeks. Units are expressed as phosphor-stimulated luminescence (PSL) of the radiolabeled product of each GAD primer set relative to PSL of the radiolabeled product of β-actin primers on the same cDNA sample. The primers for mGAD65 (*A*) and mGAD67 (*C*) demonstrated cross-reactivity with hGAD65 DNA in these experiments. ■, negative segregant; ▨, autosomal; □, Y line.

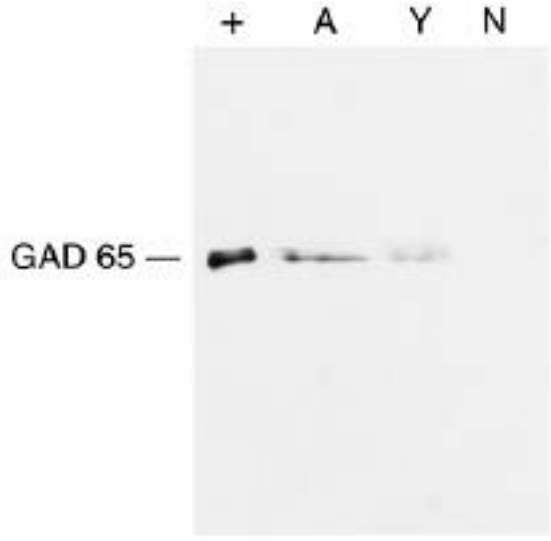

FIG. 2. Analysis of GAD65 protein expression in islets of transgenic mice by Western blotting. The positive control (+) used was 2 µg of rhGAD65. Extracts from 300 pooled islets from five A-line transgene-positive mice (A), five Y-line transgene-positive mice (Y), and five transgene-negative littermates of the A line (N), representing 126, 93, and 58 µg protein, respectively, per lane were subjected to SDS-PAGE, followed by Western blotting and immunostaining. GAD6, a murine monoclonal antibody specific for GAD65, was used as the primary antibody probe in a Western blot.

duced by the transgenes, as demonstrated by use of primers based on the human GAD65 sequence (Fig. 1*B*). Thus, despite a higher hemizygous transgene copy number on chromosome Y, the smaller number of hemizygous transgene copies on chromosome 15 produced a higher level of transcripts.

Evidence that transgenes might be expressed in neonatal thymus was assessed by RT-PCR analysis of neonates from a single A-line litter containing three transgene-positive and three transgene-negative pups. A cDNA preparation from adult brain amplified with hGAD65 primers served as a positive control. No transcripts could be detected by RT-PCR of thymic mRNA in any of the six neonates, whereas product was produced from brain cDNA.

Western blot analysis confirmed the findings from semiquantitative RT-PCR analysis of mRNA transcript levels. Analysis of extracts of 300 islets from both A- and Y-line transgene-positive male donors showed that both lines expressed significant levels of GAD65, whereas no expression was detected in nontransgenic A-line male islets (Fig. 2).

TABLE 1
High-level GAD enzymatic activity in A- and Y-line transgenic, but not standard, NOD/Lt collagenase-isolated islets

Strain	Number of islets	pmol GABA/90 min	pmol GABA · µg^{-1} protein · 90 min^{-1}
NOD/Lt	530	0.29	16
Y line	500	3.9	70
A line	350	2.3	130

Islets were isolated from male donors aged 6 weeks and assayed as described in METHODS.

After normalizing for the amount of protein in each sample (840 vs. 620 µg/ml protein in A- and Y-line islet lysates, respectively) and the densitometry readings from the Western blot (A-line OD, 0.625; Y-line OD, 0.187), the relative density of the bands (A/Y = 0.652/0.253) was estimated at 2.6-fold increase in the A-line lysate. However, the islet pools extracted could not be matched for viable β-cell mass, which potentially could be less in the Y-line islet pool. Immunostaining of a Western blot using an antiserum recognizing both the GAD65 and GAD67 isoforms gave identical results as was observed for the GAD65-specific monoclonal antibody depicted in Fig. 2; that is, GAD protein was detected only in islet lysates from both transgenic lines. Thus the expression of transgenic hGAD65 in lysates from 300 islets from both the A and Y lines was far in excess of any endogenous β-cell GAD isoforms, the level of the latter being below the detection limit of the Western blot-ECL method.

Measurement of GAD enzyme activity supported both the RT-PCR analysis of mRNA levels and Western blot results. (Table 1). Islets from nontransgenic NOD/Lt males aged 6 weeks produced 16 pmol GABA/µg protein in a 90-min assay, compared with 70 and 130 pmol/µg protein GABA in islets from age-matched Y- and A-line male islet donors. The elevated levels of GAD in β-cells of transgenic mice did not influence nonfasting plasma levels of insulin or glucagon (Table 2). Further, no significant differences in glucose clearance after glucose loading of fasted A-line NOD-*scid/scid* females aged 12 weeks were observed when compared with transgene-negative NOD/LtSz-*scid/scid* females (data not shown). Hence no autocrine effects of high β-cell GABA production on insulin secretion or paracrine effects on glucagon secretion were demonstrable.

Diabetes incidence and insulitis. Weanling mice from the NOD-transgenic A and Y lines were put into comparative diabetes incidence studies. For the A-line study, mice were identified as positive or negative for the transgene using PCR primers for hGAD65; in the Y line, all males were transgene positive and all females were transgene negative. The cumulative diabetes incidence at age 50 weeks for the A-line mice (Fig. 3*A*) was 2 of 14 (14%) for transgene-positive females compared with 9 of 9 (100%) for their transgene-negative littermates ($P < 0.0001$). Of the A-line transgene-positive males (Fig. 3*A*), only 2 of 13 (15%) developed IDDM compared with 7 of 14 (50%) of their transgene-negative male sibs ($P < 0.05$). In contrast, as shown in Fig. 3*B*, IDDM incidence in transgene-positive Y-line males (6 of 11 or 55%) was not significantly different from that in transgene-negative NOD/Lt male segregants in the A-line (Fig. 3*A*). The IDDM incidence was 90% (9 of 10) in the cohort of transgene-negative female sibs of Y-line males (Fig. 3*B*).

The number of splenic T- and B-cells assessed by flow cytometry was not significantly different when either transgenic line was compared with standard NOD/Lt mice (data not shown). Analysis of insulitis in 11 A-line females at age 9 weeks showed a mean insulitis index ± SD (0.83 ± 0.60) that was not significantly different from the insulitis index for 3 transgene-negative female littermates (1.50 ± 0.32). All stages of insulitis were noted, from noninvasive peri-insulitis to end-stage insulitis associated with nearly complete elimination of the β-cell mass of an individual islet. Likewise, a diverse spectrum of insulitic lesions was observed in three A-line males and four nontransgenic NOD/Lt males at ages 20–22 weeks. The mean insulitis index ± SE was 0.22 ± 0.07

TABLE 2
RIP7hGAD65 transgene (tg) expression does not alter nonfasting plasma insulin and glucagon levels in prediabetic males

Plasma assay	A line		Y line	NOD/Lt
	tg^-	tg^+	(tg^+)	
Insulin (ng/ml)	2.0 ± 0.2 (5)	2.3 ± 0.4 (6)	2.2 ± 0.3 (7)	2.1 ± 0.4 (3)
Glucagon (pg/ml)	68 ± 4 (5)	72 ± 3 (6)	64 ± 3 (5)	55 ± 5 (3)

Data are means ± SE (n). A-line, Y-line, and NOD/Lt males were ages 10, 12, and 9 weeks, respectively.

for the A-line and 0.36 ± 0.14 for the nontransgenic male segregants. Hence the diabetes-protective effect of A-line transgene expression in mice of both sexes was accompanied not by elimination but rather retardation of insulitis.

Proliferation assay. NOD-transgenic A-line mice were not tolerant to immunization with recombinant human GAD65 in complete Freund's adjuvant. In a GAD priming and recall assay, both NOD/Lt and NOD-transgenic A-line popliteal lymph node cells responded to in vitro recall with recombinant hGAD65. (Fig. 4). Hence expression of hGAD65 in β-cells did not elicit T-cell tolerance in this T-cell subset. We have previously shown that the $CD4^+$ T-cell subset is responsible for the primed T-cell responses to hGAD65 in NOD/Lt mice (28).

GAD and insulin antibody production. Males from the A- and Y-line and nontransgenic NOD/Lt males were bled and their sera tested by ELISA for antibodies to GAD65 and insulin. The spontaneous response to GAD65 and insulin in NOD/Lt mice in our colony was predominantly of the IgG2b isotype (27). Serum from the A-line founder, aged 20 weeks, had an anti-hGAD65 IgG2b level of 0.32 OD405 units and serum from the Y-line founder at the same age had an IgG2b level of 0.80 OD405 units. Three NOD/Lt females aged 11 weeks had an average level of 0.442 ± 0.07 OD405 units. Younger (aged 8 weeks) transgenic males of both lines had lower anti-hGAD65 serum IgG2b levels (average level 0.19 ± 0.04 OD405; n = 4); three age-matched NOD/Lt mice had an average level of 0.21 ± 0.05 OD405. Insulin antibody levels were sampled in the serum of two A-line mice (1 female, 1 male) aged 10 weeks. The IgG2b anti-insulin levels were 0.47 and 0.27 OD405 units, respectively, vs. 0.69 and 0.22 OD405 units in two transgene-negative males. Hence humoral tolerance to hGAD65 was not elicited by transgenic expression of hGAD65 in β-cells, consistent with the finding of insulitis and the absence of T-cell tolerance to hGAD65 in the transgenic mice. No IgG1, IgG2a, IgG3, or IgM antibodies to GAD65 were detected in any group.

Adoptive transfers. Splenic leukocytes from diabetes-resistant transgenic A-line donors could transfer diabetes to NOD/LtSz-*scid*/*scid* recipients. (Fig. 5). If the prediabetic donors were A-line females aged 8 weeks (Fig. 5*A*), IDDM onset was retarded in comparison with leukocytes transferred from nontransgenic NOD/Lt females. By 8 weeks after transfer, none of the seven recipients of A-line splenic leukocytes and three of the seven recipients of NOD/Lt splenic leukocytes had become diabetic; by 12 weeks after transfer, one of seven and five of seven, respectively, had developed diabetes. However, by 22 weeks after transfer, five of seven recipients of A-line cells and six of seven recipients of nontransgenic cells had developed diabetes. When splenic leukocytes from nondiabetic transgenic A-line males aged 22 weeks or age-matched standard NOD/Lt male donors (also nondiabetic) were transferred into NOD/LtSz-*scid*/*scid* male recipients (Fig. 5*B*), four of eight recipients of A-line cells and four of seven recipients of NOD/Lt cells developed diabetes within 10 weeks of the transfer. By 15 weeks after the transfer, seven of eight recipients of A-line cells and four of seven recipients of NOD/Lt cells had become diabetic. We interpret these data to mean that the rate of cytotoxic effector activation is slowed in the A-line transgene-positive mice, but that diabetogenic effectors are clearly generated over time.

Because insertion of A-line transgenes on proximal chromosome 15 disrupted a function essential for normal development, it is conceivable that hemizygous content of the transgenes retards the rate of T-cell cytopathic activation due to partial loss of function. To test this hypothesis, a common pool of nontransgenic NOD/Lt splenic leukocytes from prediabetic donors aged 5 weeks was adoptively transferred into standard NOD/Lt-*scid* homozygotes and into the NOD/LtSz-*scid* homozygotes congenic for the A-line transgenes (NOD/LtSz-*scid*.A line). As shown in Fig. 5*C*, the strong retardation in IDDM development mediated by splenic leukocytes from IDDM-resistant A-line donors (Fig. 5*A*) was also observed when the altered chromosome 15 was present in

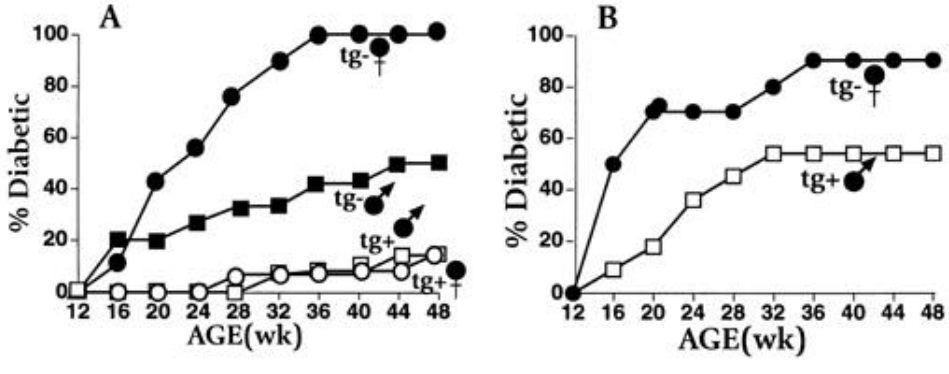

FIG. 3. Diabetes incidence in GAD A-line transgenic mice is lower than in NOD mice. *A*: Incidence study of A-line transgenic mice. A-line transgenic-positive (tg^+) females (○; n = 14), A-line transgenic-negative (tg^-) females (●; n = 9); A-line tg^+ males (□; n = 13), and A-line tg^-males (■; n = 14). *B*: Incidence study of Y-line transgenic mice: Y-line tg^+ males (□; n = 11) and Y-line tg^- females (●; n = 10).

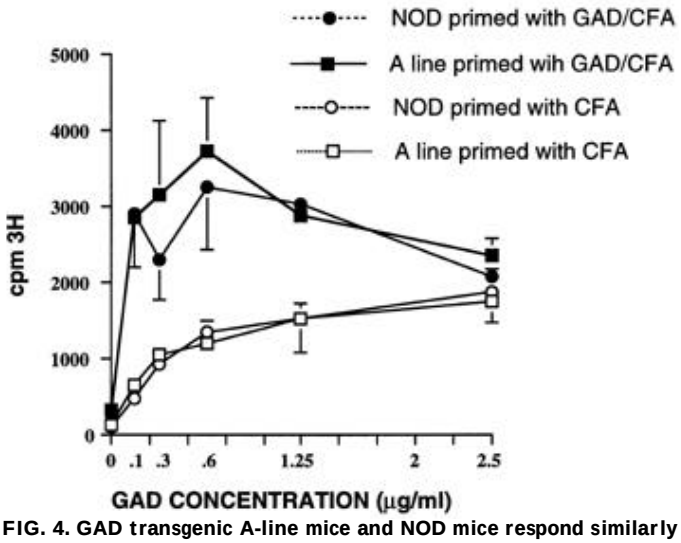

FIG. 4. GAD transgenic A-line mice and NOD mice respond similarly to priming and recall with hGAD65. Data represent the mean incorporation of ^{3}H by triplicate wells of pooled lymph node cells from two males per group. CFA, complete Freund's adjuvant.

only the host and not the donor splenocyte population. Of this latter group, five of nine recipients had not developed IDDM 16 weeks after the transfer. Splenic leukocytes from these five females were pooled and adoptively transferred into a second set of standard NOD/LtSz-*scid* homozygotes. Five weeks after the transfer, six of seven recipients were diabetic; all were diabetic by 8 weeks after the transfer (data not shown). Hence there clearly is a protective effect in a nonlymphoid compartment in these *scid* mice hemizygous for the A-line transgenes. Whether this protection results from high level A-line transgene expression in β-cells or because of insertational mutagenesis into a gene expressed in a nonlymphoid compartment, but necessary for T-cell activation, was not resolved by these adoptive transfer experiments.

RT-PCR analysis of cytokine genes expressed in insulitic infiltrates. Islets from pancreases of A-line, Y-line, and transgene-negative males aged 6 weeks were isolated, and cDNAs prepared as described in METHODS. Amplification with cytokine-specific primer sets showed that diabetes protection unique to the A-line was associated with a strong Th1→Th2 deviation (Fig. 6). In contrast to nontransgenic and Y-line islets, A-line islets showed a drastically reduced ratio of IFN-γ transcripts relative to β-actin, while exhibiting a strong upregulation of the Th2 cytokine, IL-10. Another Th2 cytokine, IL-4, was measured by RT-PCR, but no transcripts were detectable in islet cDNAs made from any of the three classes of islets. These results at the gene expression level support the observations obtained from adoptive transfer that diabetes development in A-line transgenics was associated with a retarded rate of effector cell activation.

DISCUSSION

In this study we addressed the question of whether the level of expression of an intracellular β-cell autoantigen, GAD65, affects the rate or incidence of IDDM in the NOD mouse. This antigen has been shown to be an early target of autoimmune T- and B-cell responses in the NOD mouse; administration of recombinant GAD65 protein in a tolerogenic form to young NOD females retards diabetogenesis (6–8). Although rat islets express substantially more of both GAD isoforms than can be detected in mouse islets, diabetes-prone BB rats are not protected by tolerogenic treatments with GAD65 (32). It is con-

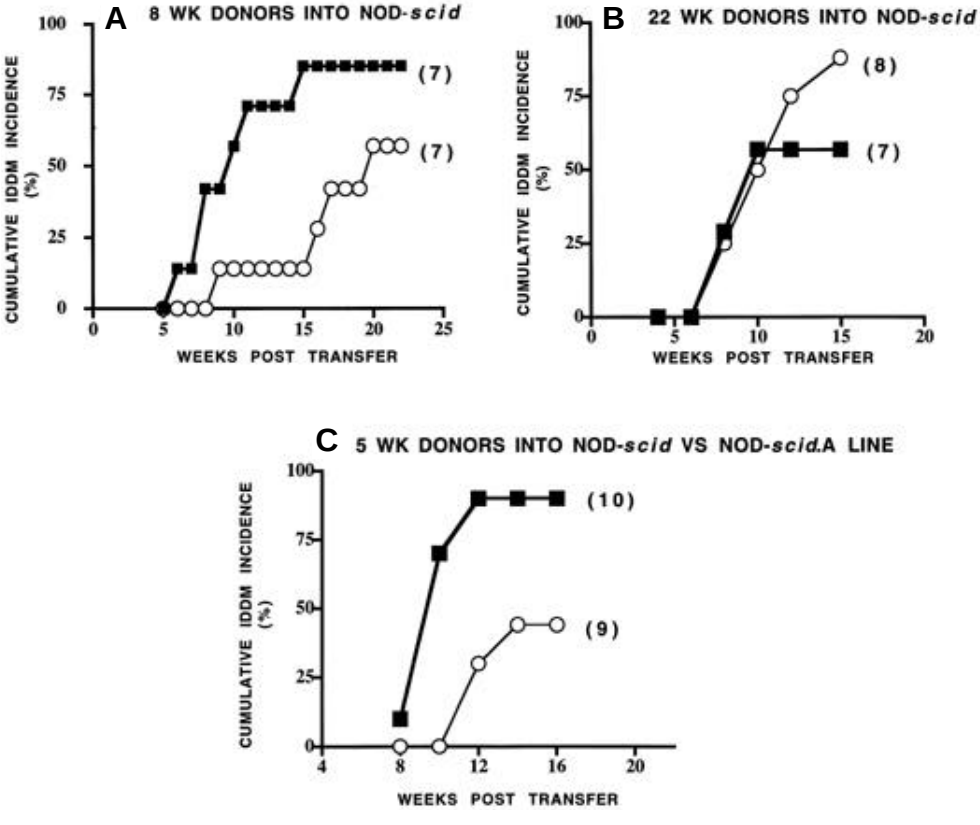

FIG. 5. Adoptive transfer studies in NOD-*scid* indicated that protection entails expression of A-line transgenes in a nonlymphoid compartment. A significantly retarded rate of IDDM adoptive transfer (P = 0.05) was mediated by T-cells from young (aged 8 weeks) (A), but not older (aged 22 weeks) (B) nondiabetic A-line transgenic donors as compared with T-cells from standard NOD/Lt donors. Number of NOD-*scid* recipients of standard NOD (■) or NOD A-line (○) donor cells is shown in parentheses. When the same pool of prediabetic splenic leukocytes from young (aged 5 weeks) nontransgenic NOD/Lt +/+ females were transferred into NOD-*scid* (■) or NOD-*scid* females congenic for the A-line transgenes (○), a significant (P = 0.004) retardation in IDDM transfer was observed in the *scid* mice congenic for the transgenes (C).

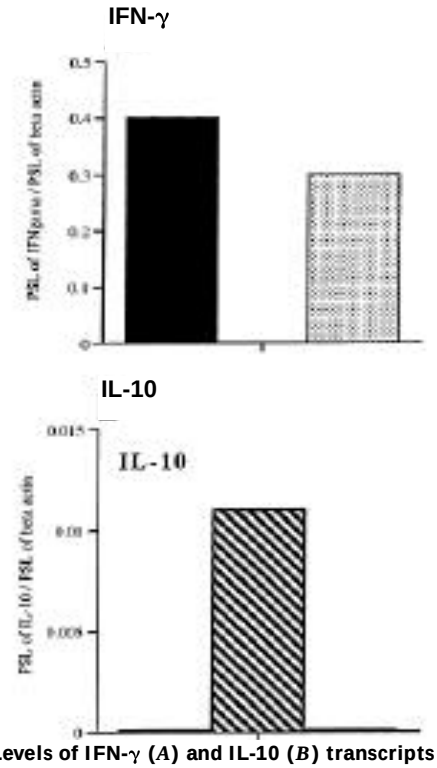

FIG. 6. Levels of IFN-γ (*A*) and IL-10 (*B*) transcripts detected by RT-PCR in islets of A-line, Y-line, and transgene-negative male mice age 6 weeks. RT-PCR was performed and radiolabeled PCR products were semiquantified on a Fuji analyzer. Units are expressed as phosphorescence stimulated luminescence (PSL) using the cytokine primers, relative to PSL using β-actin primers on the same islet cDNA samples. ■, negative segregant; ▨, A line; ⊡, Y line.

ceivable that peripheral T-cells may not be able to acquire tolerance to the trace amounts of GAD present in standard NOD/Lt islets. If pancreatic β-cell–expressed GAD was an essential autoantigen necessary for activating the T-cell–mediated attack on NOD islets, we would have expected that transgene-driven expression of hGAD65 at levels far exceeding constitutively expressed levels of GAD67 in NOD/Lt islets would have modulated diabetogenesis by one of several mechanisms. We did anticipate that acceleration of the autoimmune attack would occur if the transgenic expression resulted in an increased presentation of GAD65 peptides to $CD8^+$ T-cells by major histocompatibility complex (MHC) class I antigens on the surface of the β-cell target and/or to the Th1 subset of $CD4^+$ T-cells in the islet by MHC class II antigens on antigen-presenting cells. It should be noted that although a report has appeared describing GAD65 peptide-reactive class I–restricted cytotoxic T-cell in humans (33), only $CD4^+$ GAD peptide reactive T-cells have thus far been reported in NOD mice (14). In fact, we observed no acceleration of diabetes in either line; rather, IDDM retardation was limited to the A line. That this retardation was correlated with a reduced level of IFN-γ transcripts in the early islet lesions is not surprising, since a variety of treatment protocols capable of immunodeviating NOD Th1-biased cytokine responses to a more balanced Th1/Th2 profile are commonly associated with IDDM protection in NOD mice (34). However, our inability to detect IL-4 transcripts at this early insulitic stage, coupled with the absence of an isotype switch in GAD autoantibodies to the IgG1 subclass, does not allow us to conclude that IDDM resistance in the A line was the direct consequence of a Th1→Th2 immune deviation. Indeed, we favor the interpretation that these cytokine shifts reflect a reduced rate of T-effector cell activation, and consequently a retarded rate of β-cell loss in the A-line islets. Although islets from A-line transgenic mice apparently expressed higher levels of hGAD65 than did islets from Y-line males, islets from both lines expressed GAD65 quantities greatly exceeding the level in standard NOD/Lt β-cells. Hence it is difficult to prove that quantitative differences in hGAD65 production between the two lines represent the basis for immunoprotection in one line but not the other. Similarly, the demonstration of T-cell responses in both transgenic lines after priming with GAD65, coupled with the continued presence of humoral responses, argues against the possibility that peripheral anergy was elicited by hGAD65 presentation in the periphery without an adequate second signaling.

Alternatively, diabetogenesis could be retarded or prevented if expression of GAD65 from the insulin promoter results in expression in the thymus and the development of central tolerance. Recent studies have indicated that intrathymic activation of the insulin promoter can tolerize mice to transgenes ligated to the promoter (35). Indeed, more recent studies in mice have indicated low level expression by a special class of intrathymic stromal cells of genes encoding endocrine prohormones such as proinsulin and prosomatostatin as well as GAD (36). This may be a mechanism for eliciting $CD4^+$ T-cell tolerance to low abundance endocrine proteins made by a relatively small number of endocrine cells and secreted at high levels only several weeks postpartum (36). Support for the concept that intrathymic expression of islet endocrine prohormones may be an essential feature of tolerance induction has come from the analysis of genetics of IDDM in humans. The high-risk *IDDM2* region is presumed to be a variable number tandem repeat (VNTR) upstream of the insulin promoter. The high-risk VNTR allele seems to be less expressible in human fetal thymus than the VNTR allele associated with IDDM resistance (37,38). In this regard, we recently demonstrated that a single intrathymic injection of hGAD65 into NOD/Lt mice produces significant retardation of IDDM that is correlated with a Th1→Th2 deviation at the level of cytokine mRNA transcripts expressed by islet infiltrating leukocytes (39). However, in the present study, there was no evidence of ectopic transcriptional expression of the RIP7hGAD65 construct in newborn thymus as assessed by RT-PCR. Although thymic expression of transgenes has been demonstrated from a shorter version of the insulin promoter (35,36), transgenes expressed from the RIP7 promoter have not yet been detected in the thymus (this study; D. Hanahan, personal communication). This failure to detect transgene expression in a newborn thymus is consistent with the finding that adult A-line mice failed to develop central deletional tolerance to GAD65, since both spontaneous GAD65 antibody responses and recall GAD65-specific T-cell responses were detected in adult A-line mice. Although deletional tolerance was not detected in GAD65-specific B-cells or $CD4^+$ T-cells, the possible development of tolerance in the $CD8^+$ T compartment was not addressed in this study (split tolerance).

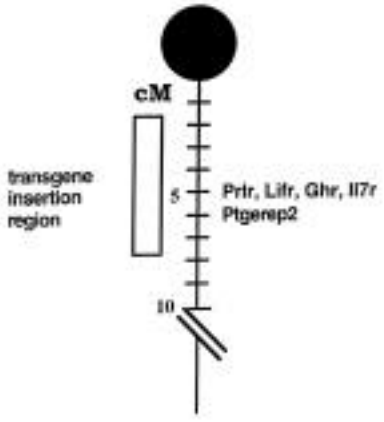

FIG. 7. The hGAD65 transgenes are inserted on proximal chromosome 15 adjacent to the centromere. Some candidate genes in the region are the prolactin receptor (*Prlr*), leukemia inhibitory factor receptor (*Lifr*), growth hormone receptor (*Ghr*), IL-7 receptor (*Il7r*), and prostaglandin E2 receptor (*Ptgerep2*).

Glucagon-secreting pancreatic α-cells have GABA-A receptors and can be inhibited by GABA (40). This in turn may suppress hepatic glucose output and thereby possibly suppress the symptoms of hyperglycemia. However, neither the A-line nor the Y-line transgenic males were distinguished from prediabetic nontransgenic NOD/Lt males in terms of plasma glucagon or insulin levels. Hence paracrine effects of transgene expression are not indicated.

As discussed above, many of the immune intervention studies in NOD mice entailing administration of candidate autoantigens or peptides derived from them in the prediabetic phase have involved downregulation of IFN-γ mRNAs expressed by islet-infiltrating leukocytes (34). We observed that splenic leukocytes from young prediabetic A-line transgene-positive donors adoptively transferred IDDM more slowly into NOD-*scid* recipients when compared with young transgene-negative donors. We favor the interpretation that a slower rate of cytopathic activation occurred in islet-infiltrating leukocytes in the former. In a colony of NOD mice in which IDDM pathogenesis was markedly retarded in males in comparison with females, the male gender-biased protection was correlated with a higher level of Th2 cytokine gene expression (41). In the present study, we observed reduced IFN-γ transcript levels when comparing the rapidly progressing destructive insulitis in NOD and Y-line males to the more slowly activating insulitis in A-line males. This reduction was correlated with an increase in the IL-10 transcript level, which could reflect a Th1/Th2 deviation or the generation of a recently designated Tr1 regulatory subset that secretes IL-10 but not IL-4 and represses Th1 development (42). As shown by the data in Fig. 5*C*, transgene integration into the genome of effector T- or B-cells is not required for protection, since integration of the A-line transgenes into the NOD/LtSz-*scid* genome was sufficient to retard the rate at which splenic T-cells from nontransgenic NOD/Lt donors adoptively transferred IDDM into these transgene-bearing *scid* recipients.

Finally, we would like to address the possibility that the site of insertion on chromosome 15 of the A-line transgenes may be involved in the IDDM protection observed in this line. The potential for transgenes to disrupt endogenous gene function is only now being recognized. Transgenic reintroduction of the NOD's I-A^{g7} gene into NOD mice produced a reduction in IDDM incidence associated with a subtle reduction in B-cell numbers (43). In another study, a transgene ligated to the rat insulin promoter integrated into the *scid* locus on chromosome 16, producing severe combined immunodeficiency (44). In the present study, neither of the two RIP7hGAD65 transgenic lines were T- or B-cell deficient as assessed by flow cytometry. The cytogenetic band in which the IDDM protective A-line transgenes are localized indicates that they integrated within 6 cM from the centromere. The diagram presented in Fig. 7 shows a number of interesting candidate genes in this area whose function may have been disrupted by insertional mutagenesis. These include the leukemia inhibitory factor receptor (*Lifr*), the growth hormone receptor (*Ghr*), the prolactin receptor (*Prlr*), the IL-7 receptor (*Il7r*), and prostaglandin E2 receptors (*Ptgerep2*). Although many transgenic manipulations in NOD mice that reduce IDDM incidence have been reported previously, this is the first report in which the chromosome location of the integrated transgenes under investigation has been identified. Insertional mutagenesis is rarely, if ever, considered as the basis for any diabetes protection that may be observed in transgenic NOD progeny. Yet the overall frequency of insertion mutations created by transgenesis has been estimated to be 5–10% (45), and the above-cited example of a transgene designed for expression in β-cells leading to disruption of the DNA-dependent protein kinase gene at the *Prkdc*^{scid} locus (44) emphasizes the need to consider insertional mutagenesis as the basis for the reduced rate of diabetogenic activation of T-cells in A-line transgenics.

In summary, we compared islet-specific GAD transgene expression in two independent lines of NOD/Lt mice that both express at high levels, but only one of which was protected from IDDM development. We demonstrated that protection in the A line was associated with higher protein and enzyme activity levels and correlated with shifts in the pattern of cytokine genes expressed by islet-infiltrating leukocytes. We raised the possibility that the protection in the A-line is the consequence of either an increased presentation of the GAD65 autoantigen or insertional mutagenesis. The latter possibility seems worth pursuing, since the A-line transgenes integrated into and disrupted a gene required for normal development. This study underscores the need to map transgene integration positions when evaluating mechanisms of transgene-conferred protection.

Note Added in Proof: A study has recently appeared reporting accelerated IDDM in males in one line of transgenic NOD mice in which GAD65 expression was driven by an MHC class I promoter (Geng LP, Solimena M, Hayday AC, Flavell RA, Sherwin RS: Widespread expression of an autoantigen-GAD65 transgene does not tolerize nonobese diabetic mice and can exacerbate disease. *Proc Natl Acad Sci U S A* 95:55–60, 1998).

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Author Queries (please see Q in margin and underlined text)

Au: Please check throughout manuscript that B-cell and β-cell (beta) are used correctly.

Q1: Please spell out OD.
Q2: In "(B6 X SJL) F1," OK to add space after second parenthesis?
Q3: Has dNTPs been correctly spelled out?
Q4: Has ME been correctly spelled out?
Q5: Please give manufacturer name and location for hybridization/detection kit.
Q6: Please spell out DAPI.
Q6a: Is "GAD6" OK here or GAD65?
Q7: Please give location for Kontes.
Q8: Please give full name and location of manufacturer of scintillation counter.
Q9: Is the correct measure 25 µg/l hGAD?
Q10: In sentence beginning "The h65GAD," should this be changed to "The hGAD65"?
Q11: Please spell out CTP.

Q13: In last half of sentence beginning "FISH analysis," change to "the transgene was inserted" correct?
Q13a: Correct that A-line tg+ males are represented by the open square?

Q14: Please spell out CFA.

Q16: Please spell out CTL.

Q17: Does NCI stand for National Cancer Institute?

Q18: Ref. 20: Please clarify which journal is meant.