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Immunology

T-cell tolerance toward a transgenic β-cell antigen and transcription of endogenous pancreatic genes in thymus

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ABSTRACT **Transgenic mice expressing T antigen (Tag) in pancreatic β cells establish systemic tolerance toward this self-protein. The self-tolerance in two families of rat insulin promoter (RIP)-Tag mice, expressing different levels of Tag protein, has been characterized. These mice have impaired antibody responses to Tag, show diminished Tag-specific T-cell proliferation, and evidence an inability to generate Tag-specific cytotoxic T cells. The existence of systemic tolerance toward a β-cell-specific protein motivated examination of transgene expression in the thymus. Indeed, low levels of Tag mRNA were detected intrathymically. Remarkably, this expression is a valid property of the insulin gene regulatory region, since insulin RNA was also expressed in the thymus of nontransgenic mice. RNA for other pancreatic genes was also detected in the thymus, thus raising the possibility that many tissue-specific genes could be expressed intrathymically during immunological development and induction of self-tolerance. These results raise important questions for future research into the role of the thymus in tolerance induction toward so-called tissue-specific antigens.**

Much of our knowledge about the cellular mechanisms of self-tolerance comes from studies of the interaction of T cells with major histocompatibility antigens (1–3). It is evident from these studies that the thymus is the primary site for induction of self-tolerance, wherein mechanisms including programed cell death and functional inactivation serve to eliminate self-reactive T lymphocytes. Yet the existence of organ-specific autoimmunity suggests that tolerance toward proteins with restricted patterns of expression may be established by alternative mechanisms (3, 4). Many recent studies have focused on the concept of "peripheral tolerance," whereby nonresponsiveness toward organ-specific antigens is proposed to be elaborated extrathymically, in mature T cells. In one approach, transgenic mice expressing novel antigens in a restricted pattern have been used to study peripheral tolerance (4–6). For example, the insulin gene regulatory region has been utilized to target a variety of antigens to the pancreatic β cell in transgenic mice (7–12). The β cell, a rare cell type localized within the pancreatic islets, is the target cell for immune-mediated destruction in type I diabetes.

The initial demonstration that transgenes utilizing the rat insulin promoter (RIP) could be used to study interactions of the β cell with the immune system came from studies on a viral oncoprotein, simian virus 40 (SV40) T antigen (Tag). Lines of RIP-Tag mice showed two alternative immunological phenotypes, tolerance, or nontolerance and spontaneous autoreactivity (8), which correlated with distinct patterns of transgene expression. The tolerant mice began to express Tag in islet cell progenitors concomitant with activation of the endogenous insulin genes during embryogenesis (13). In contrast, the nontolerant mice activated the transgene in adulthood (8), resulting in failure to establish tolerance and spontaneous autoimmunity (14). With regard to the developmental-onset RIP-Tag mice, previous work documented humoral tolerance toward Tag and a lack of leukocyte infiltration of the pancreatic islets (8, 15). Several other studies that targeted viral antigens to the β cells illustrate the diversity of possible immune responses to this rare cell type (9–12).

In the present study we have characterized T-cell responsiveness in two developmental-onset lines of RIP-Tag mice that express distinctive amounts of Tag. Remarkably, humoral, proliferative, and cytotoxic responses toward this β-cell antigen are dramatically impaired. The existence of profound systemic T-cell tolerance raises a perplexing question: How is it that T cells throughout the body are rendered nonresponsive toward a pancreatic islet cell antigen? This dichotomy provoked examination of transgene and endogenous insulin gene expression in the developing thymus. We report that the RIP-Tag transgene is expressed at low levels in the thymus. Notably, this expression pattern recapitulates that of the endogenous insulin genes. Moreover, several other pancreatic genes are also transcribed intrathymically, which implicates the thymus in establishment and maintenance of tolerance to these "peripheral proteins."

MATERIALS AND METHODS

Mice. RIP1-Tag2 mice have been described (7). To generate the RIP3-Tag construct, a 9.5-kb *Eco*RI fragment 5′ to the *Eco*RI site at −451 bp was used to replace the sequences between −695 bp and −451 bp in the original RIP1-Tag construct. This construct was injected into B6D2F2 embryos. The RIP1-Tag2 mice studied were backcrossed between 9 and 12 generations to C57BL/6 and the RIP3-Tag2 mice between 6 and 8 generations. C57BL/6, B6D2F1, and BALB/c mice were purchased from either The Jackson Laboratory or Bantin & Kingman (Fremont, CA). Male and female mice were studied and were immunized at 6 weeks of age.

T Antigen Protein. Tag was purified from Sf9 or High Five (Invitrogen) insect cells infected with a baculovirus expression vector carrying the SV40 early region (16) by affinity chromatography (17).

Antibody Response. Mice were primed i.p. with 10 μg of Tag and 5 μg of β-galactosidase (β-gal) in complete Freund's adjuvant (CFA), given booster immunizations 14 days later with the same amount of protein in incomplete Freund's adjuvant, and bled on day 20. Sera were tested in a standard ELISA. Briefly, plates were coated with 10 μg of Tag or β-gal

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Abbreviations: RIP, rat insulin promoter; SV40, simian virus 40; Tag, SV40 T antigen; β-gal, β-galactosidase; CTL, cytotoxic T lymphocyte; RT, reverse transcriptase; LCMV, lymphocytic choriomeningitis virus; GP, glycoprotein; GAD65 and GAD67, two isoforms of glutamic acid decarboxylase; β_2m, β_2-microglobulin.

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per ml and blocked with 1% bovine serum albumin. Serial dilution of sera and positive control antibodies, 419 (7) and Z378 (Promega), were tested. Plates were developed with biotinylated goat anti-mouse IgG (Fisher), streptavidin-horseradish peroxidase (Vector Laboratories), and *o*-phenyldiamine (Zymed).

Proliferation Assay. This assay has been described (18). Briefly, female mice were immunized s.c. at the base of the tail with 25 or 50 μg of Tag protein in CFA. Ten days later lymph node cells were cultured in 96-well flat-bottomed plates in HL-1 medium (Ventrex Laboratories, Portland, ME) in triplicate. Wells containing 4×10^5 cells and serial dilutions of Tag protein, nonviable desiccated *Mycobacterium tuberculosis* H37RA (Difco), or medium alone were incubated at 37°C for 72 hr and pulsed with 1 μCi of [^{3}H]thymidine the last 6 hr (1 Ci = 37 GBq). Plates were harvested and counted on a Betaplate Reader (Pharmacia LKB).

Cytotoxic T Lymphocyte (CTL) Assay. This assay has been described (19). Briefly, mice were immunized i.p. with 2×10^7 C57SV, SV40-transformed fibroblasts from C57BL/6 mice. Seven to 10 days later, 2×10^7 spleen cells were cultured with 2.5×10^5 irradiated (3000 rads; 1 rad = 0.01 Gy) C57SV or 5×10^7 irradiated (3000 rads) BALB/c spleen cells. CTLs were tested using either a ^{51}Cr release assay (19) or a DNA fragmentation assay (20) on C57SV or control allogeneic P815 (American Type Culture Collection) H-2^d targets.

RNA Preparation and cDNA Synthesis. Tissues were removed from mice at various ages. To prevent potential cross-contamination of samples, dissecting instruments were sequentially rinsed in 2 M NaOH, autoclaved water, and ethanol between samples. RNA was prepared using the RNAzol (Tel-Test, Friendswood, TX) method. Aliquots (3 μg) of RNA were treated with DNase and split in half. cDNA was synthesized under standard conditions using random primers. One tube received buffer (−RT) and the other buffer plus Moloney murine leukemia virus reverse transcriptase (BRL) (+RT). For the dilution series, pancreatic RNA from a newborn RIP1-Tag2 mouse was diluted into thymus RNA from an adult C57BL/6 mouse.

Detection of RNA in Pancreas and Thymus. RNA-specific fragments were amplified from cDNA using *Taq* DNA polymerase in reaction mixtures spiked with 5 μCi of [α-^{32}P]-dATP. Amplification was carried out for 30 cycles of 1 min at 95°C, 30 s at 60°C, and 2 min at 72°C. The primers for Tag (21), β_2-microglobulin (β_2m) (22), and pancreatic genes (23) have been reported, except for trypsin (GATTCTGCCAAGATCATCCG and GTATACACCAGGAGCATCTG), elastase (TCCACGGTGAAGACGACCATG and GGCAATGACATTGTTCATCCA), and two isoforms of glutamic acid decarboxylase, GAD65 (CCTGGTGAGTGCCACAGCTG and CTGGCGCCACCTTTGAGAGG) and GAD67 (TGCAACCTCCTCGAACGCGG and CCAGGATCTGCTCCAGAGAC). For all primer sets, the two primers are in different exons. Eight percent polyacrylamide gels were run, fixed, dried, and used to expose x-ray film.

Western Blotting. Pancreata from 1-week-old mice were disrupted in 50 mM TRIS, pH 8.0/0.3% SDS/1.0% 2-mercaptoethanol/2 μM leupeptin/2 μM pepstatin/0.5 mM *p*-nitrophenyl *p'*-guanidinobenzoate. Extracts were heated for 10 min at 95°C and clarified by centrifugation. The protein level was determined and samples were run on 10% SDS/PAGE. The gel was blotted onto nitrocellulose, blocked with 5% nonfat milk, probed with monoclonal antibody 416 (7), detected with anti-mouse IgG-horseradish peroxidase (Vector Laboratories), and developed using the ECL system (Amersham).

RESULTS

RIP1-Tag2 and RIP3-Tag2 mice use different versions of the rat insulin II promoter to direct the expression of Tag to the pancreatic β cell. RIP1-Tag comprises 695 bp of 5′ flanking DNA, whereas RIP3-Tag extends ≈10,000 bp 5′ from the promoter. Both lines of mice begin expressing Tag in the pancreatic bud at embryonic day 9 (ref. 13; unpublished data). Since Tag is a nuclear oncoprotein, its expression inevitably leads to the formation of β-cell tumors (ref. 7; unpublished data). Tumors are first detected between 10 and 12 weeks of age. Therefore, mice studied in the immunological assays were immunized at 6 weeks of age and analyzed over the next several weeks, prior to overt tumor formation.

RIP-Tag Mice Develop Systemic Tolerance. To characterize the tolerance toward the β-cell antigen Tag, RIP1-Tag2 and RIP3-Tag2 mice were tested in three T-cell-dependent assays. In the first assay, mice were immunized with a combination of Tag and, as a control antigen, β-gal, and their ability to make Tag-specific antibody was assessed (Fig. 1*A*). Transgenic mice had an impaired Tag-specific antibody response relative to controls. Humoral tolerance has been described for RIP1-Tag2 mice (15) but not for RIP3-Tag2 mice. The mean anti-Tag response of transgenic mice was 350-fold lower than that of the control group.

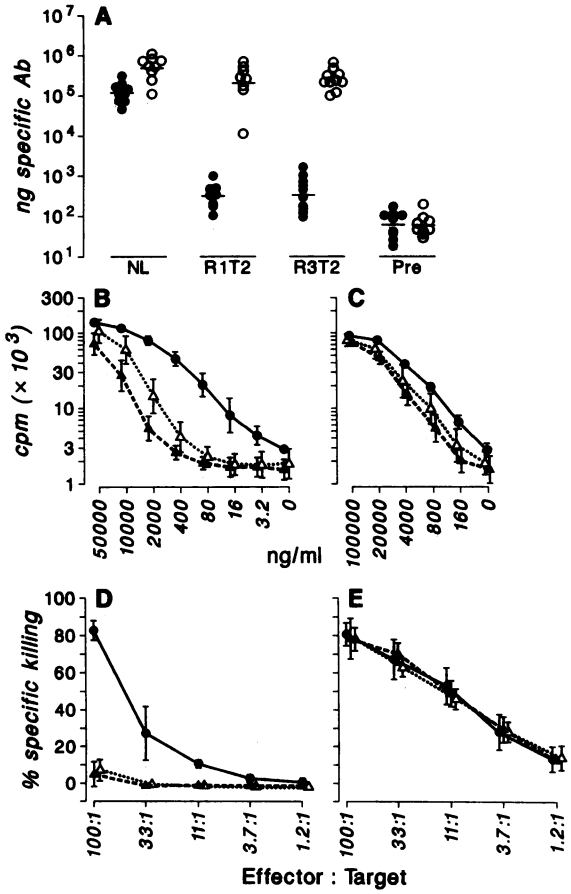

FIG. 1. RIP-Tag mice develop systemic tolerance toward Tag. (*A*) Secondary antibody (Ab) response to Tag and β-gal. The response to Tag (●) and β-gal (○) was calculated for individual mice. The mean of all animals in the group is represented by a bar. Nontransgenic mice, RIP1-Tag2, and RIP3-Tag2 are abbreviated as NL, R1T2, and R3T2, respectively. Pre indicates the values obtained from the preimmune serum of the nontransgenic littermates. (*B* and *C*) *In vitro* proliferation of RIP-Tag lymphocytes. Lymph node cells were cultured with dilutions of Tag (*B*) or *M. tuberculosis* (*C*) and pulsed with [^{3}H]thymidine. (*D* and *E*) CTL activity generated by RIP-Tag splenocytes cultured with either Tag-expressing fibroblasts or BALB/c stimulators. Cultured splenocytes were tested for their ability to kill ^{51}Cr-loaded C57SV (*D*) or allogeneic P815 (*E*) targets. In *B–D*, the mean for all animals in each group, nontransgenic littermates (●), RIP1-Tag2 (▲), and RIP3-Tag2 (△), is shown.

Next we evaluated the ability of T cells from immunized mice to proliferate in response to Tag (Fig. 1 *B* and *C*). Cultured lymph node cells from transgenic mice had diminished Tag-dependent proliferation, relative to controls. By comparing the amount of Tag protein that elicits 20% of the maximal proliferation in the control group, it can be determined that 35 and 90 times more Tag is required for RIP3-Tag2 and RIP1-Tag2 T-cells, respectively, to proliferate to an equivalent extent. Although the Tag protein was affinity purified, we cannot exclude a contribution to the observed response by minor contaminants in the preparation. In independent experiments the proliferative response of transgenic mice to other foreign antigens, hemocyanin, ovalbumin, and β-gal, was indistinguishable from that of controls (data not shown).

In the third assay, we asked whether RIP1-Tag2 and RIP3-Tag2 mice could generate Tag-specific CTLs. Spleen cells from immunized mice were tested for their ability to kill Tag-expressing fibroblasts or allogeneic targets in a ^{51}Cr release assay (Fig. 1 *D* and *E*) and a more sensitive DNA fragmentation assay (data not shown). Although control mice generated CTLs capable of killing Tag-expressing cells, no anti-Tag CTL activity was detected in cultures from transgenic mice in either assay.

In two of the assays used to characterize the tolerant phenotype of RIP1-Tag2 and RIP3-Tag2 mice, immune responses toward Tag could be detected, albeit at significantly reduced levels. Therefore we asked if these reactive T cells were potentially autoreactive toward Tag protein in β cells. Pancreata from mice that had been immunized and given booster immunizations with Tag s.c. were examined for islet infiltration and destruction by immunohistochemistry. No evidence of lymphocytic infiltration or immunopathology was observed (data not shown). Thus, RIP1-Tag2 and RIP3-Tag2 T-cells respond normally to foreign antigens and weakly to exogenous Tag but are systematically tolerant toward the endogenous antigen Tag in islet β cells.

Quantitation of Tag Protein in the Pancreas. The level of Tag in the pancreas was quantitated to provide an estimate of the amount of this protein being produced by these systemically tolerant mice. First we developed a semiquantitative PCR-based assay (Fig. 2*C*) to examine Tag mRNA expression. The pattern of Tag expression in the developing pancreas of the two lines of mice differed (Fig. 2*A*) but stabilized by 1 week of age. Therefore, we chose to quantitate Tag protein from pancreatic extracts of 1-week-old mice by Western blotting (Fig. 3). Approximately 1 ng of Tag was detected in 30 μg of pancreatic extract from RIP3-Tag2 mice. Since 1.8 mg of extract was obtained from a single pancreas on average, we estimate that 60 ng of Tag is present in a 1-week-old RIP3-Tag2 pancreas. RIP1-Tag2 pancreata contained ≈10 ng of Tag protein.

Tag Expression in the Thymus of RIP-Tag Mice. While the primary site of Tag expression is the pancreatic β cell, documentation of systemic tolerance toward this self-protein led us to investigate whether the transgene might be expressed in the thymus. Thymic cDNAs were tested for the presence of Tag-specific message by PCR (Fig. 2*B*). As expected, a Tag-specific signal was not amplified from nontransgenic thymus cDNA or from samples to which no RT had been added (−RT). Surprisingly, faint Tag bands appeared at distinct times during development in the two lineages. In RIP3-Tag2 mice Tag message was detected at 1 week, whereas in RIP1-Tag2 it was detected in newborn mice. To confirm the specificity of this expression, we examined other tissues from transgenic mice. A Tag-specific signal could be amplified from cDNA made from brain, testis, and small intestine of adult RIP1-Tag2 mice and from brain and skeletal muscle of adult RIP3-Tag2 mice, but not from adult kidney, heart, liver, large intestine, skin, lung, salivary gland, adrenal, thyroid, lymph node, or bone marrow, nor

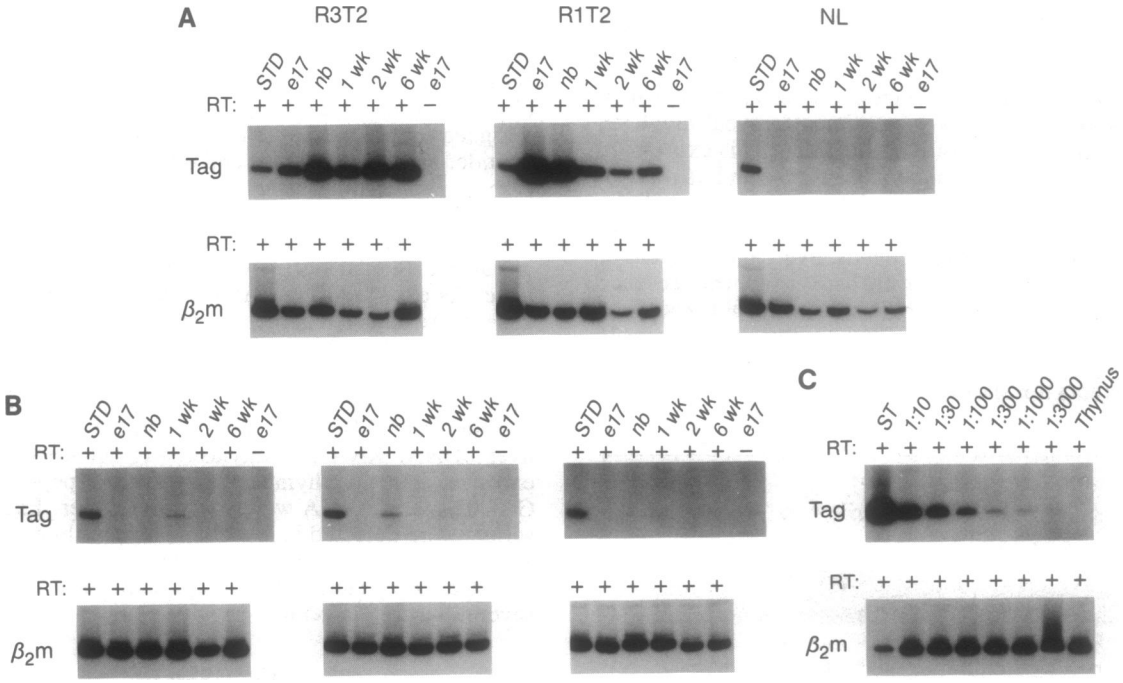

FIG. 2. Expression of Tag during development in pancreas and thymus. Pancreatic and thymic cDNAs were tested by PCR for the presence of Tag- and β_2m-specific sequences. The large T antigen band (156 bp) is shown. Ages examined are embryonic day 17 (e17), newborn (nb), 1, 2, and 6 weeks. + and − indicate whether RT had been added to the sample. (*A*) Tag expression in the pancreas of transgenic mice. (*B*) Tag expression in the thymus of transgenic mice. (*C*) Sensitivity of RNA-PCR assay. Pancreatic RNA from a newborn RIP1-Tag2 was diluted into thymus RNA of a C57BL/6 adult at the ratios indicated above the lanes prior to cDNA synthesis. The first sample (ST) is undiluted pancreatic RNA. To compare the reaction products from different experiments, the 1:100 sample from the dilution series was included as a standard (STD) in each experiment.

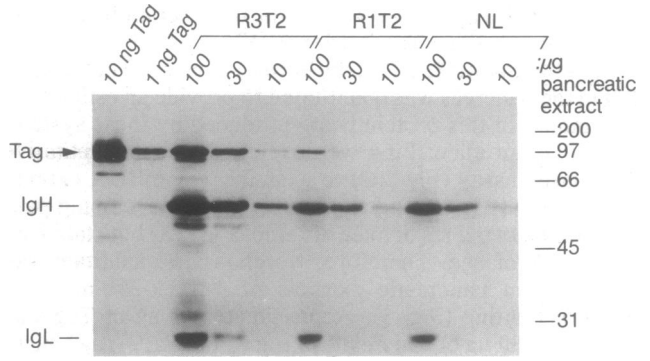

FIG. 3. Quantitation of Tag protein in RIP-Tag pancreata. Pancreatic extracts from 1-week-old RIP3-Tag2 (R3T2), RIP1-Tag2 (R1T2), and nontransgenic littermate (NL) mice were run on SDS/PAGE and immunoblotted. The Tag-specific band is marked by an arrow. Mouse IgG was present in the extracts and the heavy (IgH) and light (IgL) chains are detected by the secondary reagent.

newborn liver (data not shown). Although Tag mRNA in brain and testis of RIP1-Tag2 was easily detectable, both are considered immunologically privileged sites. The levels of Tag message in intestine of RIP1-Tag2 as well as skeletal muscle and brain of RIP3-Tag2 mice are quite low. A faint Tag-specific signal was detected in spleen from an adult RIP3-Tag2, but not a RIP1-Tag2 mouse. The inability to detect Tag RNA in most tissues supports the specificity of the thymic Tag expression. When cDNAs prepared from five times as much RNA were tested in the PCR assay, Tag-specific message was detected in newborn and 1- and 2-week-old transgenic thymus from both lineages, but not in nontransgenic littermates, thus indicating that this expression is not transient but persists until at least 2 weeks of age.

To determine whether Tag protein could be detected in the thymus, we examined thymic sections from newborn RIP1-Tag2 and 1-week-old RIP3-Tag2 mice for Tag immunoreactivity (data not shown). No Tag-expressing cells could be identified, supporting the notion that the level of Tag expressed by thymic residents is low.

Thymic Tag Expression Recapitulates Endogenous Insulin Gene Expression. The above experiments reveal low-level Tag expression in thymus of RIP-Tag mice. This expression could be an artifact of the chimeric transgene and its random integration or, rather, reflect an intrinsic property of the insulin gene regulatory region. To distinguish between these two possibilities, cDNAs from thymus, liver, and pancreas of C57BL/6 mice of various ages were assayed for the presence of insulin transcripts by RNA-PCR (Fig. 4). Insulin RNA was detected in thymus and pancreas, but not liver, of all animals tested. Insulin message was also undetectable in spleen and lymph node (data not shown). Thymic insulin expression was

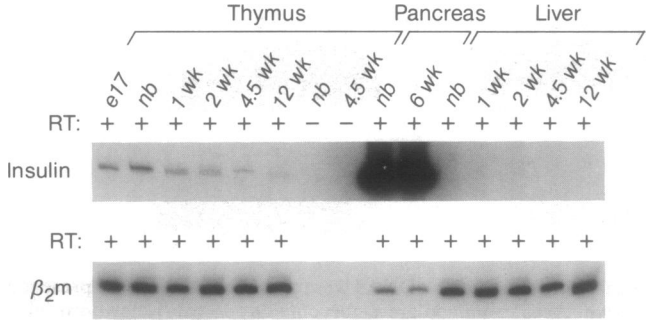

FIG. 4. Insulin expression in the thymus. cDNAs from thymus, pancreas, and liver of C57BL/6 mice were tested for the presence of insulin or β_2m-specific transcripts in independent reactions. The figure is labeled as in Fig. 2.

Table 1. Expression of pancreas-specific genes in the thymus

Gene	Tissue			
	Pancreas	Thymus	Liver	Brain
Tag	+++++	+	−	
β_2m	++++	+++++	+++++	+++++
Glucagon	++++	++	−	
Insulin	+++++	+	−	
Pancreatic polypeptide	++++	++	−	
Somatostatin	++++	++	−	
Trypsin	++++	+	−	
Amylase	+++++	−	+++	
Carboxypeptidase A	++++	−	−	
Elastase	+++++	+	+	
GAD65	−	−	−	+++++
GAD67	+	+	−	+++++

cDNAs from newborn RIP1-Tag2 and nontransgenic mice were tested for transcripts of genes normally expressed in the pancreas. All samples are from newborn mice except the brain sample, which is from a 6-week-old mouse. Data from four independent experiments are summarized. The intensity of each signal was compared to the Tag bands in the dilution series in Fig. 2*C*. Bands with intensities greater than or equal to the undiluted sample are represented as +++++. Bands greater in intensity than the 1:10, 1:30, 1:100, and 1:300 samples are designated by ++++, +++, ++, and +, respectively. Inability to detect specific mRNA is indicated by −. Although GAD65 protein has been detected at very low levels in mouse islets (24), as well as in cell lines derived from RIP1-Tag2 mice (25), we have been unable to detect GAD65 transcripts in RNA extracted from newborn or adult pancreas, from isolated islets, or from the thymus, despite its clear expression in brain.

strongest in perinatal mice although it persisted until 12 weeks of age. Thus, thymic Tag expression is not an artifact of the transgenic system but, rather, reflects a property of the insulin genes.

Expression of Pancreas-Specific Genes in the Thymus. The detection of insulin gene transcription in the thymus raised the larger issue of whether other pancreas-specific genes are expressed there. To address this question, cDNAs from thymus, liver, and pancreas were analyzed for gene transcripts selectively expressed by distinct pancreatic cell types (Table 1). Primers that amplify RNA-specific products were designed for endocrine- (glucagon, insulin, pancreatic polypeptide, and somatostatin) and exocrine- (trypsin, amylase, carboxypeptidase A, and elastase) specific genes and for GAD67 and the β-cell autoantigen, GAD65. The results from newborn RIP1-Tag2 and nontransgenic mice are summarized in Table 1. Similar results were obtained for adult C57BL/6 mice. As expected, mRNAs for all endocrine and exocrine genes were detected in the pancreas at high levels. We failed to detect GAD65 RNA in the pancreas but were able to detect low levels of GAD67 message in newborn pancreas. Weak signals for glucagon, pancreatic polypeptide, somatostatin, trypsin, and GAD67 were detected in the thymus but not in liver cDNA. RNA for amylase was detected in liver, as expected, but not thymus, while carboxypeptidase A and GAD65-specific RNA were found in neither. Elastase transcripts were detected in low abundance in thymus and liver. Thymic expression of somatostatin has been previously reported (26). Thus, the thymus expresses low levels of several pancreatic genes.

DISCUSSION

We have shown that RIP3-Tag2 and RIP1-Tag2 mice develop systemic T-cell tolerance toward the self-antigen Tag. The pancreatic β cells comprise the main site of Tag protein synthesis, containing 9 and 60 ng of Tag in 1-week-old RIP1-Tag2 and RIP3-Tag2 mice, respectively. Should the entire contents of the β cells be released into the circulation,

Tag would be present at 0.5 and 3 nM in the intravascular fluid volume of RIP1-Tag2 and RIP3-Tag2. These levels fall into the range of rare serum proteins that may be ignored by the immune system (27). There is no evidence that Tag protein is quantitatively released by the β cells. Thus the amounts released, for example, by cell death, should be several orders of magnitude lower than these maximal calculated values and therefore below the threshold for tolerization as a serum protein.

How is it then that systemic tolerance toward Tag is established? It is unlikely that all recent thymic emigrants circulate past the β cells and become tolerized in the pancreas (28). Another possibility is that Tag, released by the β cells, is carried back to the lymph nodes or thymus. As discussed above, this is also an inadequate explanation for the systemic tolerance observed. In addition to the Tag produced by the β cells, we now report expression of Tag RNA in the thymus itself. This expression is directed by the insulin gene regulatory region and is 3000–10,000 times lower than that in the pancreatic β cells. Two other groups using insulin-promoted transgenes have provided evidence that peripheral (12) and thymic (29) expression of the transgene can contribute to systemic tolerance.

The surprising observation that the endogenous insulin genes are expressed in the thymus motivated similar evaluation of other tissue-specific genes. Remarkably, thymic expression of several pancreatic genes from endocrine and exocrine cell types was detected. Whether other genes, normally thought of as being "peripheral," are expressed intrathymically awaits further study. If expression of "tissue-specific" genes is a general property of the thymus, it would support a hypothesis in which the thymus plays a role in limiting autoreactivity to proteins thought of as being peripheral. A set of observations lends credence to this notion. Oldstone *et al.* (11) described mice that express the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) in the pancreatic β cells. GP is normally ignored by the immune system. When mice are infected with LCMV, a slow onset of diabetes ensues. This is in contrast to RIP-GP mice developed by Ohashi *et al.* (10), which develop rapid onset of disease after LCMV infection. Recently, Oldstone's group has discovered GP expression in the thymus only in those mice with slow-onset disease (M. B. Oldstone and M. Vonnerrath, personal communication). These results argue that thymic expression of a peripheral protein contributes to nonresponsiveness but is inadequate to tolerize all potentially autoreactive T cells. Arnold *et al.* (4) have proposed a model in which lymphocytes undergo multiple antigen encounters that drive them further and further into a state of nonresponsiveness. The initial encounter may occur in the thymus. If thymic expression of tissue-specific genes plays a role in self-tolerance toward rare cell types, then it will be of interest to assess the possibility that defects in thymic expression are a component to organ-specific autoimmunity.

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