Thyroid Antigens, Not Central Tolerance, Control Responses to Immunization in BALB/c Versus C57BL/6 Mice

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Background: Vaccination with cDNA for the human thyrotropin receptor (TSHR) in a plasmid, without adjuvant, induces TSHR antibodies in C57BL/6 but rarely in BALB/c mice. This outcome could be due to a difference between “high” versus “low” antibody responder mouse strains. However, unlike their poor response to TSHR-DNA vaccination, BALB/c mice vaccinated with thyroid peroxidase (TPO)-cDNA readily develop antibodies to TPO. We hypothesized that insight into these conundrums would be provided by the following differences in central tolerance: (i) between two mouse strains (C57BL/6 versus BALB/c) for the TSHR; and (ii) between two thyroid autoantigens (TPO and the TSHR) in one mouse strain (BALB/c).

Methods: We studied autoantigen expression using real-time polymerase chain reaction to quantify mRNA transcripts for the TSHR, TPO, and thyroglobulin (Tg) in thymic tissue (as well as in thyroid) of young mice.

Results: Our hypothesis was not confirmed. Intrathymic TSHR transcript expression was similar in BALB/c and C57BL/6 mice. Moreover, thymic mRNA transcripts for TSHR and TPO were comparable. Unlike the 10-fold differences for the autoantigens in thyroid tissue (Tg greater than TPO which, in turn was greater than the TSHR), intrathymic transcripts for TPO and the TSHR were similar, both being slightly lower than the level for Tg.

Conclusions: Central tolerance, assessed by measuring intrathymic transcripts of thyroid autoantigens, does not explain the different outcome of TSHR-DNA vaccination in BALB/c and C57BL/6 mice, or even susceptibility versus resistance to hyperthyroidism induced by TSHR-adenovirus. Instead, differences in MHC and TSHR T-cell epitopes likely contribute to TSHR antibody development (or not) following DNA plasmid immunization. The greater immunogenicity of TPO versus TSHR probably relates to the greater number of nonhomologous amino acids in the human and mouse TPO ectodomains (78 amino acids) than in the human and mouse TSHR ectodomains (58 amino acids). Overall, the autoantigens themselves, not central tolerance, control DNA plasmid-induced immunity to TPO and the TSHR.

Introduction

GENETIC INTRAMUSCULAR IMMUNIZATION using a plasmid containing the cDNA for the thyrotropin receptor (TSHR) induces variable results. In two studies, such immunization generated thyroid stimulating antibodies in BALB/c mice (1) and hyperthyroidism in outbred strains (2). Vaccination was performed in the absence of adjuvants other than unmethylated CpG motifs in the plasmid DNA (3) and the mice were housed in a conventional animal facility. In contrast, in other studies using the same “minimal” TSHR-DNA vaccination approach, few BALB/c mice developed TSHR antibodies (4–7), particularly under pathogen-free conditions, and variable responses were observed in animals maintained in conventional facilities at different locations (8). These data clearly indicate that environmental factors influence the outcome of intramuscular TSHR-DNA vaccination (reviewed by McLachlan *et al.* [9]).

Besides the influence of the environment on the response to TSHR plasmid DNA vaccination, genetic factors are also critical. Thus, under pathogen-free conditions, we observed a striking difference between two inbred mouse strains. Unlike BALB/c, which very rarely developed TSHR antibodies (4,5), the majority of C57BL/6 mice became antibody positive after TSHR-DNA vaccination (10), with some of the latter possessing thyroid stimulating activity. No such activity was present in any of the very few BALB/c mice with TSHR antibodies. The different outcomes could be explained in terms of “high” versus “low” antibody responder mouse strains (for example, Blum and Cioli [11]). However, this possibility was precluded by our unexpected finding regarding DNA vaccination for the autoantigen thyroid peroxidase (TPO). In contrast to the low efficacy of vaccination with TSHR-DNA, immunization with TPO-DNA in a plasmid induced antibodies in 80% of BALB/c mice (12,13), excluding the possibility that BALB/c mice are “poor responders” in general. Taken together, our findings

FIG. 1. Contrasting response to vaccination with TSHR DNA in a plasmid by C57BL/6 versus BALB/c mice and to TPO DNA vaccination in BALB/c mice. Data are presented as the percentage of mice positive (by ELISA) for the respective antibody (black bars) versus the percentage of antibody negative animals (white bars). The number of animals in each group is shown in parentheses. Data for mice positive for TSHR antibodies by ELISA are from Pichurin *et al.* (5) and Schwarz-Lauer *et al.* (10) and data for TPO antibodies are from Guo *et al.* (12,13). TSHR, thyrotropin receptor; TPO, thyroid peroxidase.

from “minimal” immunization with plasmid DNA (summarized in Fig. 1) pose intriguing questions about tolerance to thyroid autoantigens and/or immunogenicity of the autoantigens themselves.

Central tolerance is determined by T cell education in the thymus. Immature T cells that bind with high affinity to peptides derived from autoantigens, expressed ectopically in the thymus, undergo apoptosis and are deleted (14). The magnitude of thymic autoantigen expression plays a role in autoreactive T-cell deletion and may affect the development of disease. For example, a type I diabetes susceptibility locus in humans maps to VNTR (variable number of tandem repeats) upstream of the insulin gene (15,16). These VNTR influence the level of intrathymic insulin expression and may, therefore, be protective of disease by maintaining tolerance to insulin. Moreover, intrathymic expression of a number of autoantigens (including insulin) are controlled by the Auto-immune regulator (Aire) and autoreactive T cells and auto-antibodies develop spontaneously in mice defective for Aire (for example, Anderson *et al.* [17] and Ramsey *et al.* [18]).

The different outcomes in BALB/c and C57BL/6 mice to TSHR-DNA plasmid vaccination, as well as the preferential response to TPO-DNA versus TSHR-DNA vaccination in BALB/c mice, could be related to differences in central tolerance. To explore these possibilities, we used real-time polymerase chain reaction (PCR) to quantify intrathymic expression of thyroid autoantigens, namely TPO, the TSHR, and thyroglobulin (Tg) in BALB/c and C57BL/6 mice.

Materials and Methods

Mice and tissue harvesting

BALB/cJ and C57BL/6J mice (4 weeks old) were obtained from Jackson Laboratories (Bar Harbor, Maine). Thymuses and thyroids were harvested from 30-day-old mice and trans-

ferred to RNeasy lysis buffer (Qiagen, Valencia, CA). For each strain, pools were prepared of thymic tissue from five mice (about 25 mg per mouse) and thyroids from three mice. Total RNA was extracted and used to isolate mRNA (RNeasy Midi Kit and Oligotex mRNA Midi Kit, respectively, both from Qiagen). To avoid genomic DNA contamination, mRNA samples were treated with TURBO DNase (Applied Biosystems/Ambion, Austin, TX). Reverse transcription was performed using oligo(dT) with AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA). Control reactions were performed without reverse transcriptase.

Isolation of mRNA and protocol for real-time PCR

Expression of mouse thyroid autoantigens, TSHR, TPO, Tg as well as the autoantigen insulin, were quantified by real-time polymerase chain reaction (PCR). Amplification of the house-keeping gene β -actin was performed for data normalization in thyroid tissue. Keratin-8, which is specifically expressed in thymic epithelial cells (19,20), was amplified to normalize the data for thymic tissue. In addition, we studied expression of Aire, a transcription factor that controls intrathymic expression of many autoantigens (reviewed by Mathis and Benoist [21]). The primer pairs used for amplification (Table 1) were selected from a broader panel based on their efficacy in real time PCR. Primer pairs for TSHR-1 and for TPO were designed in our laboratory. Primer pairs for TSHR-2 were the mouse homologs of primers reported for the rat TSHR (22). All primers span introns.

Real-time PCR was performed using FastStart SYBR Green Master mix (Roche, Basel, Switzerland) on 2.5% of each cDNA (total volume 25 μ L). Reactions were run in iCycler Thermal Cycler with iQ5 Real-Time PCR Detection System module (Bio-Rad Laboratories, Hercules, CA). The amplification program included an initial denaturation step at 95°C for 10 minutes, followed by denaturation at 95°C for 30 seconds, and annealing and extension at 55°C for 30 seconds, for 40 cycles. Relative gene expression levels were calculated using the comparative CT method ($\Delta\Delta C_t$), according to the Pfaffl model (23). Each sample was run in three parallel reactions, including the corresponding control lacking reverse transcriptase.

The data are expressed in two ways: (a) For thymus: values were normalized to keratin-8 (to control for the amount of cDNA) using Bio-Rad iQ5 2.0 software and expression in BALB/c for each antigen was calculated relative to values of 1.0 in C57BL/6; (b) Relative amounts of each antigen in thymus (or thyroid): calculations were based on the mean C_t threshold values for the housekeeping genes, keratin-8 (for thymic epithelial cells) and actin (for thyroid). For each gene of interest in the thymus, we calculated C_t [keratin] minus C_t [gene of interest]. This value was divided by 3.3 because, with oligonucleotide efficiency of 98%, a 10-fold difference in mRNA concentration results in a difference of 3.3 cycles. Finally, this value was converted to the anti-log to provide data for each gene relative to keratin-8. A similar approach was used for thyroid genes using C_t [actin] as the reference value.

Statistical analyses

The mean $\pm$ 2 standard deviations (SD) was used as the cutoff for statistically significant differences in real-time PCR values.

TABLE 1. PRIMER SETS USED TO AMPLIFY THYROID AUTOANTIGENS IN MOUSE THYROID AND THYMIC TISSUE. ALSO INCLUDED ARE PRIMERS FOR THE MURINE AUTOANTIGEN INSULIN, THE THYMIC TRANSCRIPTION FACTOR AIRE, AND β -ACTIN

Transcript ^a	Primer name	Sequence (5' to 3')	Product	Reference
TSHR-1	mTSHR-F56 For	CTGCTTGTTCTGCTGCTC	94 bp	—
	mTSHR-R149 Rev	CTCTGAAGTCGTCCTCCTG		
TSHR-2	TSHR-For	TGGAGGAGTATACAGTGGACCAA	89 bp	22
	TSHR-Rev	CTTTGAGTTGCTCCAGGCC		
Tg	p2340_For	AGGGTACCCTGCTGGACCAAGTG	94 bp	39
	p2340_Rev	TGTCTAGATGTCACACGCTGAGG		
TPO	TPO-F3	GGTCCTCCTGTGCGAATAG	~130 bp	—
	TPO-R3	CTGGTGTCTCAAGTCTCTG		
Insulin	Ins2_For	GACCCACAAGTGGCACAAC	Not known	40
	Ins2_Rev	TCTACAATGCCACGCTTCTG		
keratin-8	K2_8 For	AGGAGCTCATTCCGTAGCTG	~120 bp	20
	K2_8 Rev	TCTGGGATGCAGAACATGAG		
Aire	Aire-F2	CAGCAACTCTGGCCTCAAAG	284 bp	41
	Aire-R2	CTTCGAACCTGTTGGGTGTATAA		
β -actin	actin-S	GGTTCCGATGCCCTGAGGCTC	~340 bp	42
	actin-AS	ACTTGCGGTGCACGATGGAGG		

^aTwo different primer pairs were tested for TSHR amplification. The predicted sizes of the product are shown as well as the references to primer sequences from other studies. Note: TSHR modified from primers for rat TSHR (22).

Tg, thyroglobulin.

Results

Thyroid

As measured by real-time PCR, mRNA transcript levels in thyroid tissue were highest for Tg, 10 times higher than for β -actin, the reference standard for other autoantigens (Fig. 2). TPO transcript levels, which were comparable to those for β -actin, were 10-fold higher than those for the TSHR. There were no significant differences between BALB/c and C57BL/6

FIG. 2. Expression of Tg, TPO, and TSHR mRNA in thyroid tissue from BALB/c and C57BL/6 mice. Thyroid antigen expression was measured by real-time PCR and normalized to the values for the house keeping gene β -actin (value of 1.0, indicated by dotted line). Note the logarithmic scale for thyroid autoantigen expression. PCR, polymerase chain reaction.

mice. These thyroid data provide a base-line for comparing thyroid autoantigen expression in thymic tissue from the two mouse strains.

Thymus

When studying thymic tissue, we encountered a problem using one of two primer sets tested for TSHR amplification (Table 1). Both TSHR primers sets were similarly efficient for real-time PCR amplification for thyroid tissue and (as shown above for one set), the level of TSHR expression was similar in BALB/c and C57BL/6 mice. However, when tested for thymic cDNA, the TSHR-2 primer set showed a major strain difference: significantly higher TSHR transcript levels in C57BL/6 than BALB/c mice. Although the melting curves were similar in both mouse strains for the TSHR-1 primer set, they were different for C57BL/6 versus BALB/c mice using the TSHR-2 primer set. Moreover, in a regular PCR, an unexpected 220-bp product was generated from C57BL/6 (but not BALB/c) thymic cDNA. On sequencing, this product was not the TSHR. Consequently, for all thymic studies, we used TSHR-1 primer set. We present these findings as a cautionary note regarding the possibility that a primer set used for thymic studies in one mouse strain may not be suitable for another strain.

Turning to the major issue in this study, namely intrathymic transcript levels of all three thyroid autoantigens, we found no significant differences between BALB/c and C57BL/6 mice for Tg, TSHR or TPO (Fig. 3A). For interspecies comparison, transcript levels for each autoantigen were normalized to those for C57BL/6 (the latter expressed as 1.0). Although intrathymic TPO expression tended to be higher in BALB/c than C57BL/6 mice, because of greater variability the difference was not statistically significant. In contrast, and unexpectedly, expression of the autoantigen insulin and the thymic transcription factor Aire were significantly reduced in BALB/c versus C57BL/6 mice.

FIG. 3. Intrathymic expression of thyroid autoantigens (TSHR, Tg, and TPO), the autoantigen insulin (Ins) and the thymic transcription factor Aire in BALB/c and C57BL/6 mice. Expression levels measured by real-time PCR were normalized to keratin-8 (expressed in thymic epithelial cells). (A) Expression of each autoantigen relative to its level in C57BL/6 mice, the latter normalized to 1.0 (dotted line). The data shown are the mean $\pm$ SEM of triplicate measurements from one of three or more representative experiments. *, values significantly lower for Aire KO versus WT mice (<2 SD for WT animals, see Methods). (B) Comparison of intrathymic expression levels of different self antigens. Data for the autoantigens shown in panel A were normalized to expression of keratin-8 for each strain (shown as 1.0). Except for TPO, the data are the mean $\pm$ SD of two experiments (each performed in triplicate).

In addition to comparing differences between mouse strains for each thyroid autoantigen, we recalculated the data to determine absolute mRNA transcript values relative to keratin-8, the reference protein for thymic epithelial cells (as performed for the thyroid, Fig. 2). In both BALB/c and C57BL/6 mice, mRNA transcript levels of all three thyroid autoantigens were three orders or more lower than for keratin-8 (value of 1.0, Fig. 3B). Aire and insulin expression was higher than for any of the thyroid autoantigens, but even these values were one hundredth of the keratin-8 transcript level. Despite these low values for thyroid autoantigens, Tg was the most highly expressed, with TPO and the TSHR in similar amounts. In particular, the dramatic 10-fold differences in thyroid tissue ($Tg > TPO \gg TSHR$) were muted in

the thymus, being only partly recapitulated for Tg and absent for TPO and the TSHR.

Discussion

In this study, we sought an explanation for two conundrums (Fig. 1). First, BALB/c mice, unlike C57BL/6, rarely produce antibodies in response to TSHR-DNA plasmid vaccination. Second, despite their inability to generate TSHR antibodies, BALB/c mice develop TPO antibodies after vaccination with TPO-DNA, excluding the possibility that BALB/c mice are simply poor antibody responders to immunization. These observations, made in the same laboratory, were performed with the same immunization protocol and, because the mice were housed in pathogen-free facility, the contribution of environmental factors was excluded (4,5,10,12,13).

Differences in central tolerance to the TSHR could explain the puzzling outcomes of DNA plasmid immunization. Thus, higher intrathymic TSHR expression in BALB/c than C57BL/6 mice would lead to greater TSHR tolerance and greater difficulty for breaking tolerance and inducing TSHR antibodies in the former than in the latter mouse strain. Along the same lines, in BALB/c mice, higher intrathymic expression of the TSHR versus TPO would ensure more profound tolerance to the former thyroid autoantigen than to the latter and, as a result, greater difficulty for inducing TSHR than TPO antibodies. To test these hypotheses, we compared intrathymic transcripts of TSHR and TPO in these two mouse strains. For completeness, we included Tg, insulin (as another autoantigen), and the thymic transcription factor Aire.

Our hypotheses were not confirmed. We found no significant difference for intrathymic expression of the TSHR, TPO, or Tg between BALB/c and C57BL/6 mice. In the thyroid, mRNA transcripts were markedly different for the three autoantigens, being highest for Tg, 10 times higher than for TPO and 100 times higher than for the TSHR. These differences would be expected, Tg being the most abundant thyroid protein, with lesser amounts of TPO, and much lower levels of the TSHR. In contrast, in the thymus, expression levels of the three thyroid autoantigens were of similar magnitude, with slightly more Tg and comparable amounts of TPO and the TSHR. Expression of the thyroid autoantigens was similar in the thymus and thyroid for both BALB/c and C57BL/6 strains. In contrast, intrathymic mRNA transcripts for Aire, as for the autoantigen insulin, were significantly lower in BALB/c than in C57BL/6 mice. In BALB/c mice, we have observed that the absence of Aire enhances the TSHR antibody development after immunization with TSHR adenovirus (24). Because of the difference in Aire expression, we speculate that the lack of Aire may have a greater impact in C57BL/6 than in BALB/c mice for responses induced by immunization with thyroid autoantigens. A very recent study, published after ours was completed, confirms our suggestion that the function of Aire is generally stronger in C57BL/6 than in BALB/c mice (25).

It should be noted that mRNA transcripts do not always correlate with protein levels and variable RNA translation could reveal intrathymic differences between TSHR protein in BALB/c versus C57BL/6 mice (or for TPO versus TSHR) that were not detected by real-time PCR. However, previously we observed that intrathyroidal mRNA transcripts of transgenic

human TSHR A-subunit correlated with protein levels in high- versus low-expressor mice (26). Moreover, the greater proportion of intrathyroidal Tg than TSHR transcripts demonstrated in the present study are consistent with considerably more Tg than TSHR A-subunit protein, approximately 300 μ g versus 3 μ g per thyroid gland, respectively (27). Against this background, it is unlikely that translational differences would alter our conclusion that central tolerance, as reflected by intrathyroidal transcript levels, is similar for the TSHR in the two mouse strains, and for the TSHR versus TPO in one mouse strain.

If central tolerance does not play a role, what other factors could contribute to the vaccination differences we observed for the TSHR in two mouse strains, or for TPO versus the TSHR in BALB/c mice? Factors controlling innate immunity, particularly Toll-like receptors (TLRs), may be involved in the strain specific differences. TLRs function by recognizing repeated molecular patterns present in infectious organisms, such as unmethylated CpG motifs (common in bacterial or viral DNA but rare in mammalian DNA) that bind to TLR9 (28,29). There was no difference between BALB/c and C57BL/6 mice in acute allograft rejection after CpG administration (30). However, expression of TLR9 in the liver was reduced in BALB/c and increased in C57BL/6 mice during *Trypanosoma cruzi* infection (31). It is possible, therefore, that the variable responses of the two mouse strains to the plasmid could play a role in the preferential development of TSHR antibodies in C57BL/6 than in BALB/c mice (Table 2). Most likely, differences in adaptive immune responses contribute to the outcome of TSHR-DNA plasmid vaccination. TSHR antibodies in BALB/c and C57BL/6 mice recognize the same linear peptide epitope. In contrast, these mouse strains differ markedly in the major histocompatibility molecules (MHC) and TSHR epitopes recognized by their T cells (Table 2). Moreover, the TSHR amino acid sequences encompassed by these T-cell epitopes differ between the TSHR in humans and mice (26). Overall, differences in MHC pre-

sentation to T cells are likely to be involved in the different outcomes of TSHR-DNA vaccination in C57BL/6 versus BALB/c mice.

In our studies, immunization was performed with cDNA encoding the human TSHR or human TPO, proteins that are not identical (although ~90% homologous) to the corresponding mouse proteins (32–34) (Table 2). Despite similar overall homologies, the larger ectodomains of human and mouse TPO contain 78 nonhomologous residues whereas the smaller ectodomains of the human and mouse TSHR include only 58 nonhomologous residues. There are, therefore, more discordant amino acids that can be processed and presented to T cells in TPO than in the TSHR, possibly contributing to the greater immunogenicity in mice of human TPO versus the human TSHR.

Finally, our findings have broader implications for the model of Graves' disease in mice. Graves'-like hyperthyroidism is most effectively induced by immunizing mice with adenovirus expressing the TSHR, particularly the TSHR A-subunit (7,35). Adenovirus has a more potent adjuvant effect (36) than DNA plasmid CpG motifs (3). Both BALB/c and C57BL/6 mice develop TSHR antibodies in response to TSHR- or TSHR A-subunit adenovirus immunization. However, BALB/c mice are susceptible, and C57BL/6 mice are resistant, to developing hyperthyroidism (7,37). Using recombinant inbred strains derived from intercrosses of BALB/c and C57BL/6 strains, we found that MHC region genes contribute to induction of TSHR antibodies but a different set of chromosomes and loci are associated with development of hyperthyroidism (38). The nature of the genes responsible for thyroid function in these CXB recombinant inbred mice have not been identified. Our current findings likely exclude a role for central tolerance in the genetic basis for susceptibility versus resistance, at least in these two strains of mice.

In summary, intrathyroidal thyroid autoantigen expression, a potential determinant of central tolerance, does not explain the different outcome of TSHR-DNA vaccination in BALB/c and

TABLE 2. CHARACTERISTICS OF BALB/c AND C57BL/6 MICE THAT MIGHT IMPACT THEIR RESPONSE TO VACCINATION WITH TSHR-DNA IN A PLASMID. ALSO, DIFFERENCES IN TSHR AND TPO THAT COULD INFLUENCE THE OUTCOME OF DNA VACCINATION WITH TSHR VERSUS TPO IN BALB/c MICE

Characteristic	BALB/c	C57BL/6	Reference
Innate immunity (TLR9)			
TLR9 in <i>Trypanosoma cruzi</i> acute infection	<i>Decreased</i> (liver)	<i>Increased</i> (liver)	31
CpG administration for GVHD rejection	Accelerates	Accelerates	30
Adaptive immune response			
TSHR T-cell epitopes (human TSHR amino acid residues)	<i>52–71</i> <i>67–86</i> <i>157–176</i>	<i>112–121</i> <i>157–176</i>	43,44 ^a
MHC class I (and class II)	<i>H2 d (IA–d)</i>	<i>H2–b (IA–b)</i>	
Linear antibody epitope	22–41	22–41	10
Amino acid homologies of autoantigens			
Nonhomologous amino acids in mouse vs. human TSHR ectodomains (397 residues)	<i>52 residues</i>	52 residues	32
Nonhomologous amino acids in mouse vs. human TPO ectodomains (~820 residues)	<i>78 residues</i>		33,34

Bold italics reflect differences likely to contribute to the differences between the two strains for the TSHR and for the different outcome for TPO versus the TSHR in BALB/c mice.

^aReference (43) for BALB/c and (44) for C57BL/10, also IA-b.

TLR, toll-like receptor; CpG, immunostimulatory motif in bacterial DNA; GVHD, graft versus host disease; MHC, major histocompatibility antigen.

C57BL/6 mice, or susceptibility versus resistance to hypothyroidism induced by TSHR adenovirus. Instead, different MHC and TSHR T-cell epitopes likely contribute to this difference in mice maintained under pathogen-free conditions. Moreover, the greater immunogenicity of TPO versus the TSHR probably relates to the greater discrepancy between human- and mouse-TPO ectodomain (78 of 838 amino acids) than between human and mouse TSHR (58 of 397 residues). In conclusion, the autoantigens themselves, not central tolerance, control DNA plasmid-induced immunity to TPO and the TSHR.

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Disclosure Statement

No competing financial interests exist.

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