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ORIGINAL ARTICLE

Immunoglobulin heavy chain variable region genes contribute to the induction of thyroid-stimulating antibodies in recombinant inbred mice

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Graves' hyperthyroidism is an autoimmune disease occurring spontaneously in humans and caused by autoantibodies that stimulate the thyrotropin receptor. In mice, inducing Graves'-like hyperthyroidism requires in vivo expression of the thyrotropin receptor using plasmid or adenovirus vectors. However, mice with different genetic backgrounds vary markedly in their susceptibility to induced hyperthyroidism. Further, in some strains major disparities exist between the induction of hyperthyroidism and detection of thyroid-stimulating antibodies. To break tolerance, virtually all Graves' mouse models involve immunization with human thyrotropin-receptor DNA and the standard thyroid-stimulating antibody bioassay uses cells expressing the human thyrotropin receptor. We hypothesized, and now report, that disparities between hyperthyroidism and thyroid-stimulating antibody bioactivity are explained, at least in part, by differential antibody recognition of the human vs the mouse thyrotropin receptor. The genetic basis for these species differences was explored using genotyped, recombinant-inbred mouse strains. We report that loci in the immunoglobulin heavy chain variable region as well as in the major histocompatibility complex region contribute in a strain-specific manner to the development of antibodies specific for the human or the mouse thyrotropin receptor. The novel finding of a role for immunoglobulin heavy chain variable region gene involvement in thyroid-stimulating antibody epitopic specificity provides potential insight into genetic susceptibility in human Graves' disease.
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Introduction

The thyroid is the organ most commonly affected in human autoimmunity. Hashimoto's thyroiditis is associated with autoantibodies to thyroid peroxidase and thyroglobulin and hypothyroidism affects 4.6% of the US population.[1] Hyperthyroidism in Graves' disease, prevalent in 0.5–1% of women,[1,2] is caused by autoantibodies to the thyrotropin receptor (thyroid-stimulating hormone receptor, TSHR) that mimic thyroid stimulation by thyrotropin (TSH) (reviewed in Rapoport and McLachlan[3]). Autoimmune hyperthyroidism only develops spontaneously in humans. Nevertheless, in other autoimmune diseases, induced animal models have provided important clues to elucidating the pathogenesis and genetic basis of their human counterparts, such as experimental autoimmune encephalomyelitis[4] (a model for multiple sclerosis) and collagen-induced arthritis.[5]

Induction of hyperthyroidism in mice is not as straightforward as the two foregoing induced autoimmune diseases. Even though highly purified, disease-specific antigen (unequivocally the TSHR) is available, conventional immunization with TSHR protein and adjuvant cannot induce hyperthyroidism. Although this approach generates high titers of TSHR antibodies, these are incapable of activating the TSHR on the surface of intact cells (reviewed in McLachlan *et al*.[6]). To produce Graves' hyperthyroidism in mice, it is necessary to express *in vivo* the TSHR or its A-subunit using plasmid or adenovirus vectors (reviewed in McLachlan *et al*.[6]). However, mice with different genetic backgrounds vary markedly in their susceptibility to induction of hyperthyroidism.

Virtually all mouse models of induced hyperthyroidism involve *in vivo* expression of the TSHR without additional adjuvant (for example, Nagayama *et al*.[7] and Chen *et al*.[8]). Human TSHR cDNA is generally used, because of its availability and because its use bypasses the need to overcome self-tolerance with potent adjuvants. (In one study, hyperthyroidism was induced by injecting B cells expressing mouse TSHR with the

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adjuvant cholera toxin B[9].) Importantly, after immunization with the human TSHR, the induced thyroid-stimulating antibodies (TSAb) must stimulate the mouse TSHR to produce hyperthyroidism *in vivo*.

It has been recognized for 50 years that TSAb in human Graves' patients stimulate the mouse (m) TSHR *in vivo*[10] and early bioassays for TSAb used a rat thyroid cell line (FRTL5[11]). After the molecular cloning of the TSHR, Chinese hamster ovary (CHO) cells expressing the recombinant human (h) TSHR have progressively supplanted rat thyroid cells in TSAb assays.[12] In BALB/c mice made hyperthyroid by immunization with human TSHR adenovirus, serum thyroxine levels correlated with TSAb activity when measured with FRTL5 rat thyroid cells.[7] Unexpectedly, with the human hTSHR bioassay, TSAb activities in hyperthyroid mice correlated poorly with serum thyroxine levels. For example, some euthyroid BALB/c mice had high TSAb activity whereas other hyperthyroid animals were TSAb negative.[13] Similarly, some C3H/He mice were hyperthyroid despite very low levels of TSAb assayed using hTSHR-expressing CHO cells.[14] BALB/c mice are far more susceptible than C57BL/6 mice to hTSHR-adenovirus-induced hyperthyroidism.[15] Nevertheless, when assayed with hTSHR-CHO cells, TSAb activities were comparable in these two mouse strains.[15]

To explore the foregoing inconsistencies in the relationship between the degree of murine hyperthyroidism and TSAb activity, we generated mouse TSHR-expressing CHO cells.[16] Unlike with hTSHR-CHO cells, when assayed with mTSHR-CHO cells TSAb levels were higher in BALB/c than in C57BL/6 mice, more consistent with the greater susceptibility of the former to induced hyperthyroidism.[16] With this background, in the present study we hypothesized that TSAb generated in genetically diverse strains of mice and assayed with hTSHR- and mTSHR-CHO cells as separate traits would provide insight into the genetic basis for variability in TSHR antibody functional activity in different mouse strains. We report that loci in the major histocompatibility complex (MHC) region as well as in the immunoglobulin heavy chain variable region contribute in a strain-specific manner to the development of antibodies specific for the human or the mouse TSHR.

Results

TSAb in sera from parental and recombinant inbred strains bioassayed using cells expressing the mouse and human TSHR

C3H and B6 mice were immunized three times with adenovirus encoding the human TSHR A-subunit. Sera were drawn 1 week after the second injection and 1 month after the third injection and were tested for TSAb in bioassays using eukaryotic cells expressing the mouse or the human TSHR. As we observed previously,[14] TSAb levels determined using the human TSHR were far lower in C3H than in B6 parental strains after the second A-subunit immunization (Figure 1a). This observation is paradoxical because C3H mice are more susceptible to hyperthyroidism than B6 mice.[14] A possible explanation for this phenomenon was that despite immunization with the human TSHR A-subunit the TSAb generated in the C3H mice preferentially recognized the mouse TSHR. This hypothesis was confirmed when the same sera were tested with cells expressing the mouse TSHR. In the latter assay, TSAb activities were comparable in C3H and B6 parental strains (Figure 1b). This difference for each strain can be expressed as the ratio of TSAb activities determined with the mouse and human TSHR assays (Figure 1c). Similar data were obtained with the sera obtained at the time of euthanasia, 4 weeks after the third immunization (Supplementary Figure S1).

On the basis of the foregoing differences between B6 and C3H mice in TSAb determined in mouse and human TSHR assays, we performed similar assays on 13

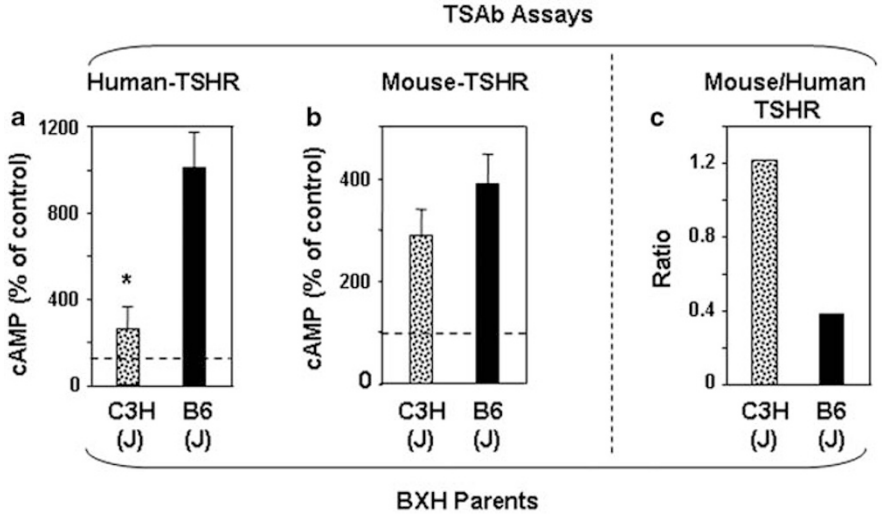

Figure 1 Thyroid-stimulating antibodies (TSAb) specific for the mouse thyroid stimulating hormone receptor (TSHR) and the human TSHR in parental BXH strains, C3H and B6 mice. Sera were tested 1 week after two immunizations with A subunit adenovirus. (**a**) Human TSHR TSAb (previously reported by McLachlan *et al*.[14] (**b**) Mouse TSHR TSAb. (**c**) Ratio of mouse TSHR/human TSHR TSAb. TSAb values (mean + s.e.m.) are expressed as percent cAMP generated by sera from control adenovirus-immunized mice; indicated by dashed lines (**a**, **b**); number of mice C3H ($n = 10$); B6 ($n = 10$); *$P = 0.006$ (rank-sum test). The TSAb ratios were calculated from the means for mouse and human TSHR TSAb.

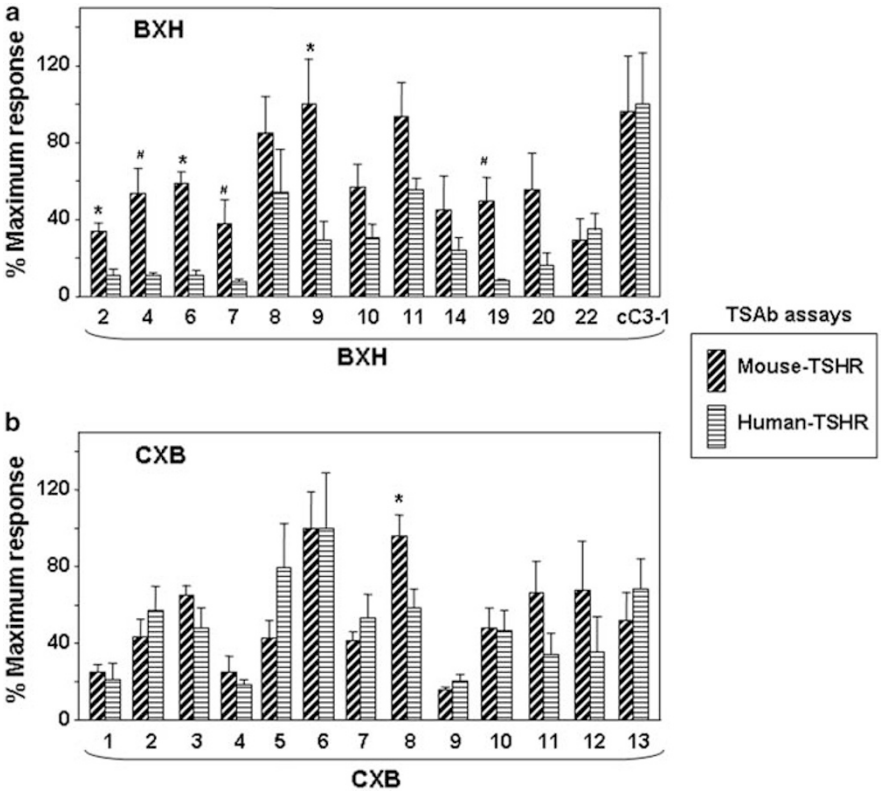

Figure 2 Comparison of mouse-TSHR TSAb vs human-TSHR TSAb in recombinant inbred strains BXH (**a**) and CXB (**b**) after two immunizations with A-subunit (human) adenovirus. The data are shown as paired bar graphs (mean + s.e.m.) of mouse-TSHR TSAb and human-TSHR TSAb for each strain. Because the maximum stimulation was higher with the human TSHR than with the mouse TSHR, TSAb values were expressed as a percentage of the mean maximum levels attained as follows: BXH strains, mouse TSHR 485% (BXH9) and human TSHR 1229% (BXHcC3-1); CXB strains: mouse TSHR 555% (CXB6) and human TSHR 945% (CXB6). Significant differences between mouse and human TSHR TSAb: (**a**) $^{*}P < 0.019$ (*t*-test), $^{\#}P < 0.010$ (rank-sum test); (**b**) $^{*}P = 0.030$ (*t*-test).

recombinant inbred BXH strains derived from the two parental strains. BXH strains had been immunized and tested as described above for the parental B6 and C3H mice. Because maximum stimulation levels differed in the two bioassays (Figure 1) and also because TSAb activities induced in the different strains varied widely, to facilitate comparison the data were normalized to the recombinant strain giving the highest mean TSAb value, expressed as 100% (see Materials and methods). Six of the 13 BXH strains (BXH2, -4, -6, -7, -9 and 19) developed significantly higher mouse TSAb than human TSAb activity after two immunizations (Figure 2a).

In earlier studies, TSHR A-subunit adenovirus immunization of BALB/c(J) and B6(J) mice generated similar TSAb activities when measured in the standard human TSHR assay.[15] Recombinant inbred CXB strains were derived from parental BALB/c and B6 mice of the Bailey strains (By, rather than J). We had previously investigated the 13 CXB recombinant inbred strains,[14] but had not assayed TSAb activities in these mice following immunization with TSHR A-subunit adenovirus. However on doing so in the present study, we found that, in sera tested 1 week after the second immunization, most CXB strains had comparable TSAb activities measured in the mouse TSHR and human TSHR assays and only one strain (CXB8) had significantly higher TSAb activity in the assay using the human vs the mouse TSHR assay (Figure 2b).

Linkage analysis in BXH and CXB strains for TSAb determined in mouse and human TSHR assays

For these analyses, we used the raw TSAb data (not the values normalized to 100% for better visualization, as shown in Figure 2). Putative quantitative trait loci (see Materials and methods) were analyzed for the following traits after the second immunization with human TSHR A-subunit adenovirus: (1) TSAb assayed with human TSHR-expressing cells; (2) TSAb assayed with mouse TSHR-expressing cells and (3) the ratio between these two TSAb assay values.

In 13 BXH recombinant inbred strains, the whole-genome scan revealed highly significant linkage between the ratio of TSAb values determined using the mouse vs human TSHR assays and a locus on chromosome (Chr 12; Figure 3a). The linkage ratio statistic (LRS) of 20.433 is equivalent to a logarithm of the odds (LOD) score of 4.432 (Table 1). Similarly, whole-genome scanning for the 13 CXB strains revealed the strongest linkage between a Chr 12 locus and TSAb assayed with mouse TSHR-expressing cells (Figure 3b). However, data from the same CXB sera assayed for activity using the human TSHR provided evidence for linkage between human TSHR TSAb and loci on Chr 17 and Chr X (Figure 3c and Table 1). The linkage data obtained in this study, taken together with our previous findings[14,17] (summarized in Table 1), permit the following general conclusions:

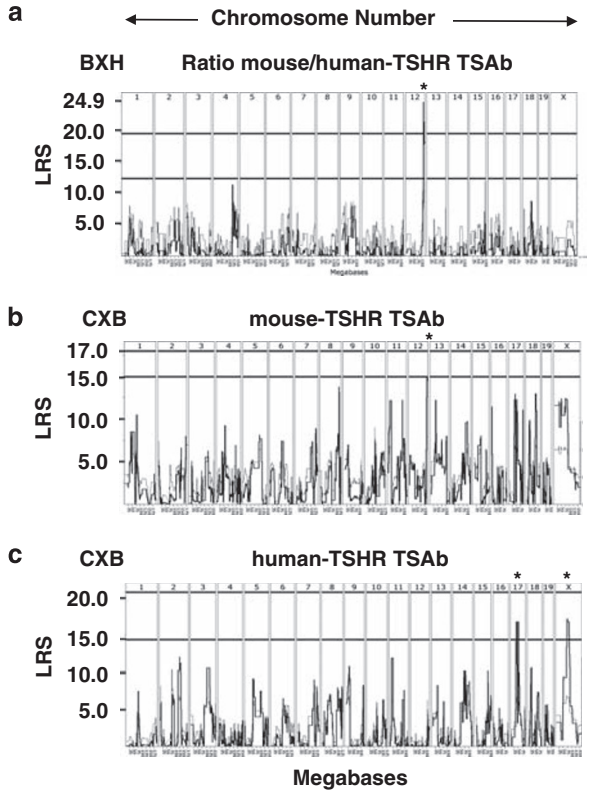

Figure 3 Whole-genome interval mapping in BXH and CXB strains for thyroid-stimulating antibodies (TSAb) traits after two A-subunit-Ad immunizations: (**a**) human-TSHR TSAb in CXB mice; (**b**) mouse-TSHR TSAb in CXB mice; (**c**) ratio mouse/human (m:h) TSHR TSAb in BXH mice. Chromosomes 1–9 and X are indicated at the top of each panel and likelihood ratio statistics (LRS) values on the vertical axis. The horizontal lines (**a**, **c**) indicate the LRS values above which a trait is associated with a particular chromosome (indicated by asterisk(s)). In (**a**), the upper horizontal line indicates the level for a significant association. GeneNetwork identifiers: (**a**) GN 10683; (**b**) GN 10685; (**c**) GN 10157.

First, loci on the terminal portion of Chr 12 are linked to the following traits involving TSAb after two immunizations with human TSHR A-subunit adenovirus:

(a) TSAb measured in the human TSHR assay for BXH mice (Figure 4a) and in the mouse TSHR assay for CXB mice (Figure 4a).
(b) The mouse/human TSHR TSAb ratio in BXH (Figure 4b) and
(c) The human/mouse TSHR ratio in CXB mice (Figure 4b).

Second, loci on other chromosomes were shared by TSAb and non-TSAb traits:

(a) In CXB mice, there is linkage between human TSHR TSAb values and loci on Chr 17 (Figure 4c) and Chr X (4d);
(b) Similar loci on these Chr previously identified in CXB mice for TSHR antibodies measured by TSH-binding inhibition (TBI)[17] are indicated in Figures 4c and d.

Combined linkage analysis in BXH and CXB mice
Combining the data for BXH and CXB strains strengthened the linkage between TSAb values (using either the mouse or human TSHR or the ratio between these assays) with loci on Chr 12 (Table 2). This combined analysis provides statistical data as LOD (not LRS) scores. LOD scores of 3.49–3.56 were obtained for the Chr 12 region from 109.73 to 118.161 Mb, which is dominated by immunoglobulin H chain genes (Supplementary Table S2). The highest LOD scores (>5.8) were obtained for TSAb ratios linked to Chr 12 loci between 110.306 and 111.967 Mb, a region which includes the gene for type 3 deiodinase (*Dio3*).[18]

Linkage analysis in BXH and CXB strains for TSAb following the third immunization
Thyroid-stimulating antibody assays performed on sera 1 month after the third immunization replicated the foregoing data with sera obtained after the second immunization. However, with greater stimulation to the immune system, in addition to the loci on Chr 12, 17 and X, a number of other loci in BXH and CXB mice were linked to TSAb traits, for example, on Chr 9 and 10 (Supplementary Information Table S1). The potential significance of these loci is unknown because the genes therein are unidentified.

Discussion

Before considering our present findings, it should be noted that two assays are used to assess TSHR antibody levels in Graves' patients and induced animal models of this disease. Clinically, the most standardized and cost-effective assay involves antibody-mediated TBI. This assay uses purified human or porcine TSHR. A bioassay for functional TSAb is more complex and involves measuring the cAMP response to stimulation in non-thyroidal cells expressing the recombinant human TSHR. Early studies with the animal model of Graves' disease revealed that background genes in different mouse strains had a major effect on the development of hyperthyroidism. Thus, although TSHR antibodies (measured as TBI) were induced in several mouse strains by immunization with TSHR-expressing adenovirus, BALB/c mice were the most susceptible in developing hyperthyroidism.[7,19] C57BL/6 mice, which develop high TSHR antibody levels, rarely become thyrotoxic.[7]

Because of this strain difference and the dominance of the BALB/c phenotype in F1 offspring of the BALB/c $\times$ B6 cross,[15] we studied recombinant inbred CXB mice (derived from these parental strains) for genetic factors that control the variability in TSHR antibodies and hyperthyroidism. The findings of our study suggested that loci on different chromosomes were responsible for TSHR antibody induction vs thyroid function.[17] Extending these studies to BXH mice, derived from C3H/He and B6 parental strains, we confirmed the importance of different gene sets controlling immune responses vs thyroid function. In this second study, in addition to measuring TBI, we assayed functional TSAb. We were puzzled to observe that, despite very low TSAb levels, C3H/He mice and some BXH strains became hyperthyroid.[14] Data from the present study resolve this conundrum. Remarkably, despite genetic immunization with a vector expressing the human TSHR A-subunit, C3H/He mice and some BXH strains develop TSAb that preferentially recognize the mouse TSHR, not the human TSHR

Table 1 Chromosomal linkage for TSAb in CXB and BXH mice immunized twice with TSHR A-subunit adenovirus

Strain	*LRS*	*Chr*	*Locus*	*Mb*	*Candidate*	*Hu*
Human TSHR TSAb						
BXH[a]	13.628	12	rs13459138	113 270	*Igh V* genes	14
			rs3705923	117 869	*Igh V* genes	
			rs3692361	118 161		
			rs3679276	120 329		
CXB	16.787	17	gnf17.035.152	34 663	MHC region	6
			rs6395893	38 786	genes	
			D17Mit66	47 186		
			rs3690039	48 900		
	16.787	X	rs13483834	72 576	*Il1rapl1*	X
			rs13483877	83 201		
Mouse TSHR TSAb						
CXB						
	14.518	12	rs13481658	113 595	*Igh V region*	14
			rs3711281	118 633	*genes*	
TSAb ratio						
BXH m:h[b]	20.433	12	CEL-12_10454502266.496	110 245		14
			CEL-12_10470961366.496	110 409		
			D12Mit133	110 746	(*Dio3*)	14
			rs13481651	111 769		
	18.323	12	rs13459138	113 271	*Igh V genes*	14
			rs3705923	117 869	*Igh V genes*	
			rs3692361	118 161		
			rs3679276	120 329		
CXB h:m	11.552	12	D12Mit132	107.374	(*Dio3*)	14
			rs13481636	108.001		

TSAb activity was measured with mouse-TSHR cells and human-TSHR cells; these data were used to calculate the TSAb ratios (human:mouse TSHR, h:m or mouse: human TSHR, m:h). Loci and chromosomal locations (megabases, Mb) are presented for LRS scores suggestive of linkage. Possible candidate genes are included with the corresponding (or likely) human (Hu) chromosome in parentheses.

[a]Previously reported.[14]

[b]Similar observations for h:m TSHR TSAb (but lower LRS values).

Candidate genes and abbreviations: MHC region including *C2* (34.470663), *Hspa1* (34.577246); *Tnf* (34.807461 and *Ltb* (34.802573); *Il1rapl1*, interleukin 1 receptor accessory protein-like 1; Dio3, deiodinase type 3 (110.727 Mb); *Igh V* region genes, immunoglobulin heavy chain variable region genes.

LRS = LOD × 4.61. GeneNetwork (GN) Identifiers:- human-TSHR TSAb, BXH GN10147; CXB GN10683; mouse-TSHR TSAb, CXB GN10685; TSAb Ratio: BXH m:h GN10157; CXB h:m GN10686. No linkage was observed for BXH mouse-TSHR TSAb GN10155.

used in the conventional bioassay. Of course, hyperthyroidism can only develop by activation of the mouse TSHR *in vivo*. Better antibody recognition of a related antigen than the immunogen, termed 'heteroclicity', is a well-established phenomenon; for example, preferential antibody recognition of bovine or porcine insulin after immunization with human insulin[20] and greater affinity early in the immune response for mouse than for pigeon cytochrome *c* after immunization with the latter antigen.[21]

In humans, TSAb cross-reactivity with TSHR from different species was first recognized in the late 1950s, before the immunoglobulin nature of TSHR antibodies was recognized. A component of Graves' serum was found to stimulate the guinea pig thyroid with a prolonged time course compared with TSH, hence the name 'long acting thyroid stimulator'.[10] When the assay was adapted for use in mice, sera from some patients did not cross-react with the mouse thyroid and were long acting thyroid stimulator negative. The first commercialized TBI assay also involved antigen cross-reactivity, namely porcine TSHR.[22] Correlation between TBI measured with the porcine TSHR and the recombinant human TSHR is generally good (for example, Kakinuma *et al*.[23]). In retrospect, the few exceptions, involving better recognition of porcine than human TSHR, likely represent antibody heteroclicity.

Parenthetically, we wish to comment on our present findings of relatively minor differences between TSAb measured in the human and mouse TSHR assays in the CXB recombinant inbred strains (BALB/c crossed with B6 mice). These data would appear to conflict with our previous report that, following human TSHR immunization, mouse-specific TSAb predominated over human TSAb activity in BALB/c mice, and *vice versa* for B6 mice.[16] The explanation for this apparent discrepancy is that the latter studies used BALB/c and B6 both of the Jackson substrain,[16] whereas CXB mice are derived from the Bailey strains of BALB/c and B6. The clear-cut difference between BALB/c susceptibility and B6 resistance to hyperthyroidism in Jackson substrains[7,15] is muted in the Bailey substrains.[17] Moreover, in a direct

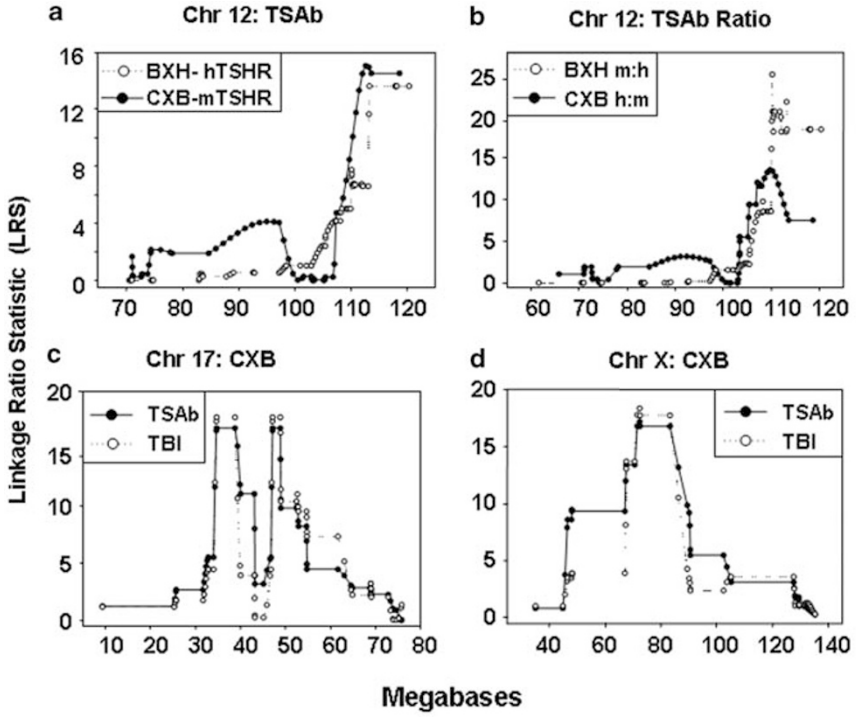

Figure 4 Individual chromosomes in CXB and BXH mice associated with thyroid-stimulating antibodies (TSAb) activity or TSH binding inhibition (TBI) after two A-subunit-Ad immunizations in the present and previous studies[14,17]: (**a**) Chr 12: mouse-thyroid-stimulating hormone receptor (TSHR) TSAb for CXB mice and human TSHR TSAb for BXH mice; (**b**) Chr 12: ratios for mouse/human-TSHR-TSAb for BXH mice and human/mouse TSAb for CXB mice; (**c**) Chr 17: TBI (inhibition of TSH binding) for CXB mice and human TSHR-TSAb; (**d**) Chr X: TBI (inhibition of TSH binding) for CXB mice and human-TSHR TSAb. Chromosomal distances (Mb) are on the horizontal axes and likelihood ratio statistics are on the vertical axes. GeneNetwork identifiers: (**a**) CXB GN 10685 and BXH GN 10147; (**b**) BXH GN10157 and CXB GN 10686; (**c**) GN 10512 and GN 10683; (**d**) GN 10512 and GN 10683.

Table 2 Combined linkage analysis for TSAb in BXH and CXB recombinant inbred mice (26 strains) immunized twice with TSHR A-subunit-adenovirus

Trait	*Chr*	*Chr interval (Mb)*	X^2	P	*LOD*
TSAb					
CXB mouse-TSHR TSAb and	12	109 730–110 187	20.95	0.000324	3.490
BXH human-TSHR TSAb		110 245–110 306	20.05	0.000488	3.312
		110 882–111 769	19.44	0.000643	3.192
		113 181–113 187	19.92	0.000519	3.285
		113 595–118 161	21.13	0.000298	3.526
Ratio					
CXB human/mouse TSHR TSAb and	12	108 577–109 137	21.06	0.00030858	3.511
BXH mouse/human-TSHR TSAb		110 306–110 409	33.80	8.19781E−07	6.086
		110 882–111 967	32.53	1.49236E−06	5.826

Abbreviations: TSAb, thyroid-stimulating antibodies; TSHR, thyroid-stimulating hormone receptor.

comparison, fewer Bailey BALB/c than Jackson BALB/c (BALB/cByJ vs BALB/c/J) mice developed hyperthyroidism in response to TSHR A-subunit adenovirus immunization.[24] The Bailey substrains of BALB/c and B6 mice have been bred separately from their Jackson counterparts for more than 50 years.[24]

The most important outcome of our study, with potential implications for the genetics of human Graves' disease, is the discovery of novel candidate genes besides the previously observed linkage to the MHC gene region.[14,17] In BXH strains, preferential TSAb recognition of the mouse vs human TSHR in BXH mice was significantly linked to loci on Chr 12 between 110.245 and 111.769 Mb (LRS of 20.433; LOD of 4.43). This region includes the gene for *Dio3*, an enzyme involved in thyroid hormone inactivation.[18] An increase the power of genetic analysis by combining the TSAb species specificity data for the BXH and CXB mice increased the LOD score for this Chr 12 region to >5.8.

Potentially of greater pathophysiological importance were loci slightly further upstream on Chr 12. Thus, LOD scores of 3.49–3.56 were obtained for the Chr 12 region

from 113.270 to 120.329 Mb. Combining linkage data for the CXB and BXH strains narrowed this region to 113.595–118.161 Mb. Remarkably, in this broad region, 32 of 66 (48%) of the genes markers encode the variable regions (IgV H) of immunoglobulin heavy chains (Supplementary Table S2), which are logical candidate genes for influencing antibody specificity. Moreover, the high frequency of *VH* genes in this locus, taken together with earlier findings that an IgV H gene controlled antibody heteroclicity (reactivity to steryl-oxazolone vs the immunogen furyl-oxazolone[25]), strongly suggests that *VH* gene differences between mouse strains underlie the susceptibility (or lack thereof) to develop antibodies capable of activating the TSHR.

In early studies, it was reported that two genes, linked to immunoglobulin heavy chains (Gm) and HLA, controlled susceptibility to Graves' disease.[26] Because the chromosomal region encompassing genes for immunoglobulin heavy chain variable and constant regions is large (~3 million bases in mice[27]), these observations for humans are potentially similar to ours in mice. However, similar associations were made for Hashimoto's thyroiditis,[28] a condition in which TSAb are absent. Moreover, later studies found no evidence for genes associated with the immunoglobulin H chain loci in Graves' or Hashimoto's diseases.[29,30]

Why have immunoglobulin *VH* germ-line gene loci not previously been identified in genetic linkage and genome-wide association studies in human Graves' disease? In our opinion, ascertainment of traits for inclusion in these studies has been too broad. Greater stratification than a simple diagnosis of Graves' disease is necessary. In Graves' patients, TSHR antibodies are present at very low concentrations and are rarely (if ever) detected in family members without clinical disease. In addition, TSHR antibody levels are modulated by, and may disappear after, therapy with antithyroid drugs, surgery or radioiodine (for example, McGregor *et al*.[31] and Chiovato *et al*.[32]). For these reasons, to the best of our knowledge, no studies in humans have tested the genetic susceptibility for TSHR antibodies separately from hyperthyroidism, as has been done for autoantibodies to thyroid peroxidase and thyroglobulin.[33] One advantage of the Graves' animal model, using genetically identical mouse strains subject to identical environmental factors, is the ability to separate susceptibility to TSHR antibody development from variations in thyroid function.[14,17] The data from the present study suggest that insight into the genetics of human Graves' disease would be facilitated by restricting genetic analysis to untreated patients which are subdivided according to TSAb levels or even divided into TSAb antibody subsets including perhaps differential TSAb activity for the human vs the mouse TSHR.

Besides our novel findings pointing to *VH* germ-line genes in Chr 12 as candidates for genetic susceptibility to TSAb generation, data from our present and previous linkage studies[14,17] provide evidence for other loci underlying this trait:

(i) TSHR antibodies (determined in TSAb and TBI assays) in CXB mice were linked to the same Chr 17 loci including MHC classes I and II, complement components, tumor necrosis factor and lymphotoxin. These data support observations in humans in which MHC class II has long been associated with autoimmune thyroid disease (reviewed in Jacobson and Tomer[34]) and very recently MHC class I.[35]

(ii) The highest LRS or LOD scores for TSAb species specificity were associated with the Chr 12 region including the gene encoding *Dio3*,[18] a selenoenzyme that catalyzes the conversion of thyroid hormones to inactive metabolites. However, because it is difficult to envisage how *Dio3* could contribute to the pathogenesis of Graves' disease, TSAb activity is more likely linked to another closely associated, presently unidentified gene.

(iii) TSAb generation in CXB mice linked to Chr X loci between 72.5 and 83.3 Mb, supports our previous finding with TSHR antibodies detected in the TBI assay.[17] There are no obvious candidate genes in this region, with the possible exception of interleukin-1 receptor accessory protein-like 1 (*Il1rapl1*). In addition to the foregoing distal Chr X loci, TSHR antibody linkage to more proximal Chr X loci (32–44 Mb) was noted for TBI in BXH mice.[14] Again, there are no logical candidate genes in this region. As noted previously, although not confirmed in all data sets,[36,37] associations with the X chromosome have been reported for human Graves' disease.[38–40]

In conclusion, measuring mouse TSAb vs human TSAb in mice with different and known genetic backgrounds provides novel insight into the genetic basis for susceptibility to induction of antibodies capable of activating the TSHR. Most important, we provide the first evidence suggesting involvement of immunoglobulin *VH* genes in this process. These data suggest that reexamination of these candidate genes in human Graves' disease is warranted, using improved subject ascertainment and stratification, for example, by including only patients with untreated disease of recent onset, of the same gender, and focusing on TSHR antibodies rather than a simple history of disease.

Materials and methods

Mouse strains and immunization

Adenoviruses, mouse strains and immunization protocols were previously described.[14,17] Briefly, we used adenovirus encoding the human A-subunit (TSHR amino acids 1–289; A-subunit-Ad)[8] and null adenovirus (Con-Ad).[41] Propagation, purification and determination of particle virus number were reported previously.[7] Female mice (5- to 8-week old) of the following strains were obtained: (1) C3H/HeJ and C57BL/6J (parental BXH strains); (2) RI CXB1/ByJ–CXB7/ByJ; CXB8/HiAJ–CXB13/HiAJ; (3) RI BXH2-, -4, -6 to 11, -14-, -19 TyJ, BXH20/KccJ, BXH22/KccJ and B6cC3-1/KccJ (Jackson Laboratory, Bar Harbor, ME, USA). Parental strains are referred to as C3H, B6 (Jackson or Bailey strains, J or By) and RI strains as CXB1, CXB2 or BXH2, BXH4 and so on.

Mice were immunized with A-subunit-Ad (10^8 particles per injection) on three occasions at 3 weekly intervals. Blood was drawn 1 week after the second injection and mice were killed 4 weeks after the third immunization. Six mice were studied for each CXB or BXH strain (except for CXB5; only two animals were available). The number of parental animals immunized with A-subunit-Ad was 10 C3H/J and 10 B6/J mice.

Additional parental strain mice were immunized with Con-Ad (10^8 particles per injection): five C3H/J and five B6/J. All studies were approved by the institutional animal care and use committee and performed with the highest standards of care in a pathogen-free facility. All sera had previously been characterized for thyroxine and TSHR antibodies measured by inhibition of TSH binding (TBI) or enzyme-linked immunosorbent assay.[15,17]

TSAb activity measured using mouse TSHR- and human TSHR-expressing cells

Thyroid-stimulating antibody was assayed by stimulation of cAMP generation in CHO cells expressing the human TSHR[14] and the mouse TSHR.[16] TSHR-CHO cells in 96-well plates were incubated (80–90 min, 37 °C) with test sera diluted 1:20 in Ham's F12 containing 10 mM HEPES (pH 7.4) and 1 mM isobutylmethylxanthine. After aspirating the medium, intracellular cAMP was extracted with ethanol, evaporated to dryness and resuspended in Dulbecco's phosphate-buffered saline. Samples (12 µl) were assayed using the LANCE cAMP kit (PerkinElmer, Boston, MA, USA). Sera from CXB mice and their parental strains were analyzed for TSAb using mTSHR and hTSHR bioassays; sera from BXH mice and their parental strains were tested for TSAb using mTSHR-CHO cells (data for hTSHR-CHO cells were previously reported[14]).

Human and mouse TSAb levels were expressed as a percentage of cAMP values attained with sera from control adenovirus-immunized mice and these values were used for linkage analysis (see below). An additional trait used in linkage analysis is the TSAb ratio for mTSHR/hTSHR (BXH strains), and hTSHR/mTSHR (CXB strains). Because maximum cAMP induction differed in the two bioassays, for visual comparisons TSAb values for individual mice were normalized by assigning 100% to the mean value for the strain with the highest response. These are as follows: BXH strains: mouse-TSHR 485% (BXH9) and human-TSHR 1229% (BXHcC3-1); CXB strains: mouse TSHR 555% (CXB6) and human TSHR 945% (CXB6).

Statistical analyses

Significant differences between responses in different groups were determined by Mann–Whitney rank-sum test or, when normally distributed, by Student's *t*-test. Multiple comparisons were performed using analysis of variance. Tests were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA, USA). Data in bar graphs are shown as mean + s.e.m.

Genetic linkage analysis

Putative quantitative trait loci involved in TSAb induced by A-subunit-Ad immunization of CXB and BXH strains were mapped using the genotype files for these recombinant inbred strains generated by Williams *et al*.[42] available at http://www. nervenet.org and embedded in GeneNetwork (www.genenetwork.org). The probability of linkage between our traits and previously mapped genotypes was estimated at ~1 cM intervals (~2 Mb) along the entire genome, except for the Y chromosome. To establish criteria for suggestive and significant linkage, we performed a permutation test was performed (1000 permutations at 1 cM intervals).[43] This test compares the peak likelihood ratio statistics (LRS = LOD × 4.6, where LOD is the logarithm of the odds) obtained for a given data set with the peak LRS score obtained for 1000 random permutations of the same data set.

The primary phenotype data have been entered into the mouse CXB and BXH phenotype databases in GeneNetwork (www.genenetwork.org) under the trait accession identifiers: CXB10683–101690; and BXH10147, 10148; 10155–10160). These data can be found by searching the CXB or BXH databases for the name 'McLachlan'. In the Results section, we refer to the identification (GN) specific trait numbers so that readers can verify and extend the data analysis. Because GN interval maps connect directly to the University of California Santa Cruz (UCSC) Genome Browser, it is possible to explore the gene complement of chromosomal intervals together with the quantitative trait loci profile.

CXB and BXH data were combined (when appropriate) to provide a data set from 26 RI strains sharing one parental strain (B6). As described for BXH and BXD RI mice[44] and CXB and BXH mice,[14] we calculated the probability associated with a X^2 value equal to: $2(\ln P_{BXD} + \ln P_{CXD})$ with 4 degrees of freedom, where $\ln P_{BXD}$ and $\ln P_{CXD}$ are the natural logarithms of the probabilities derived independently for the two RI strains in the same chromosomal interval. For the combined analysis, the Gene Network program only provides an LOD score; for comparison with the LRS scores provided by the standard analysis using this program, an LRS value of 4.61 is equivalent to an LOD score of 1.0.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)