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Exceptional Hyperthyroidism and a Role for both Major Histocompatibility Class I and Class II Genes in a Murine Model of Graves’ Disease

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Abstract

Autoimmune hyperthyroidism, Graves’ disease, can be induced by immunizing susceptible strains of mice with adenovirus encoding the human thyrotropin receptor (TSHR) or its A-subunit. Studies in two small families of recombinant inbred strains showed that susceptibility to developing TSHR antibodies (measured by TSH binding inhibition, TBI) was linked to the MHC region whereas genes on different chromosomes contributed to hyperthyroidism. We have now investigated TSHR antibody production and hyperthyroidism induced by TSHR A-subunit adenovirus immunization of a larger family of strains (26 of the AXB and BXA strains). Analysis of the combined AXB and BXA families provided unexpected insight into several aspects of Graves’ disease. First, extreme thyroid hyperplasia and hyperthyroidism in one remarkable strain, BXA13, reflected an inability to generate non-functional TSHR antibodies measured by ELISA. Although neutral TSHR antibodies have been detected in Graves’ sera, pathogenic, functional TSHR antibodies in Graves’ patients are undetectable by ELISA. Therefore, this strain immunized with A-subunit-adenovirus that generates only functional TSHR antibodies may provide an improved model for studies of induced Graves’ disease. Second, our combined analysis of linkage data from this and previous work strengthens the evidence that gene variants in the immunoglobulin heavy chain V region contribute to generating thyroid stimulating antibodies. Third, a broad region that encompasses the MHC region on mouse chromosome 17 is linked to the development of TSHR antibodies (measured by TBI). Most importantly, unlike other strains, TBI linkage in the AXB and BXA families to MHC class I and class II genes provides an explanation for the unresolved class I/class II difference in humans.

Introduction

Susceptibility to Graves’ disease has long been associated with genes of the major histocompatibility complex (MHC; HLA in humans)[reviewed in [1]]. Hyperthyroidism in Graves’ patients is caused by autoantibodies to the thyrotropin receptor (TSHR) that mimic the stimulatory activities of the ligand (TSH) on the thyroid gland [reviewed in [2]]. Associations between particular autoimmune diseases and MHC amino acid sequences [3] likely reflect the ability of the MHC class II binding pocket to accommodate peptides that stimulate autoreactive T cells, as shown recently for thyroglobulin [4]. A similar mechanism would be expected to play a role in MHC class II binding for peptides of the thyrotropin receptor (TSHR), the autoantigen in Graves’ disease. Consistent with this possibility, MHC class II region genes were most tightly linked to susceptibility in a genome-wide association scan in thyroid autoimmune disease, which included an expanded cohort of Graves’ patients [5]. However, numerous early studies reported associations between class I (B8) in addition to class II (DR3) genes (for example [6]). Besides MHC class I and II, a recent study found “a novel and major association of HLA-C in Graves’ disease that eclipses the classical HLA-DRB1 effect” [7].

Graves’ disease can be induced in mice by injecting cells expressing the human TSHR or immunization with the human TSHR DNA in plasmid or adenovirus vectors [reviewed in [8]]. Surprisingly, in several mouse models of induced Graves’ disease, initial studies suggested that MHC genes were less important susceptibility factors than non-MHC genes [for example [9]; reviewed in [10]]. However, later linkage studies with recombinant inbred (RI) strains - essentially families of related strains of mice - provided an answer to this apparent discrepancy in terms of the MHC gene contribution to human versus murine Graves’ disease susceptibility. In two RI families (CXB and BXH), development of TSHR antibodies was linked to loci in the MHC region whereas genes on different chromosomes were linked to hyperthyroidism [11,12]. These studies in CXB and BXH strains demonstrated a role for MHC region genes in controlling the generation of TSHR antibodies, at least as measured by inhibition of TSH binding to its receptor [TBI]. However, more detailed mapping of genes within the MHC region was not performed for these two small RI families because each only contained 13 strains.

The AXB and BXA families of strains (here abbreviated AXBXA) were derived from parental strains A and C57BL/6 (B6). B6 mice are also one parental strain in the CXB and BXH sets.
Mice of the B6 strain are “good antibody responders” to immunization with adenovirus expressing the TSHR or its A-subunit but rarely develop hyperthyroidism [14,15]. Mice of the A strain have not previously been examined for their response to TSHR immunization. However, A strain mice have been investigated for antibody induction to a variety of antigens including phosphorylcholine [16], staphylococcal nuclease IV [17] and hen-egg lysozyme [18] and for their responses to infectious organisms such as Plasmodium falciparum [19].

The A strain is particularly interesting in terms of its MHC genes compared with those in B6, C3H/He and BALB/c mice. The latter two (C3H/He and BALB/c) are the non-B6 parents of the BXH and CXB families, respectively. The MHC class II genes of A mice are of the k-haplotype, as in C3H/He mice, in contrast to the d- and b-haplotypes of B6 and BALB/c mice. Moreover, the MHC class I genes of A strain mice are a mixture of k- and d-haplotypes [20]. For these reasons, and because the collected AXBXA set is significantly larger (26 strains) than the previously studied families, we investigated the outcome of immunizing AXBXA strains with adenovirus expressing the TSHR A-subunit. Our findings provide several unexpected and intriguing results, particularly in relation to the contribution of MHC region genes to the generation of TSHR antibodies.

**Results**

**TSHR antibodies and hyperthyroidism induced in A and B6 mice and their F1 offspring**

Mice of the A/J strain (referred to as “A”) immunized three times with TSHR A-subunit-Ad developed significantly lower TSHR antibody levels (measured by TSH binding inhibition, TBI) than either B6 mice or the F1 hybrids (B6XAF1) between the two parental strains (Fig. 1A; p < 0.05, ANOVA). In terms of thyroid function, no A strain mice and only one B6 mouse had elevated serum T4 compared with Con-Ad immunized animals of the same strain. However, two of 20 B6XAF1 mice immunized with TSHR A-subunit-Ad were clearly hyperthyroid (Fig. 1B).

**Response of recombinant AXBXA strains to A-subunit adenovirus immunization**

After immunization with A-subunit-Ad, 15 of 26 AXBXA strains developed low TBI levels, from almost negative to <50% inhibition of TSH binding (Fig. 2A; strains ranked left to right for TBI, lowest to highest). The TBI response in the other 11 strains was more robust (>50%). TBI values were similar after two or three immunizations (Fig. 2A, speckled versus solid bars, respectively). In contrast, when measured by ELISA, TSHR antibody levels for some strains were lower after the third versus the second immunization (Fig. 2B). Moreover, the rank order for TSHR antibodies measured by ELISA differed from that for TBI. Focusing on the strains with strong TBI responses, TSHR antibodies measured by ELISA were disproportionately low, indeed almost undetectable, in strains BXA2, BXA13 and BXA1 (labeled B2, B13 and B1) (Fig. 2B versus 2A).

Turning to thyroid function, T4 levels were virtually unchanged in AXBXA strains after two immunizations (data not shown). However, after the third immunization, serum T4 was markedly increased in BXA13, BXA16 and AXB13 strains (labeled B13, B16 and A15), as reflected in the increase above pre-immunization levels (“delta” T4 values; Fig. 2C). Indeed, one BXA13 mouse had the highest absolute T4 level (22 μg/dL) that we have ever observed over 8 years in any mouse strain. Thyroid histology confirmed that thyroid hyperplasia in BXA13 far exceeded that routinely observed for other hyperthyroid AXBXA strains (Fig. 2F versus 2E; euthyroid tissue in Fig. 2D) or even hyperthyroid mice of the susceptible BALB/c strain (for example [21]). In BXA13 strains (which had the largest increase in serum T4), there was a striking discordance between the very strong serum TBI activities...
TSAb activity
Thyroid stimulating antibody (TSAb) activity was measured in AXBXA strains after the third A-subunit-Ad immunization. Separate assays were performed using CHO cells stably expressing the human-TSHR or the mouse-TSHR. The rank order for TSAb values corresponded approximately to that for TBI. Strains with the highest TSAb values, specific for the human- or the mouse-TSHR, generally had high TBI levels (Fig. 3A and B versus Fig. 2A). Another measure of TSAb activity, the ratio of human:mouse-TSHR responsivity (Fig. 2C), indicated preferential recognition of the human versus the mouse-TSHR consistent with the immunogen being the human-TSHR A-subunit.

Linkage analysis in AXBXA mice
Quantitative trait loci (QTL) were mapped using the AXBXA strain database files [13,22] for the following parameters: TSHR antibodies measured by TSH binding inhibition (TBI), ELISA, TSAb activity (specific for the human-TSHR, the mouse-TSHR, or the human:mouse TSAb ratio); T4 levels and the difference (“delta”) between T4 after immunization and baseline.

Figure 2. TSHR antibodies, T4 and thyroid histology in 26 AXBXA strains immunized three times with TSHR A-sub-Ad. Data are ranked from left to right according to TBI activity (after the third immunization) from the strain with the lowest TBI (AXB1, abbreviated A1) to the strain with the highest TBI activity (AXB13, A13). Grey boxed areas with an asterisk link panels A,B and C for the strains with the greatest increase (delta) in T4, namely BXA13, BXA16 and AXB13 (B13, B16 and A13, respectively). A) TBI (inhibition of labeled TSH binding to its receptor) and B) TSHR antibody measured by ELISA (OD 490 nm). Bar graphs represent the mean +SEM (5–6 mice/strain) after 2 immunizations (2x, speckled bars) or 3 immunizations (3x, solid bars). The vertical dashed line indicates the cut-off between strains with less than 50% TBI (to the left) and greater than 50% TBI (right of the line). C) T4 increase (delta T4 3x). Data shown as bars graphs for the mean + SEM. D, E, F) Representative thyroid histology (magnification 10x) for euthyroid mouse (D), hyperthyroid mouse with T4 10 μg/dL (E) and extremely hyperthyroid mouse with T4 ~20 μg/dL (F, as in BXA13 strains).

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The most striking linkage was between TBI and a locus on Chr 17 (twin peaks at \(33\) and \(37\) Mb) with likelihood ratio statistic (LRS) values of \(24.2\) (after two immunizations) and \(32.5\) (after three immunizations) (Table 1). The latter LRS value (corresponding to LOD score 7.05) greatly exceeds the LRS cut-off of \(20–25\) usually required to reach genome-wide significance of \(p_{0.05}\). TSHR antibodies measured by ELISA (after two and three immunizations) were linked to loci on Chr 1, with a higher LRS value after the second than after the third immunization (Table 1), likely associated with higher ELISA antibody values after two than after three adenovirus injections (Fig. 2B). TSAb specific for the mouse-TSHR (m-TSAb) was linked to the same loci on Chr 17 region as TBI (Table 1).

Linkage for TSHR antibodies in AXBXA, CXB and BXH sets compared with previous reports on AXBXA responses to other immune challenges

We compared linkage data for induced TSHR antibodies in the AXBXA set with our previous findings in CXB and BXH sets [11,12,23] and with information on the responses in the AXBXA set to other immune challenges [20,24]:

(a) The most notable similarity is the near identical linkage between TBI and the Chr 17 locus (present study) and induced antibodies to human Factor IX (anti-Factor IX) [20] (Fig. 4A). In addition, the linkage on Chr 17 for TBI overlapped in all three RI sets. However, for BXH and CXB mice, LRS values were lower, broader and spanned the two AXBXA peaks (Fig. 4B). Variability in the response of AXBXA strains to infection with *Histoplasma capsulatum* (measured as spleen weight) was also linked to the Chr 17 locus but the major peak was distal to the peaks for TBI and anti-factor IX (Fig. 4C versus Fig. A and B).

(b) TSAb activities, specific for the human-TSHR, or the h:m-TSAb ratio, in AXBXA, BXH and CXB sets were all linked to loci on the distal end of Chr 12 (Fig. 4D).

(c) Linkage to Chr 1 loci for TSHR ELISA antibodies in AXBXA mouse strains overlapped to a minor extent with the values for BXH strains (Fig. 4E) or versus anti-factor IX antibodies in AXBXA strains (Fig. 4F).

Contribution of parental B6 alleles versus non-B6 alleles

Information on the contribution of parental alleles is provided by the additive effects for linkage. Positive additive effects indicate the contribution from A-strain alleles (A strain mice) whereas
negative additive effects indicate the contribution from B-alleles (B6 strain mice) (see Methods).

(a) TBI: the negative additive effects (~20) indicate the contribution from B6 variant genes (Fig. 5, top left; red ellipse). In contrast, the strong positive additive effects (>8000) demonstrate that anti-Factor IX levels are increased by alleles inherited from the A-strain (Fig. 5, bottom left, purple ellipse).

(b) TSAb, in AXBXA, BXH and CXB sets, TSAb measurements involving the human-TSHR were linked to Chr 12 loci. For AXBXA strains, the negative additive effects indicate the contribution of B alleles (red ellipse, Fig. 5 top right); in CXB and BXH strains, the positive additive effects reflect the contribution of alleles from BALB/c and C3H/He strains (blue and pink ellipses, respectively; Fig. 5, right middle and bottom panels). Incidentally, in the AXBXA set, TSAb specific for the mouse-TSHR was linked to the same Chr 17 locus as TBI (Table 1) and, as for TBI, B alleles increased this trait (data not shown).

LRS values for TSAb activities in the three RI sets individually are relatively low (Table 1; [23]). However, combined linkage analysis increased the LOD scores for linkage between TSAb (specific for the human-TSHR or the human:mouse-TSAb ratio) and Chr 12 (Table 2).

**Table 1. Chromosomal linkage for TSHR antibodies and thyroid function in AXBXA RI mice immunized with TSHR A-subunit-Adenovirus.**

| Trait         | LRS | LOD* | Chr | Locus       | Mb     | h-Chr b |
|---------------|-----|------|-----|-------------|--------|---------|
| TSHR Antibodies |     |      |     |             |        |         |
| TBI (3x)      | 32.52 | 7.05 | 17  | rs3672987   | 33.247 | 19      |
|               |       |      |     | rs13482968 | 37.269 |         |
| ELISA (2x)    | 20.19 | 4.38 | 1   | rs4222856   | 180.251 | 1       |
|               |       |      |     | CEL-1       | 180.396 |         |
|               |       |      |     | 178482360   |        |         |
| ELISA (3x)    | 15.08 | 3.27 | 1   | D1Mit356    | 174.819 | FcR-like d |
|               |       |      |     | rs4136041   | 177.367 |         |
| m TSAb (3x)c  | 14.10 | 3.06 | 17  | rs3672987   | 33.247 | 19      |
|               |       |      |     | rs13482968 | 37.269 |         |
| h TSAb (3x)c  | 15.11 | 3.28 | 12  | D12Nd2      | 115.135 | 14      |
|               |       |      |     | rs3686531   | 120.968 |         |
| Thyroid Function |     |      |     |             |        |         |
| T4 (3x)       | 14.52 | 3.15 | 16  | rs4192837   | 60.329 | 3       |
|               |       |      |     | rs4195972   | 64.435 |         |
| Delta T4 (3x) | 10.45 | 2.27 | 16  | rs4202837   | 73.290 | 3       |
|               |       |      |     | rs4203607   | 73.919 |         |

Loci and their chromosomal locations (megabases, Mb) are given for traits with the highest LRS (Likelihood Ratio Statistic) scores after two or three (2x or 3x) immunizations; significant linkage in bold.

*LOD scores: calculated from LRS/4.61.

aInformation on the corresponding human (h) Chr or likely Chr is included.

bLinkage analysis performed using SEM; Lower LRS values: TBI (2x); LRS 24.21 (33.247, 37.2686 Mb); TSAb h:m ratio (3x): LRS 14.85 (115.135, 120.968 Mb).

dFc receptor-like 6 (174.819 Mb). Note: Lower LRS values: TBI (2x): LRS 24.21 (33.247, 37.2686 Mb);TSAb h:m ratio (3x): LRS 14.85 (115.135, 120.968 Mb).

**Discussion**

Recombinant inbred AXBXA strains immunized with TSHR A-subunit adenovirus provided insight into two aspects of Graves’ disease. First, we observed more severe hyperthyroidism in one of these strains than we previously observed in any other inbred strain. Second, our data are the first to implicate MHC class I genes as contributing to the variability in the TSHR antibody response in an induced mouse model of Graves’ disease. Neither of these outcomes was anticipated from studying the parental strains and the F1 obtained by crossing A to B6 strains. Briefly, TSHR antibody responses (measured as TBI) were low in A-strain mice and high in the F1, as in B6 mice, demonstrating that high responder B6 parental genes dominate the low responder A-strain parental variants. Moreover, although uncommon, some F1 mice became hyperthyroid. Consequently, it seemed likely that studying the relatively large AXBXA set (26 members) would contribute to understanding the genetic susceptibility to induced Graves’ disease. However, the overall impact was far greater than we had expected.

Very severe hyperthyroidism associated with extreme thyroid follicular cell hyperplasia (Fig. 2F) was observed in BXA13 mice. We offer what we believe to be a likely explanation for this phenomenon. TSHR antibody assays for inhibition of TSH binding (TBI) and TSHR activation (TSAb) measure pathogenic (‘functional’) antibodies. Immunization of BALB/c and B6 mice with A-subunit adenovirus induces both functional TSHR antibodies detectable by TBI and TSAb as well as antibodies measured by ELISA (for example [15]). Pathogenic (or ‘functional’) TSHR antibodies, including the human monoclonal stimulating antibody M22 [25], have little or no recognition of the purified, recombinant TSHR A-subunit protein coated on an ELISA plate. Despite high TBI values, BXA13 mice were virtually negative for TSHR-ELISA antibodies.

It should be noted that neutral TSHR antibodies have been detected in Graves’ sera by flow cytometry [26] or by inhibiting monoclonal antibody binding to ELISA plates coated with peptides corresponding to the TSHR cleavage region, amino acid residues 316–366 [27]. Such neutral antibodies may play a role in exacerbating the autoimmune response in Graves’ disease [27]. In contrast, ELISA-type TSHR antibodies induced in mice bind predominantly to the extreme amino-terminus of the TSHR [28]. In fact, the A-subunit used for immunization lacks the cleavage region recognized by most neutral TSHR antibodies [27].
Moreover, as described below, TSHR-ELISA antibodies in mice protect, rather than exacerbate, responses to the TSHR. Pre-treating BALB/c mice with TSHR A-subunit protein before A-subunit adenovirus immunization attenuated hyperthyroidism by “deviating” the humoral response from pathogenic (TBI and TSAb positive) towards TSHR-ELISA antibodies [29]. Consistent with the extreme degree of hyperthyroidism, the qualitative balance in the BXA13 strain was almost entirely towards functional TSHR antibodies, with minimal ELISA-positive antibodies. Even BALB/c mice, the most susceptible to developing hyperthyroidism in our induced Graves’ disease model, develop high levels of TSHR-ELISA antibodies as well as functional TSHR antibodies (for example [21]). Therefore, the BXA13 strain may resemble human Graves’ patients more closely than do similarly immunized BALB/c mice. Unlike some other autoimmune diseases such as type 1 diabetes, Graves’ disease occurs only in humans and there are no spontaneous animal models of the disease. The importance of our present finding is that the use of BXA13 mice may provide a major enhancement in studying induced Graves’ disease.

Our initial goal for investigating mice of the AXBXA set was to expand our studies of the genetic basis for susceptibility to induced hyperthyroidism and TSHR antibodies. In this set, elevated serum T4 was linked to a locus on Chr 16. This linkage was not observed in comparable studies of BXH and CXB strains (Chr 3, 13)[11,12]. As previously observed [30], linkage of baseline T4 in AXBXA strains (Chr 2)[30] is distinct from linkage in BXH and CXB strains (Chr 1, 11 and 13). Similarly, TSHR antibodies measured by ELISA were linked to Chr 1 but the loci were different from those previously observed in CXB and BXH strains [11,12]. In contrast, TSAb activity was linked to the same distal region on Chr 17 noted for BXH and CXB strains [23]. Indeed, combined linkage for the three RI sets (AXBXA, CXB and BXH) increased the LOD score from 3.54 in two RI sets [23] to 5.71 in three RI sets over a broad interval (113.595–117.869 Mb). More than half the genes within this interval (54%) encode immunoglobulin heavy chain V region genes. The high frequency of VH genes in this locus suggests that VH gene differences between mouse strains underlie the susceptibility (or lack thereof) to develop antibodies capable of activating the TSHR (as discussed previously [23]).

The most striking observation for the AXBXA set was the very strong linkage between TSHR antibodies measured by TBI and a broadly defined MHC region on Chr 17 with LOD scores rising from 5.25 after two immunizations to 7.05 in mice immunized three times. The same Chr 17 locus was linked in AXBXA strains immunized to develop antibodies to Factor IX [20] and, albeit to a lesser extent, to spleen responses after infection with Histoplasma capsulatum [24]. Linkage to Chr 17 is not a general characteristic of...
AXBXA strains to immunization: the major linkage peak for Histoplasma susceptibility is downstream of the MHC region. Moreover, variability in anti-cardiac sacrolemmal antibodies that develop in AXBXA mice after Coxsackie infection is linked to Chr 5 and 7 [31].

Linkage between Chr 17 loci and both TBI and antibodies to human Factor IX raised the following question: because both antibodies were induced using human-adenovirus vectors, do these linkages merely reflect susceptibility to adenovirus? This issue was refuted by information on additive effects: B-alleles (negative additive effects) increase TBI whereas A-alleles (positive additive effects) increase anti-Factor IX. Moreover, the major locus for susceptibility to mouse-adenovirus (which resembles human adenoviruses in structure and genome organization) maps to Chr 15 [32], not to Chr 17. Together with the additive effect data, this information supports the conclusion that linkage between TBI and Chr 17, or between anti-Factor IX and the same Chr 17 locus, is unrelated to the adjuvant effects of adenovirus and is specific for the two very different immunogens.

Finally, it was possible to putatively assign MHC genes linked to TBI in AXBXA strains (present study) and in CXB and BXH strains [11,12]. In the AXBXA set, TBI is strongly linked to Dmb1 (class II) and H2-Q6 and H2-M5 (class I) and to a lesser extent to H2-Ab1 (class II). Although the role of class I antigens is not understood, strong linkage between TBI and Dmb1 suggests that peptide loading to MHC class II antigens plays a critical role in AXBXA responses to A-subunit immunization. Potentially interesting is TBI linkage to the class III genes heat shock protein (BXH set) and a lymphocyte antigen-6 family member (CXB set). The large family of lymphocyte antigen-6 proteins (27 in humans and 37 in mice), have putative immune roles [33]. Heat shock proteins have previously been associated with Graves’ disease (for example [34,35]), although their specific role in thyroid autoimmunity (as distinct from cellular processes in general) is enigmatic [36]. It is intriguing that, at least in some mouse strains, heat shock proteins may be involved in the development of TSHR antibodies.

In conclusion, investigations in AXBXA recombinant inbred mice immunized with TSHR A-subunit adenovirus provided unexpected insight into several aspects of Graves’ disease. First, extreme thyroid hyperplasia and hyperthyroidism in one AXBXA strain likely reflects its inability to generate ELISA-type TSHR antibodies of the type not observed in Graves’ patients. For this reason, BXA13 strain immunized with A-subunit-adenovirus may provide the most suitable mouse strain for investigating human Graves’ disease. Second, linkage data from AXBXA mice strengthen the case for the contribution of immunoglobulin heavy chain variable region genes to the generation of thyroid stimulating antibodies. Third, genes within and outside the

Figure 5. Influence of parental genes (B6 or non-B6) on linkage between TBI and Chr 17 (left panels) and between TSAb and Chr 12 (right panels) in AXBXA, CXB and BXH sets. Also included are data for antibodies to Factor IX in AXBXA mice. LRS values are plotted together with the “Additive effect” which is defined as: half the difference in the mean phenotype of all cases homozygous for one parental allele at this marker minus the mean of all cases homozygous for the other parental allele at this marker. For AXBXA strains: positive additive effects indicate that A alleles increase trait values; negative additive effects indicate that B6 alleles (red ellipse) increase trait values. Chromosomal location is on the X-axis (Mb); LRS values on the left Y-axis; additive values on the right Y-axis. Ellipses highlight Chr locations for peak LRS values. Left: TBI activity in AXBXA mice (upper panel) and anti-human factor IX (lower panel, from [20]. Additive effects:- positive, A alleles (purple ellipse); negative, B6 alleles (red ellipse). Right panels: Upper right, TSAb specific for the human TSHR in AXBXA mice; middle right, TSAb human: mouse (h:m) ratio in CXB strains (from [23]; lower right, TSAb mouse: human (m:h) ratio in BXH mice (from [12]). Additive effects:- positive, C3H/He alleles (blue ellipse) or BALB/c (pink ellipse); negative, B6 alleles (red ellipse).
MHC region are linked to the generation of TSHR antibodies (measured by TBI). Moreover, the finding that TBI in AXBXA strains is linked to both class I and class II MHC region genes may provide an explanation for conflicting findings regarding the two classes of these genes in different human populations.

**Materials and Methods**

**Immunization of mice with TSHR A-subunit adenovirus**

Female mice of the following 26 strains (5 to 8 weeks of age; The Jackson Laboratory, Bar Harbor, Maine) were studied: A/J and B6AF1J [hereafter referred to as “A” and “B6AF1”]; AXB/PgnJ strains 1, 2, 4, 5, 6, 8, 10, 12, 13, 15, 19a (formerly AXB18), 23, 24; BXA/PgnJ strains 1, 2, 4, 7, 8, 11, 12, 13, 14, 16, 24, 25, 26. Adenovirus expressing the human TSHR A-subunit (amino acid residues 1–289, A-subunit-Ad) [21] and control adenovirus lacking an inset (Con-Ad)[37] were propagated in HEK293 cells (American Type Culture Collection, Manassas, VA), purified on CsCl density gradients and viral particle concentration was determined from the absorbance at 260 nm [38].

A and B6AF1 strains were immunized intramuscularly three times with A-subunit-Ad (10^8 particles per injection) (10–20 mice/strain) or Con-Ad (5 mice/strain) on three occasions at three weekly intervals as described [21]. Data previously obtained for B6 mice immunized in the same way [15] are included for comparison. Baseline T4 values in AXBXA strains were published recently [30]: briefly, before immunization, blood samples were drawn from individual mice in each AXB or BXA strain (5–6 mice/strain); sera were pooled for each strain and analyzed in duplicate for T4. Prior studies in CXB and BXH mice had demonstrated consistency for individual mice in pre-immunization samples [11,12]. Subsequently, for the current study, AXBXA strains were immunized with A-subunit-Ad (10^8 particles per injection) three times at three weekly intervals. Blood was drawn one week after the second immunization and mice were euthanized four weeks after the 3rd injection to harvest blood and thyroid glands.
Assays for TSHR antibodies

TSHR antibodies were investigated using three assays: TSH binding inhibition (TBI), ELISA using TSHR A-subunit protein, and a bioassay for thyroid stimulating antibody (TSAb). TBI was determined using a commercial kit (Kronus, Boise, ID); serum aliquots (25 µl) were incubated with detergent solubilized TSHR. 125I-TSH was added and the TSHR-antibody complexes were precipitated with polyethylene glycol. TBI values were calculated from the formula: - [1- (TSH binding in test serum - non-specific binding)/TSH binding in normal serum - non-specific binding] X 100.

TSAb activity specific for the human-TSHR or the mouse-TSHR was assayed as described previously [23]. Briefly, Chinese hamster ovary cells expressing human TSHR (or the mouse-TSHR) were plated in 96 well plates and, when confluent, incubated (60 min, 37°C) with test sera diluted 1:20 in Ham’s F12 supplemented with 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 PBS. Samples (20 µl) were assayed using the LANCE cAMP kit (PerkinElmer, Boston, MA). TSAb activity was expressed as a percentage of cAMP values attained with sera from control, immunized mice.

Serum thyroxine and thyroid histology

Total thyroxine (T4) was measured in undiluted mouse serum (25 µl) using a radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). T4 values were computed from standards in the kit and expressed as µg/dL at base-line and after two immunizations (2x) or three immunizations (3x). In addition, to incorporate baseline thyroid function, we calculated the change (delta) after two or three immunizations. Thyroid glands were fixed in buffered formaldehyde (pH 7.4), paraffin-embedded and serial sections were stained with hematoxylin and eosin (Research Animal Diagnostic Laboratory, University of Missouri, Columbia, MO).

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 (ANOVA). Tests were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA).

Genetic linkage analysis

Putative quantitative trait loci (QTL) involved in traits of the AXBXA set before and after A-subunit-Ad immunization were mapped using the genotype files for AXB/BXA RI strains generated by Williams et al. [13] embedded in GenoNetwork. The genotype file consists of 2446 markers with unique strain distribution patterns (www.genenetwork.org/dbdloc/AXBXAGeno.html). The probability of linkage between our traits and previously mapped genotypes was estimated at ~1 centiMorgan intervals (~2 megabase; Mb) along the entire genome, except for the Y chromosome. To establish criteria for suggestive and significant linkage, a permutation test was performed (1000 permutations at 1-centimorgan intervals)[41]. This test compares the peak likelihood ratio statistics (LRS; LRS = LOD x 4.6, where LOD is the logarithm of the odds) obtained for the properly ordered data with the distribution of peak LRS scores obtained from 1000 random permutations of the same data. Additive effects were also estimated. In AXBXA strains, a positive additive effect indicates that an A allele increases trait values at a particular locus or marker; a negative additive effect indicates that a B allele increases trait values. The primary phenotype data (10 traits) have been entered into the mouse AXB/BXA Phenotype database on GenoNetwork (www.genenetwork.org) under the trait accession identifiers GN 10130 to 10166, 10172 to 10175 and can be found by searching for the name ‘Gravesian’.

For some parameters, we combined the LRS values for AXB/BXA strains with our previous findings for CXB or BXH strains [11,12] to provide linkage data for an entire collection of 52 RI strains that all share a B6 parental strain. Following the combined linkage analysis approach for neuron number in two or more families [42], we calculated the probability associated with a x2 value equal to: -2 (lnPAXBXA + lnPCXB + lnPBXH) with 6 degrees of freedom, where lnPAXBXA, lnPCXB and lnPBXH are the natural logarithms of the probabilities derived independently for the three RI families in the same chromosomal interval. Combined linkage data are provided as LOD scores (convertible to LRS values by multiplying by 4.61, as described above). In the combined analyses, “point-wise” p values are provided for the single points examined (as opposed to genome-wide tests which examine few hundred points).

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Author Contributions

Conceived and designed the experiments: SMM BR. Analyzed the data: SMM BR RWW. Wrote the paper: SMM RWW BR. Immunized and tail bled mice: HAA. Performed assays for T4, TBI, ELISA: HAA. Euthanized mice: C-RC. Drew blood by cardiac puncture: C-RC. Removed thyroid glands for histology: C-RC. Analyzed TSAb: HAA. Performed assays for T4: HAA. Drew blood by cardiac puncture and euthanized mice: SMM. Bled mice: HAA. Performed assays for T4, TBI, ELISA: HAA. Euthanized mice: C-RC. Drew blood by cardiac puncture: C-RC. Removed thyroid glands for histology: C-RC. Analyzed TSAbs: BR.

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