Interacting Quantitative Trait Loci Control Loss of Peripheral Tolerance and Susceptibility to Autoimmune Ovarian Dysgenesis After Day 3 Thymectomy in Mice

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Day 3 thymectomy (D3Tx) results in a loss of peripheral tolerance mediated by CD4+CD25+ T cells and the development of autoimmune ovarian dysgenesis (AOD) in A/J and (C57BL/6J × A/J)F1 (B6AF1) hybrids but not in C57BL/6J mice. Quantitative trait loci (QTL) linkage analysis using a B6AF1 × C57BL/6J backcross population verified Aod1 and Aod2 that were previously mapped as qualitative traits. Additionally, three new QTL intervals, Aod3, Aod4, and Aod5, on chromosomes 1, 2, and 7, respectively, influencing specific subphenotypes of AOD were identified. QTL linkage analysis using the A × B and B × A recombinant inbred lines verified Aod3 and confirmed linkage to H2. Aod5 colocalized with Mater, an ovarian-specific autoantigen recognized by anti-ovarian autoantibodies in the sera of D3Tx mice. Sequence analysis of Mater identified allelic, strain-specific splice variants between A/J and C57BL/6J mice making it an attractive candidate gene for Aod5. Interaction analysis revealed significant epistatic effects between Aod1–5 and Gasa2, a locus associated with susceptibility to D3Tx-induced autoimmune gastritis, as well as with H2. These results indicate that the QTL controlling D3Tx-induced autoimmune phenomena are both organ specific and more generalized in their effects with respect to the genesis and activity of the immunoregulatory mechanisms maintaining peripheral tolerance. The Journal of Immunology, 2002, 169: 1640–1646.

The thymus plays a critical role in the selection of self-compatible T cells and the elimination of potentially deleterious autoreactive T cells through positive and negative selection. Disruption of the delicate balance between these two mechanisms can lead to autoimmune disease. For example, neonatal mice thymectomized at 3 days of age (D3Tx) develop in adulthood, autoimmune prostatitis, epididymovasitis, orchitis, thyroiditis, gastritis, and oophoritis. The cellular basis of D3Tx-induced autoimmune dysgenesis is the subject of intense investigation (reviewed in Refs. 3–6). A paucity of CD4+CD25+ T cells is found after D3Tx; furthermore, the specific removal of this subpopulation of cells from naive mice induces organ-specific autoimmunity. Additionally, adoptive transfer of CD4+CD25+ T cells into D3Tx animals prevents the development of the autoimmune disease if done within 14 days. Thus, the thymus appears to be essential for the establishment of peripheral tolerance and immune regulation mediated by these cells, and disruption of their ontogeny is a contributing factor in the pathogenesis of autoimmunity. A mechanistic understanding of these immunoregulatory cells, their ontogeny and release from the thymus, and their maintenance in the periphery are therefore extremely important in understanding peripheral tolerance, immunoregulation, and autoimmune phenomenon.

Different inbred strains of mice exhibit varying patterns of organ involvement and incidences and severity of disease (2). For example, 87% of D3Tx female A/J mice develop oophoritis but only 6% develop gastritis, whereas 33% of female BALB/cJ mice develop gastritis and 17% show signs of oophoritis after D3Tx. Thus, genetic studies have the potential to provide additional insight into the mechanisms controlling the genesis, establishment, and maintenance of CD4+CD25+ immunoregulatory T cells in the periphery and their tissue-specific responses. Genetic factors within and outside H2 are important in controlling susceptibility to D3Tx-induced autoimmune disease. For instance, A (H2a)-strain mice are susceptible to oophoritis after D3Tx, whereas A.By (H2b)- and B10.A (H2b)-congenic mice are resistant (2, 7). Silveira et al. (8) showed that two loci on chromosome 4, Gasa1 and Gasa2, control susceptibility to autoimmune gastritis after D3Tx. In a previous study, we reported that susceptibility to autoimmune ovarian dysgenesis (AOD) is controlled by loci on chromosome 16 (Aod1) and chromosome 3 (Aod2) (9, 10).

Our previous studies, however, focused on susceptibility to AOD as characterized by a qualitative measure (affected vs unaffected). In the present study, we report a quantitative trait locus (QTL) linkage analysis using composite interval mapping (11–14) and interaction analysis (15) of D3Tx-induced AOD as a model for

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the induction and maintenance of peripheral tolerance mediated by CD4+ CD25+ immunoregulatory T cells (3–6). This genetic study establishes that multiple interacting QTL control susceptibility to ovarian autoimmunity and represents an important step toward delineating the genetic mechanisms underlying D3Tx-induced autoimmune phenomenon.

Materials and Methods

Animals

Female (C57BL/6J × A/JF1, B6AF1) and female and male A/J and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female B6AF1 × C57BL/6J backcross (BC1) mice were generated at the State University of New York. Breeding stock for the A × B recombinant inbred lines (RIL) (3). 5, 6, 7, 8, 9, 12, 13, 14, 15, 17, 18, 20, 23, and 24 and the B × A RIL 8, 11, 12, 14, 20, and 20 used in this study were obtained from either Dr. E. Skamene or Dr. M. Neshit. Animals were maintained in accordance with the Animal Welfare Act and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Thymectomy

Thymectomy of A/J, C57BL/6J, BC1, and A × B and B × A recombinant inbred mice was performed at 3 days of age under ether anesthesia on female mice with a suction pipet technique (16). D3Tx females were killed at 60 days of age and were inspected for residual thymus. Animals with residual thymus at 60 days were excluded from the study. Phenotypes from 87 BC1 mice with complete thymectomies were used for linkage analysis. but genotypes from 138 BC1 mice (complete and incomplete thymectomies) were used to generate recombination fractions for the genetic map. For each RIL, three to eight animals were evaluated.

Analysis of AOD phenotypes

The ovaries were extracted, fixed in Bouin’s fixative, and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. Multiple step sections were evaluated in a double blind manner and were scored for oophoritis and atrophy. Ab titers for anti-ovarian Abs were calculated for oophoritis and atrophy. Ab titers for anti-ovarian Abs were scored blinded with severity score that ranged from 1 to 4, as described previously (10). Ab titer ranged from 1 to 8 representing the number of multiple step sections were evaluated in a double blind manner and were scored for oophoritis and atrophy. Ab titers for anti-ovarian Abs were measured in 2-fold serial dilution. The quantitative scored phenotypes representing oophoritis (1, 2, 17), atrophy (1, 2, 17, 18), and anti-ovarian Abs (1, 2, 7, 16–20) were based on pathological analysis of lesion severity and Ab visualization and titer. Ovarian inflammation (oophoritis) and loss of ovarian follicles (atrophy) were scored and analyzed independently. Each was scored blinded with severity score that ranged from 1 to 4, as described previously (10). Ab titer ranged from 1 to 8 representing the number of 2-fold serial dilution (1 = 1/50). No score (0) was given with absence of pathology (17, 21).

Genotypic analysis

Genomic DNA from 138 BC1 mice was isolated, and PCR with 245 microsatellite markers was used to obtain genotypic information covering the entire genome with no more than 20-cM intervals between markers (22–25). A linkage map was estimated from recombination fractions of the BC1 mice using the Kosambi map function and the MAPMAKER/EXP computer package (26–27).

QTL analysis

Composite interval mapping (CIM) analysis was performed using model 6 of the Zmapqtl module of QTL-Cartographer at 2-cM increments across the genome (http://stagene. ncus.edu/qcart/). Refs. (11–14). Background markers for CIM were chosen using the SRmapqtl module in QTL-Cartographer in a stepwise selection procedure and a p value of 0.10 for entry and maintenance of markers in the model. For ovarian atrophy and anti-ovarian Ab traits, 10 background markers were used, whereas for the oophoritis trait, 8 background markers were used in CIM. A 10-cM window was used to condition out QTL effects beyond the testing interval. Likelihood ratio tests (LRT) were calculated at each incremental analysis point to test the likelihood of a QTL at that position in the genome according to our linkage map. All centimorgan distances are according to the Mouse Genome Database (http://www.informatics.jax.org/) and relative to the centromere of the acrocentric mouse chromosome.

QTL linkage analysis using the A × B and B × A RIL was conducted for oophoritis using Map Manager QTX and the database for typed loci (http://www.nervenet.org/main/dictionary.html). Atrophy and anti-oocyte Ab titers were not scored in the RIL studies. The database was edited to remove errors, extinct or contaminated strains, questionable loci, loci with incomplete genotyping data, and loci with identical strain distribution patterns that interfere with interval mapping and permutation tests of significance. CIM was conducted at 1-cM increments and the single background marker unlinked to any of the chromosomes implicated in AOD systems by the most significant association was used to control for background QTL effects. A 1-cM window was used to condition out QTL effects beyond the testing interval. Analysis conducted with varying window sizes did not significantly alter the outcome.

Permutation-derived critical values

Significant linkage between QTL and marker loci was determined by permutation threshold methodology (12, 28). Experiment-wise analysis takes into account all markers on all chromosomes, whereas chromosome-wise analysis only uses the markers on a particular chromosome (29, 30). Significant (α = 0.05) and suggestive (α = 0.10) values were calculated for either experiment-wise or chromosome-wise analysis and were determined using the distribution of maximum LRT statistics from 1000 permutations.

Interaction, correlation, and least significant difference analysis

Interactions between putative QTL were investigated using a general linear model in SAS (PROC GLM) (SAS Institute, Cary, NC) and genetic markers linked to disease after D3Tx. No genetic map information was incorporated into this analysis. General linear models for each individual phenotype (anti-oocyte Ab, oophoritis, and atrophy) were constructed by incorporating maximally linked markers (QTL) as independent variables. A phenotypic model included the QTL specific for that AOD phenotype as well as additional significant independent variables. Additional independent variables were statistically selected in a stepwise manner in SAS from the AOD QTL not specific for the phenotype, markers representing Gasal (D4Mit16), Gasa2 (D4Mit14), and H2 (D17Mit50) as well as all possible interactions among Aod, Gasa, and H2 loci. Model assumptions were verified in each case.

DNA sequencing

Total RNA was isolated from ovary of A/J and C57BL/6J mice using TRIzol reagent (Life Technologies). An additional acid phenol (phenolchloroform 1:1, pH 4.3) extraction was performed before RNA precipitation to remove trace amounts of DNA contamination. transcription (RT) was performed using −2.0 μg of the total RNA, 40 pmol of oligo(dT) primer (5′-ATGAGTCCTACAGCGCTCTTTTTTTTTTTTTTT), and 200 U of Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) in a total volume of 30 μl. The RT product was diluted five times in H2O, and 2.0 μl of the diluted solution were used as a template for subsequent PCR with gene-specific primers. The amplified PCR products were TA cloned into pCR 2.1 TOPO-TA cloning vector (Invitrogen, San Diego, CA) following the manufacturers’ specifications. To facilitate TA cloning, the full-length cDNA for Mater was PCR amplified as three overlapping fragments, each −1.2 kb long.

For DNA sequencing, positive TA clones were PCR screened using the same primer pair as for the initial PCR amplification of the target gene. Plasmid DNA was prepared from the positive clones using QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). DNA sequencing reactions were performed using BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and T7 and M13 reverse vector primers. The reaction product was resolved on an ABI 377 automated DNA sequencer at the DNA sequencing core facilities of the University of Illinois. Six random clones were sequenced from both termini for each clone insert, and at least 18 random clones were sequenced to cover the full length of Mater cDNA for each mouse strain. Three to six random clones were sequenced from both fragment of interest. DNA sequencing data were assembled and analyzed using SeqEd 0.3 (Applied Biosystems) and Macvector 7.0 (Oxford Molecular, Princeton, NJ) programs. Each potential nucleotide sequence polymorphism was confirmed by comparison of chromatography from multiple clones, and additional independent clones were sequenced when necessary.

Results

Previous qualitative analysis linked Aod1 on chromosome 16 to susceptibility to AOD (any of the measured phenotypes of AOD) and susceptibility to the specific phenotypes of oophoritis and anti-ovarian autoantibody responsiveness. Aod2, on chromosome 3, was linked to ovarian atrophy, the most severe destruction of the ovaries associated with the loss of primordial oocytes (9, 10). Interestingly, Aod2 mapped to the same location as Idd3, a locus

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controlling insulin-dependent diabetes mellitus (IDDM). These results indicated the possibility that Aod2 plays a role in immunoregulation in general (10) and that Aod1 controls multiple phenotypes associated with AOD.

Incidence of AOD phenotypes in the experimental backcross population

D3Tx animals with complete thymectomy were examined for phenotypes of AOD at 60 days, an experimentally determined time point giving a progressive picture of AOD in B6AF1 mice (1, 16). After D3Tx, the pathological effects on ovaries of susceptible mice include inflammatory infiltrates of lymphocytes, macrophages, neutrophils, and plasma cells around and within ovarian follicles to a complete destruction of the ovaries (17). With progression of AOD, the ovaries become atrophic with destruction of the follicles that appear luteinized. In our population of 87 D3Tx B6AF1 $\times$ C57BL/6J mice with complete thymectomies as examined at sacrifice, 66% had oophoritis with an average pathology index (PI, an quantitative indication of the severity of each phenotype) of 1.3, 27% had ovarian atrophy with an average PI of 1.2, and 70% had Abs to ovarian tissues with an average PI of 2.8 (Table I). For comparison, other studies have shown an incidence of oophoritis in 87% of A/J mice, 8% of C57BL/6J mice (2), and 78% of B6AF1 D3Tx mice affected with AOD at 60 days (16), although quantifiable measures of severity were not done in these studies. Our results show that A/J mice have pathology indices for oophoritis, ovarian atrophy, and autoantibody titer of 2.8, 3.3, and 2.7, respectively, whereas C57BL/6J mice show pathology indices of 0.3 for oophoritis, 0.4 for atrophy, and 0 for anti-ovarian Ab response (Table I).

Severity of oophoritis linked to chromosomes 1, 16, and 17

Quantitative trait analysis was performed on the BC1 population to identify the predisposing QTL controlling the oophoritic response after D3Tx. Using CIM, we identified a locus on chromosome 1 at 63 cM and designated this QTL as Aod3 (Table II). At Aod3, the increased oophoritic response comes from the susceptible A/J allele and accounted for 14% of the experimental variance. No other loci were found that passed the experiment-wise permutation-derived threshold.

Similarly, CIM for oophoritis in the A $\times$ B and B $\times$ A RIL resulted in significant linkage to chromosome 1 (Table II), thereby confirming Aod3. Susceptibility to oophoritis was also associated with an increase in A/J alleles. The QTL accounted for 51% of the experimental variance in the RIL. Additionally, CIM revealed suggestive linkage to H2 on chromosome 17, verifying a previous report of oophoritis linkage to H2 (2, 7).

Because previous analysis had found linkage of susceptibility to AOD and oophoritis on chromosome 16 (Aod1), we examined only chromosome 16 using a chromosome-wise threshold (29, 30). Loci on chromosome 16 at 23 and 28 cM accounted for 5.1 and 5.2% of the experimental variance in oophoritis, respectively. When the window size in CIM was reduced to 5 cM to prevent the effects of one locus on the other, peak linkage was found at 23 cM; therefore, only one locus (Aod1) was declared on chromosome 16, verifying previous qualitative linkage to this chromosome.

Quantitative trait of atrophy linked to chromosomes 1, 2, and 3

A histological examination of the ovarian destruction showed that 21 of 57 BC1 animals with oophoritis progressed to ovarian atrophy and destruction of primordial follicles. Quantitative trait analysis using CIM indicated linkage of ovarian atrophy to 58 cM on chromosome 1 (Table II). As with oophoritis, mice carrying one allele at this locus from the A/J parent had more severe ovarian atrophy, and this QTL accounted for 16% of the phenotypic variance. Although oophoritis and atrophy were maximally linked to different markers on chromosome 1, a 4-cM interval (as measured by recombination fractions) containing these maximally linked markers and passing the 95% (significant) permutation threshold was shared for both traits in BC1 mice. Because this interval may contain more than one gene affecting AOD phenotypes and we are unable to separate these QTL given the present data, the results from this study link both oophoritis and atrophy to Aod5.

The ovarian atrophy trait was also linked to chromosomes 2 and 3. A QTL at 79 cM on chromosome 2 was linked to atrophy and designated Aod4 (Table II). Once again, the A/J allele at Aod4 was responsible for increased susceptibility to atrophy, and this locus accounted for 16% of the backcross population trait variance of ovarian atrophy. The qualitative linkage to Aod2 (10) was verified on chromosome 3 in our quantitative study. Although an interval of 15 cM of chromosome 3 was linked to atrophy (data not shown), maximal linkage was between 19 and 22 cM from the centromere in the region encoding Idd3 and I2. This locus accounted for 14% of the variance in our experimental model and, as with all other Aod loci, the A/J allele engendered increased ovarian atrophy.

Linkage of anti-ovarian Abs

A quantitative analysis of the ovarian Ab titer showed no significant (95% cutoff, 16.1) experiment-wise linkage to any locus tested in the genome. Mater, a gene encoding an ovarian autoantigen found after D3Tx, has been identified on chromosome 7 and is required for embryo survival beyond the two-cell stage (31, 32). Based on this information, chromosome-wise analysis on chromosome 7 found significant loci on the centromeric end at 1.1 and 6.6 cM (Table II). This may indicate two loci on the end of chromosome 7 affecting the ovarian Ab response. CIM with a 5-cM window found two loci as well. Because of the relatively small centromeric interval and few recombinants in the region, physical mapping techniques will be needed to further characterize the gene(s) at the locus we now identify as Aod5.

Segregation distortion

Linkage analysis of the anti-ovarian Ab response revealed no QTL on chromosome 16, contrary to previous reports (9). In fact, the highest LRT for anti-ovarian Ab linkage was 2.43, far below the $\alpha = 0.10$ critical value for our experiment-wise permutation. We therefore examined the genotypes on chromosome 16 and found segregation (transmission) distortion at 23 cM (maximal marker,

<table>
<thead>
<tr>
<th>Disease incidence of BC1, A/J, and C57BL/6J mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mice</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>BC1</td>
</tr>
<tr>
<td>A/J</td>
</tr>
<tr>
<td>C57BL/6J</td>
</tr>
</tbody>
</table>
D16 Mit89; \( p = 0.0001 \)) near Aod1. Segregation distortion was also found when animals with complete or incomplete thymectomies were tested together (maximal marker, D16 Mit89; \( p = 0.002 \)). In the original qualitative analysis of Aod1, a \( x^2 \) goodness of fit test against a 1:1 predicted ratio of affected animals carrying A/J and C57BL/6J alleles on chromosome 16 was used to predict linkage (9). Transmission distortion significantly altered the allele frequencies on chromosome 16 of the affected animals. Therefore, the lack of linkage of anti-ovarian Ab to chromosome 16 may be explained by segregation distortion.

Interaction analysis of loci involved in AOD

The phenotypes seen in the progression of ovarian destruction after D3Tx are highly correlated upon histological and statistical examination, even in the BC1 population (17, 21; data not shown). Because of the progression of disease, the close histological and statistical relationship of phenotypes, and the sharing of Aod3 by two phenotypes, we anticipated other Aod loci might influence multiple AOD phenotypes as well or other D3Tx-linked loci might influence AOD. Previous reports linked Gasal–2 and H2 to the development of gastritis and oophoritis, respectively (2, 7, 8). Additionally, the results of our QTL linkage analysis using the A \( \times \) B and B \( \times \) A RIL confirmed the linkage of oophoritis to Aod3 and H2. Because these loci were not detected in the initial genome scans for each phenotype, these loci may only affect an additional phenotype as they interact with another locus. This idea is consistent with the results obtained with the RIL. Thus, the complex nature of immunoregulation may be further understood by looking at interactions between loci that have been previously identified in D3Tx model diseases.

To test for interactions of additional loci contributing to each phenotype of AOD, we constructed a statistical linear regression model. Genotype information for Aod1–5, as well as the markers in our experiment closest to Gasal–2 and H2, were used as independent variables and as interaction variables (15). Linear regression analysis for the oophoritis phenotype showed that in addition to Aod1 and Aod3, interaction between Aod1 and Aod4, as well as interactions between Aod3 and Gasa2, Aod5, and H2 influenced this phenotype and together explained 35.8% of the oophoritis trait variance. For ovarian atrophy, atrophy, our regression analysis demonstrated that in addition to the previously identified Aod2–4, interactions between Aod2 and H2 as well as Aod1 and Aod4 were important for this phenotype and jointly accounted for 46.3% of the trait variance. Linear regression analysis showed that in addition to the two markers representing Aod5, interaction between the Aod2 and Aod4 loci were important for the anti-ovarian Ab phenotype and together accounted for 13.9% of the trait variance (Table III).

**Sequence of Mater**

Because Aod5, linked to the anti-ovarian Ab response after D3Tx, colocalized with Mater, an oplasmic autoantigen found after D3Tx, we sequenced Mater in both A/J and C57BL/6J mice. Four splice variants were seen for Mater in A/J mice with sequence lengths varying from 3297 to 3609 bp, and giving expected aa lengths of 1059–1163 (Table IV). In the C57BL/6J mouse at the Mater locus, two splice variants were observed with lengths of 3451 and 3297 bp and expected lengths of 1111 and 1059 aa. Within splice variant B, nucleotide changes resulted in differences of three amino acids between A/J and C57BL/6J mice (Table V). Different conserved coding units were also seen within the splice variants (Fig. 1). Although our sequencing results are based on multiple independent mRNA isolates, it is possible that the number of Mater splice variants reported here may not exhaust all that exist in both A/J and C57BL/6J mice. Nevertheless, the frequency of the isolates observed is consistent with strain-specific allelic variations in their expression.

**Discussion**

It is well established that the thymus is essential in the development of the T cell repertoire associated with the adaptive arm of the immune system. The close histological and statistical relationship of phenotypes, and the sharing of Aod3 by two phenotypes, we anticipated other Aod loci might influence multiple AOD phenotypes as well or other D3Tx-linked loci might influence AOD. Previous reports linked Gasal–2 and H2 to the development of gastritis and oophoritis, respectively (2, 7, 8). Additionally, the results of our QTL linkage analysis using the A \( \times \) B and B \( \times \) A RIL confirmed the linkage of oophoritis to Aod3 and H2. Because these loci were not detected in the initial genome scans for each phenotype, these loci may only affect an additional phenotype as they interact with another locus. This idea is consistent with the results obtained with the RIL. Thus, the complex nature of immunoregulation may be further understood by looking at interactions between loci that have been previously identified in D3Tx model diseases.

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**Table II.** **Loci linked to phenotypes of AOD by quantitative trait analysis**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Locus</th>
<th>Cross</th>
<th>Phenotype</th>
<th>Marker(s)</th>
<th>cM ( ^a )</th>
<th>LRT( ^b,c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aod3</td>
<td>BC1</td>
<td>Oophoritis</td>
<td>D1Mit417</td>
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<td></td>
<td></td>
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<td></td>
<td>D1Mit45</td>
<td>58</td>
<td>29.9</td>
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<td></td>
<td>D1Mit128</td>
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<tr>
<td>2</td>
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<td>BC1</td>
<td>Atrophy</td>
<td>D2Mit452</td>
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<td>26.3</td>
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<tr>
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<td>BC1</td>
<td>Atrophy</td>
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<td>22.3</td>
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<td>D7Mit77</td>
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<td>16</td>
<td>Aod1</td>
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<td>Oophoritis</td>
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<td>6.4( ^c )</td>
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<td></td>
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<td>D16Mit59</td>
<td>28</td>
<td>6.5</td>
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<td>17</td>
<td>H2</td>
<td>RIL</td>
<td>Oophoritis</td>
<td>D17Mit62</td>
<td>17</td>
<td>11.7</td>
</tr>
</tbody>
</table>

\( ^a \) Centimorgan distance according to Mouse Genome Database (http://www.informatics.jax.org/).

\( ^b \) For BC1 crosses, LRT statistic as given by model 6 of Zmapqtl (CIM) in QTL Cartographer at the maximally linked marker; 95% experiment-wise cutoff: oophoritis, 15.6; atrophy, 15.8; and anti-ovarian Ab, 16.1. For RIL crosses, LRT statistic as given by CIM in Map Manager QTX. Significant linkage = 20.4 and suggestive linkage = 10.8 for oophoritis.

\( ^c \) 85% chromosomewise cutoff, 6.5.

**Table III.** **Interaction of loci among AOD phenotypes**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Trait-Specific QTL( ^a )</th>
<th>Interacting Loci( ^b )</th>
<th>Variation</th>
<th>( F )</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oophoritis</td>
<td>Aod1</td>
<td>Aod3 and Gasa2</td>
<td>35.8</td>
<td>6.2</td>
<td>&lt;0.0001</td>
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<tr>
<td></td>
<td>Aod3</td>
<td>Aod3 and Aod5</td>
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<td>Aod3</td>
<td>Aod3 and H2</td>
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<td>Aod1</td>
<td>Aod4</td>
<td></td>
<td></td>
<td></td>
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<td>Atrophy</td>
<td>Aod2</td>
<td>Aod3 and Aod4</td>
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<td>9.3</td>
<td>&lt;0.0001</td>
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<tr>
<td></td>
<td>Aod3</td>
<td>Aod2 and H2</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Aod4</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Ab</td>
<td>Aod5</td>
<td>Aod2 and Aod4</td>
<td>13.9</td>
<td>3.6</td>
<td>0.0180</td>
</tr>
</tbody>
</table>

\( ^a \) Independent variables in linear regression model represented by loci found by quantitative trait analysis.

\( ^b \) Independent variables that represent significant interactions as found by stepwise selection.

\( ^c \) Variation, \( F \), and \( p \) are for entire model with all terms included in the linear regression model.
the immune system. However, in addition to its role in positive and negative selection, the thymus markedly influences the genesis and maintenance of peripheral T cells that have potent immunoregulatory properties (33, 34). Cellular studies using the D3Tx-induced and depletion-transfer models of autoimmunity have provided insight into the role of the thymus in establishing peripheral tolerance, but combining such studies with a genetic approach has the potential to provide additional information into these mechanisms. In this study, we report the identification of new QTL that control susceptibility to D3Tx-induced AOD and the associated component phenotypes of autoimmune oophoritis, ovarian atrophy, and anti-ovarian autoantibody responsiveness and provide evidence for the existence of interactions among QTL specific for a particular disease as well as across diseases.

Many murine models of autoimmune disease have been genetically analyzed, including IDDM, systemic lupus erythematosus, experimental allergic encephalomyelitis (EAE), and experimental allergic orchitis. In each of these experimental models, component or subphenotypes have been separately scored and analyzed for genetic linkage (25, 29, 35–38). Additional studies examining the colocalization of QTL controlling autoimmunity have shown that these loci tend to cluster together (39, 40). Thus, the loci controlling any given autoimmune disease may affect a single isolated phenotype or may have a pleiotropic effect on similarly induced diseases or on autoimmunity and immunoregulation in general.

By quantitatively analyzing the component phenotypes of AOD separately, we have shown that two QTL (Aod1 and Aod3) are important for the manifestation of oophoritis. Three loci (Aod2, Aod3, and Aod4) are important in governing the severity of ovarian atrophy, whereas Aod5 is important in anti-ovarian autoantibody responsiveness. Interestingly, Aod3 was linked to both oophoritis and atrophy, and this locus may play a role in the progression from oophoritis to atrophy.

Because D3Tx leads to the development of a number of organ-specific autoimmune disorders in adult mice, and the disease manifestations in different strains are organ specific with varying degrees of severity, it has been hypothesized that D3Tx-induced autoimmune phenomenon are under complex genetic control. Thus, it is likely that any given QTL controlling a D3Tx-induced response may play a role in more than one disease or component phenotype. Previous studies have identified H2, Aod1, and Aod2, as well as Gasa1 and Gasa2 as loci controlling D3Tx-induced oophoritis and gastritis, respectively. In this study, QTL linkage analysis of D3Tx induced AOD resulted in the identification of three additional loci: Aod3, Aod4, and Aod5 and confirmation of the linkage of oophoritis to H2. Additionally, interaction analysis using each phenotype has shown that a QTL specific for one AOD subphenotype may be important in other phenotypes of AOD. Furthermore, our results indicate that H2 interacts with Aod2 and Aod3 to increase susceptibility to oophoritis and atrophy, and that Aod3 interacts with Gasa2 suggesting that the latter is also important in controlling susceptibility to both D3Tx-induced autoimmune gastritis and oophoritis. Based on our interaction results, it is possible that Aod4, a QTL showing significant interaction in all three component phenotypes, may be important in the overall progression of AOD.

Because thymectomy between D2 and D4 postpartum leads to autoimmune disease whereas D7Tx does not, the day of thymectomy is critical in disrupting the establishment of peripheral tolerance. Thus, the genesis of CD4+CD25+ immunoregulatory T cells in the thymus as well as their subsequent survival and/or maintenance in the periphery may be affected by genetic factors. In this regard, our analysis demonstrating significant interaction between Gasa2 and Aod3 and the colocalization of Gasa2 with Idd9 suggests that Gasa2 may be important in a number of different organ-specific autoimmune diseases including IDDM in nonobese diabetic mice. CD4+CD25+ T cells have been shown to
play a significant immunoregulatory role in IDDM (41). Furthermore, candidate genes for Gasa2/Idd9.2 are Cd30 and Tnffr2, members of the TNFR superfamily, which are known to mediate cell death (42). It is possible that this QTL, or any other common QTL linked to a phenotype in which CD4+CD25+ T cells play an immunoregulatory role, may influence the development of such cells or their survival in the periphery. Under this scenario, resistance to D3Tx-induced autoimmune diseases would be due to greater numbers of CD4+CD25+ T cells being generated and released into the periphery as compared with susceptible strains. Similarly, a greater propensity for survival or terminal differentiation into suppressor effectors in the periphery would be consistent with resistance whereas the opposite would be associated with susceptibility to disease (3–7).

A more thorough analysis of the data underlying linkage to Aod1 revealed that this region of chromosome 16 exhibited significant segregation distortion that may have biased the previous qualitative results. The reason underlying this transmission distortion is curious in that it is the only region of the genome exhibiting this effect. As such, it may represent a unique region of the genome that plays an as of yet unappreciated role in neonatal thymectomy-induced inflammatory diseases in general rather than being a statistical artifact. Mice that undergo thymectomy at birth subsequently die from a rapidly progressive wasting disease that is characterized by autoimmune phenomenon and systemic, multiorgan inflammation (43, 44). This phenotype, like the organ-specific autoimmune diseases induced by D3Tx, can be reversed by adaptively transferring immunocompetent cells (45). Additionally, the incidence and severity of this wasting disease and death decline as a function of time postpartum. Therefore, the organ-specific autoimmune diseases induced by D3Tx may ontogenically reflect the organs that remain the most vulnerable to autoimmune phenomenon and wasting disease elicited by thymectomy from birth to day 3 of life. Under this scenario, a QTL such as Aod1, or a QTL linked to Aod1, controlling the differential ontogeny of resistance to wasting disease and death induced by neonatal thymectomy will be detected as a QTL that controls organ-specific autoimmune phenomenon in D3Tx mice. If this is in fact the case, then linkage would be observed only when the surviving affected progeny are used in the linkage analysis, as was the case for Aod1. In fact, a significant but limited number of D3Tx BC1 mice died well before they reached 60 days of age. These animals may be responsible for the transmission distortion associated with this region of chromosome 16.

The colocalization of Aod5 with Mater on the centromeric end of chromosome 7 suggests the importance of this ovarian-specific autoantigen, which was identified by its reactivity with anti-ovarian autoantibodies in the sera of D3Tx mice (31, 32), as a candidate gene. Our sequence analysis shows the possible importance of differential splicing and variant alleles in A/J and C57BL/6J mice. Mater also has multiple transcription start sites, and it is possible that alternative splicing of this protein may affect the induction and maintenance of tolerance in a strain-dependent manner. In this regard, differential splicing of the pancreatic autoantigen IA-2 in humans has been proposed as a possible mechanism underlying genetic susceptibility to IDDM. A unique splice variant of IA-2 was identified which lacks exon 13 (46). Several epitopes recognized by autoimmune responses in IDDM overlap with this region of the molecule (47) and differential expression of this splice variant may influence the induction and maintenance of tolerance to IA-2. Additionally, expression level differences of Aod5 and/or Mater may lead to the disparate autoantibody responses seen between resistant and susceptible strains of mice as has been suggested for the induction of tolerance to insulin (48). In fact, endogenous oocyte autoantigen is required for induction and progression of AOD following D3Tx (49).

Aod2 may be important in several autoimmune models in that it colocalizes with Id3, eae3, and I2 (10, 50, 51). A sequence polymorphism in the N-terminal region of the first exon has been identified between susceptible and resistant strains for all three diseases (50, 52). Differential glycosylation between resistant and susceptible strains of mice may account for the effect of IL-2 in IDDM, EAE, and D3Tx-induced autoimmunity in susceptible strains of mice (53). With respect to D3Tx-induced autoimmune disease, IL-2 has been shown to have a profound effect. The addition of IL-2 or anti-CD28 to cultured CD4+CD25+ T cells abrogates the immunosuppressive effects of these cells (54, 55).

QTL linkage analysis of the component phenotypes of D3Tx-induced AOD has shown the importance of genetics in susceptibility to disease, immunoregulation, and peripheral tolerance as a function of the neonatal thymus. These genetic factors may be specific to a particular phenotype and/or organ or may be part of a more generalized response to D3Tx. We have shown the importance of Gasa2 and H2 in our model and provided evidence that interactions between these loci play a role in susceptibility to AOD, supporting the concept that certain of these loci may play a more generalized role in immunoregulation. The characterization of these QTL at the molecular level will undoubtedly provide mechanistic insight into the role of the thymus in the genesis and maintenance of the immunoregulatory cells associated with peripheral tolerance.

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References


