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Aod1 Controlling Day 3 Thymectomy-Induced Autoimmune Ovarian Dysgenesis in Mice Encompasses Two Linked Quantitative Trait Loci with Opposing Allelic Effects on Disease Susceptibility

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Day 3 thymectomy (D3Tx) leads to a paucity of CD4+CD25+ suppressor T cells, a loss of peripheral tolerance, and the development of organ-specific autoimmune disease in adult mice. Importantly, D3Tx does not lead to autoimmune disease in all mouse strains, indicating that this process is genetically controlled. Previously, we reported linkage of D3Tx-induced autoimmune ovarian dysgenesis (AOD) and its intermediate phenotypes, antiovarian autoantibody responsiveness, oophoritis, and atrophy, to five quantitative trait loci (QTL), designated Aod1 through Aod5. We also showed interaction between these QTL and H2 as well as Gasa2, a QTL controlling susceptibility to D3Tx-induced autoimmune gastritis. To physically map Aod1, interval-specific bidirectional recombinant congenic strains of mice were generated and studied for susceptibility to D3Tx-induced AOD. Congenic mapping studies revealed that Aod1 controls susceptibility to oophoritis and comprises two linked QTL with opposing allelic effects. Aod1a resides between D16Mit211 (23.3 cM) and D16Mit51 (66.75 cM) on chromosome 16. Aod1b maps proximal of Aod1a between D16Mit89 (20.9 cM) and D16Mit211 (23.3 cM) and includes the candidate genes stefin A1, A2, and A3 (Sifa1-Sifa3), inhibitors of cathepsin S, a cysteine protease required for autoantigen presentation, and the development of autoimmune disease of the salivary and lacrimal glands following D3Tx. cDNA sequencing revealed the existence of structural polymorphisms for both Sifa1 and Sifa2. Given the roles of cathepsins in Ag processing and presentation, Sifa1 and Sifa2 alleles have the potential to control susceptibility to autoimmune disease at the level of both CD4+CD25+ suppressor and CD4+CD25− effector T cells. The Journal of Immunology, 2003, 170: 5886–5891.

Neonally thymectomized (NTx) adult animals, depending on the species and timing of Tx, exhibit T cell lymphopenia and are immunoincompetent (1). The severity of the induced phenotypes, however, exhibits a significant species disparity. For example, in humans and sheep the peripheral T cell pool is sufficiently large at birth so that NTx leads to only moderate T cell lymphopenia. In other species, particularly rodents, the thymus matures later in ontogeny, with the majority of the peripheral T cell pool being established postnatally (2). As a consequence, NTx in these species results in profound T cell lymphopenia. In mice and rabbits thymectomized at birth (DOTx) this lymphopenia is frequently associated with a wasting disease characterized by weight loss, lethargy, ruffled fur, hunched posture, periportal edema, skin lesions, diarrhea, and death (1). Histologically, multiorgan inflammation involving the kidney, liver, and heart as well as ulcerative colitis and anemia are seen (3–6).

The etiology of DOTx-induced wasting disease may be due in part to increased susceptibility to infection (1). Both DOTx mice and rabbits, however, exhibit autoimmune phenomenon, including Coombs’-positive erythrocytes, antinuclear Abs, and lupus-like symptoms, including kidney disease (3–10). Additionally, thymectomy on day 3 of life (D3Tx) leads to autoimmune disease of the prostate (11), testes and epididymis (12, 13), thyroid (14), stomach (13, 15), lacrimal and salivary glands (16), and ovarian dysgenesis (AOD) (13, 17–19) with little or no wasting disease. The uniquely distinguishing feature of this model is its dependency on the disruption of neonatal thymopoiesis. During neonatal development, a CD4+CD25− T cell repertoire, enriched in self-reactive T cells (20–22), is established concomitantly with peripheral seeding of autoantigen-specific CD4+CD25− T suppressor cells that control this population (23–25). D3Tx therefore appears to bias the neonatal T cell repertoire toward one that is enriched in CD4+CD25− T effector cells that expand unchecked due to a paucity of CD4+CD25+ suppressor T cells. Over time, this culminates in autoimmune disease.

Abbreviations used in this paper: Ntx, neonatal thymectomy; AG, autoimmune gastritis; AOA, antiovarian autoantibody; AOD, antiovarian ovarian dysgenesis; DOTx, thymectomized at birth; D3Tx, thymectomized at 3 days of age; IDDM, insulin-dependent diabetes mellitus; PI, pathology index; QTL, quantitative trait locus, Runx1, runt-related transcription factor-1; SLE, systemic lupus erythematosus.

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Neither D0Tx nor D3Tx leads to wasting disease and/or organ-specific autoimmune disease in all strains of mice, indicating a genetic influence on this process (1). For example, AKR/J mice develop significantly less wasting disease (15% dead at 150 days post-D0Tx) compared with C3H/He mice (87% dead at 150 days post-D0Tx). Additionally, adult D0Tx AKR/J mice exhibit greater anti-SRBC responses and responsiveness to PHA and Con A compared with C3H/He mice. D0Tx AKR/J mice are also capable of developing CTL responses to allogenic cells, whereas adult C3H/He mice are almost completely devoid of such activity. No quantitative differences are seen in any of these parameters between age-matched sham controls (26). These results establish the genetic control of D0Tx-induced wasting disease and that the rate of maturation of the peripheral T cell pool is different between these two H2–mismatched strains. Additionally, epistasis was observed in D0Tx-induced dysregulation in F1 hybrid mice. All D0Tx F1, A/J and C3H/He hybrids died by 90 days of age, whereas D0Tx A/J and C57BL/1 hybrids showed a 200-day survival of 60% (27, 28).

Different inbred strains of mice also exhibit variation in organ involvement and incidence and severity of disease following D3Tx (29). For example, 87% of D3Tx female A/J mice develop AOD, but only 6% develop autoimmune gastritis (AG), whereas 17% of female BALB/cJ mice show signs of AOD and 33% develop AG. Genetically, D3Tx-induced AOD (30–32) and AG (33, 34) have been the most well characterized. Recently, we established that AOD is controlled by multiple interacting quantitative trait loci (QTL) (Aod1–Aod5, H2, and Gasa2) (32). Interaction analyses in this study revealed QTL specific for AOD, and shared QTL involved in susceptibility to D3Tx-induced AOD and AG, and possibly insulin-dependent diabetes mellitus (IDDM) (32). The hypothesis that QTL could affect more than one autoimmune disease was first proposed by this laboratory (35). This shared QTL hypothesis was recently validated by identifying histamine H1 receptor as the autoimmune disease susceptibility locus, Bphs, which controls susceptibility to both experimental allergic encephalomyelitis and autoimmune orchitis (36).

To physically map Aod1, we generated a panel of interval specific bidirecional recombinant congenic lines encompassing the candidate genetic interval on chromosome 16. The congenic mapping studies reported herein demonstrate that Aod1 encompasses a minimum of two linked QTL (Aod1a and Aod1b) with opposing allelic effects on susceptibility to D3Tx-induced autoimmune oophoritis. Additionally, cDNA sequencing of Sfai1 and Sfai2, inhibitors of cytostane proteases required for autoantigen presentation and linked to Aod1b, identified structural polymorphisms as candidates for this QTL.

Materials and Methods

Animals

Female (C57BL/6J × A/J) F1 hybrid (B6/A) and female and male A/J and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or the National Cancer Institute (Frederick, MD). Congenic mice were bred at the College of Veterinary Medicine, University of Illinois-Urbana-Champaign (Urbana, IL) under specific pathogen-free conditions. After derivation, the congenic lines were sent to the State University of New York (Binghamton, NY) for D3Tx of the progeny. 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.

Generation of interval-specific bidirecional recombinant congenic lines for Aod1

B6A female mice were bred to C57BL/6J and A/J males to produce backcross 1 (BC1 or N2) mice. F2 mice were genotyped using tail snip DNA and PCR with previously identified microsatellite markers discriminating the C57BL/6J and A/J alleles (30–32). Animals that were heterozygous for all or a portion of chromosome 16 were selected for continued breeding. Beginning at N4 animals were also genotyped at loci unlinked to chromosome 16, and after N5 this background genome scan gave 20-cM interval coverage including markers no >10 cM from the end of each chromosome. Animals with minimal background contamination were selected after each backcross following N4 for subsequent breeding. At N7, chromosome 16 heterozygous mice (for each future congenic line) that did not have any background contamination according to our 20-cM interval background scans were brother-sister mated to fix a panel of homozygous B6.A and A.B6 recombinant congenic lines across the genetic candidate interval for Aod1. Tail samples taken from progeny mice were used to verify the congenic lines by PCR using microsatellite markers across the recombinant intervals.

Thymectomy

D3Tx of N7F2-F4 and congenic mice and A/J and C57BL/6J parental controls was conducted under ether anesthesia using a suction pipette technique (18). D3Tx females were killed at 60 days of age and were inspected for residual thymus. Animals with residual thymus were excluded from the study.

Analysis of AOD phenotypes

Ovaries were fixed in Bouin’s fixative and embedded in paraffin, and 5-μm sections were stained with H&E. Multiple-step sections were evaluated in a double-blind manner and scored for oophoritis and atrophy as previously described (30–32). Autoantibodies autoantibody (AOA) titers were measured in 2-fold serial dilutions (13, 37). The quantitative trait values representing oophoritis (0-3), atrophy (1-4), and AOA (1-8) were based upon pathological analysis of lesion severity and Ab visualization and titer. No score (0) was given with the absence of pathology or detectable Ab titer (13, 30–32, 37).

Statistical analysis

A one-way ANOVA was used to test for significant differences in quantitative trait values for AOA, oophoritis severity, and severity of atrophy among the B6.A and A.B6 lines and parental C57BL/6J and A/J mice. Post hoc comparisons (contrasts) were used to determine the significance of differences in quantitative trait values between individual congenic lines and parental strains. Logistic regression with a Wald χ2 test was used to test for significant qualitative differences between strains in incidence for each of the phenotypes.

Nucleic acid sequencing

Total RNA was isolated from the spleen of adult C57BL/6J and A/J mice using TRIzol reagent (Life Technologies, Gaithersburg, MD). RT-PCR was performed to obtain cDNA. First-strand cDNA was synthesized by RT of 1.0 μg total RNA primed with poly(dT)18 oligonucleotide and Superscript II reverse transcriptase (Life Technologies). cDNAs were PCR-amplified using Taq polymerase and specific primer pairs (Operon, Alameda, CA) flanking the mRNA coding regions of Sfai1 (forward, 5′-TCAGTGTTCCAGAGCAGCAAAGA-3′; reverse, 5′-ATTCATGCTGTAACCTTCCACT-3′), Sfai2 (forward, 5′-CAATGACTGAATCACCATAAATAAT-3′; reverse, 5′-TTTCAAATAGCTTCTTTATGGATG-3′), and Sfai3 (forward, 5′-CCTGGCCCATCAATGAGTCAAGAAAA-3′; reverse, 5′-AATGCTCTTCTTATTGAGCTGCAAT-3′). The amplified fragments were cloned and PCR-screened. Insert-positive plasmid DNAs were sequenced using vector primers and the ABI PRISM Dye Terminator Reading Reaction Cycle Sequencing Kit on a model 373A automated DNA sequencer (Perkin-Elmer, PE Applied Biosystems, Foster City, CA). PCR fragments were sequenced from both insert termini.

Results and Discussion

Quantitative differences between individual B6.A congenic (B6.A-D16Mit100-D16Mit515, B6.A-D16Mit28-D16Mit515, B6.A-D16Mit57-D16Mit515, B6.A-D16Mit211-D16Mit515, B6.A-D16Mit211-D16Mit515, and B6.A-D16Mit87-D16Mit4) and AOD-resistant C57BL/6J mice (30–32) were investigated using ANOVA. A significant difference among the strains was detected for oophoritis severity (F = 6.96, p < 0.0001, d.f. = 6). No other quantitative trait values were found to be significantly different. Post hoc comparisons (contrasts) were made to determine whether congenic lines differed in oophoritis severity from the parental line. When the B6.A congenic strains were treated as a single group and compared with the C57BL/6J parental line, a significant difference
again was found only for oophoritis ($F = 6.40, p = 0.012, \text{d.f.} = 1$). For individual comparisons between the six B6.A congenic lines and C57BL/6J mice, a Bonferroni-adjusted $\alpha$ of 0.008 (0.05/6) was used. The B6.A-D16Mit211-D16Mit51 line was significantly different from the C57BL/6J line for oophoritis severity ($F = 32.34, p \leq 0.0001, \text{d.f.} = 1$). No other B6.A congenic line was significantly different from the C57BL/6J parental line for oophoritis, atrophy, or AOA.

Similarly, quantitative comparisons between the B6.A congenic mice and the AOD-susceptible A/J parental line (30–32) were performed using ANOVA. A significant difference between strains for oophoritis severity was seen ($F = 11.27, p \leq 0.0001, \text{d.f.} = 6$). When A/J mice were contrasted with all the B6.A congenic lines treated as a group, A/J mice were found to be significantly different with respect to only oophoritis severity ($F = 6.40, p = 0.012, \text{d.f.} = 1$). The six post hoc comparisons between A/J and the individual B6.A congenic lines were again performed using a Bonferroni-corrected significance level ($p = 0.008$). B6.A-D16Mit100-D16Mit51, B6.A-D16Mit28-D16Mit51, B6.A-D16Mit211-D16Mit51, B6.A-D16Mit211-D16Mit4, and B6.A-D16Mit87-D16Mit4 mice were significantly different from A/J mice for oophoritis severity in their individual comparisons ($F = 19.7–42.2, p \leq 0.0001, \text{d.f.} = 1$), but B6.A-D16Mit211-D16Mit51 mice were not significantly different using the Bonferroni-adjusted $\alpha$ level ($F = 6.3, p = 0.013, \text{d.f.} = 1$).

A qualitative analysis testing incidence of oophoritis, ovarian atrophy, and the anti-ovarian Ab response using logistic regression provided similar results (Table 1). When the C57BL/6J mice were tested with all B6.A mice (treated as a single group), susceptibility to oophoritis was significantly different ($\chi^2 = 5.5, p = 0.019$). There was no difference in incidence for ovarian atrophy and AOA titers. Using a Bonferroni-adjusted $\alpha$ of 0.008, tests between individual B6.A congenic and C57BL/6J mice revealed a significant difference in incidence between only C57BL/6J and B6.A-D16Mit211-D16Mit51 mice. When A/J mice were compared with the B6.A congenic group, a significant difference was seen ($\chi^2 = 14.1, p = 0.0002$), and all strains except B6.A-D16Mit211-D16Mit51 were significantly different from A/J mice with individual comparisons using a Bonferroni-adjusted $\alpha$.

No significant differences for either qualitative or quantitative trait values were detected by ANOVA, logistic regression, or post hoc comparisons between A/J and A.B6-D16Mit32-D16Mit110 or A.B6-D16Mit100-D16Mit110 congenic lines.

Overall these results indicate that A/J alleles on chromosome 16 from D16Mit211 (23.3 cM) through D16Mit51 (66.75 cM) significantly influence the incidence and severity of autoimmune oophoritis, verifying the previously reported linkage of susceptibility to AOD to this region of chromosome 16 (30, 32).

The B6.A-D16Mit211-D16Mit5 congenic line, separated from the B6.A-D16Mit211-D16Mit51 line at N6, did not differ in autoimmune oophoritis susceptibility or severity after D3Tx compared with C57BL/6J mice. Therefore, a gene controlling the severity and susceptibility to oophoritis resides within the interval distal of D16Mit5 (38.0 cM) up through and including D16Mit51 at 66.75 cM. We designated this locus Aod1a (Fig. 1). Three B6.A interval-specific recombinant congenic lines, B6.A-D16Mit57-D16Mit51, B6.A-D16Mit28-D16Mit51, and B6.A-D16Mit100-D16Mit51, are also homozygous for A/J alleles at Aod1a, yet were not more susceptible to oophoritis than C57BL/6J mice. The most parsimonious explanation suggests that an additional QTL coming from the susceptible A/J strain renders these lines resistant to autoimmune oophoritis. The smallest interval in these three recombinant congenic lines with A/J alleles at Aod1a is cen tromeric of D16Mit51 (23.3 cM) and includes D16Mit110 (22.1 cM) and D16Mit57 (21.5 cM), but not D16Mit89 (20.9 cM). The region containing the A/J-derived resistance locus has been designated Aod1b (Fig. 1).

The results with the two A.B6 lines studied confirm that Aod1a is distal of D16Mit110, since the A.B6-D16Mit32-D16Mit110 and A.B6-D16Mit100-D16Mit110 lines are not significantly different from A/J for any of the three traits studied (Fig. 1). Additionally, A.B6-D16Mit32-D16Mit110 and A.B6-D16Mit100-D16Mit110 possess the susceptible C57BL/6J allele at Aod1b. Interestingly, there is a trend in both of these lines toward an increased incidence and severity of oophoritis compared with A/J. Additional animals will need to be studied to validate this trend. An extended analysis of the recombination break points defining Aod1b is presented in Fig. 2. The results indicate that Aod1b resides within the interval distal of D16Mit89 (20.9 cM) including D16Mit57 (21.5 cM) and proximal of D16Mit211 at 23.3 cM.

Our congenic mapping results indicate that Aod1a and Aod1b primarily influence autoimmune oophoritis, but not other intermediate AOD phenotypes. These results are consistent with our QTL linkage analysis, indicating that the component phenotypes of

![FIGURE 1. Aod1 interval-specific bidirectional recombinant congenic lines and average trait values for autoimmune oophoritis, atrophy, and AOA.](http://www.jimmunol.org/.../fig1.png)
AOD are under differential genetic control (30–32). Aod1 (chromosome 16) and Aod3 (chromosome 1) were shown to be important in autoimmune oophoritis, whereas Aod2 (chromosome 3), Aod3, and Aod4 (chromosome 2) are QTL important in controlling ovarian atrophy and Aod5 (chromosome 5) is primarily associated with the AOA response (32). Interestingly, Aod3 was linked to both oophoritis and atrophy and may therefore be essential for progression from oophoritis to severe atrophy.

Examining the backcross animals used in the QTL linkage analysis that carried the complete Aod1a and Aod1b alleles (32) further demonstrated the complex genetic control of the oophoritis phenotype. The majority of the D3Tx backcross animals with a single oophoritis susceptibility allele at Aod1a and one oophoritis resistance allele at Aod1b exhibited oophoritis (24 of 37 animals). This observation could be explained by the necessity for two A/J alleles at Aod1b to mediate resistance. However, a more likely interpretation is found by noting the influence of other oophoritis susceptibility loci. Twelve of the 24 affected animals heterozygous at both loci also carried the Aod3 susceptibility allele; this locus accounted for a much greater percentage of the variation in oophoritis susceptibility in the backcross population. A dominant effect of Aod3 over the Aod1b resistance allele may also account for the observation could be explained by the necessity for two A/J alleles at Aod1b to mediate resistance. However, a more likely interpretation is found by noting the influence of other oophoritis susceptibility loci. Twelve of the 24 affected animals heterozygous at both loci also carried the Aod3 susceptibility allele; this locus accounted for a much greater percentage of the variation in oophoritis susceptibility in the backcross population. A dominant effect of Aod3 over the Aod1b resistance allele may also account for the involvement of multiple loci, first detected with our interaction studies with the backcross population (32). The complex genetic control of oophoritis as well as the segregation distortion seen on chromosome 16 in the backcross population may have prevented the detection of two loci on chromosome 16 in our QTL linkage analysis. These two loci and their singular effects were only appreciated when congenic animals were made.

Similar to IDDM and systemic lupus erythematosus (SLE), AOD congenic mice have demonstrated that multiple QTL underlie the genetic contribution of what was originally attributed to be a single locus or gene. In IDDM, Idd9 has been subdivided into Idd9.1, Idd9.2, and Idd9.3 by congenic mapping (38) (http://www.taconic.com/emerging/nodmodels/chrom4a.pdf), while linkage on chromosome 3 has been resolved into four QTL, Idd10, Idd11, and Idd118 (39–41) (http://www.taconic.com/emerging/nodmodels/chrom3a.pdf). In SLE, Sle1 on the telomeric end of chromosome 1 has been resolved into three and possibly four QTL (Sle1, Sle1b, Sle1c, and Sle1d), each with slightly different effects on nephritis and loss of tolerance to chromatin (42). In SLE, susceptibility and resistance loci coming from the resistant NZW parental strain are both harbored in the susceptible NZM2410 strain (43, 44). Thus, the resolution of Aod1 into two linked QTL with opposing allelic effects coming from the AOD-susceptible A/J parental strain corroborates the genetic influence on autoimmunity seen in other disease models using congenic mapping.

Although somewhat speculative, it is worth noting that several candidate genes residing within the Aod1a and Aod1b intervals are of particular relevance to the developmental model of D3Tx-induced autoimmune disease. With respect to other autoimmune disease susceptibility within the Aod1a region, eae1 (experimental allergic encephalomyelitis-11) (45) and Pgia10 (proteoglycan-induced arthritis-10) (46) at ~41.0 and ~57.0 cM, respectively, are worth noting in light of the recent validation of the shared autoimmune disease susceptibility gene hypothesis (35, 36). Given the importance of cytokines and cytokine receptors in immunoregulation, two candidate genes of interest that reside within the Aod1a region, eae1 (IL-10 receptor α-chain) at 64.0 cM and IIL10rb (IL-10 receptor β) at 61 cM (http://www.informatics.jax.org/). The later candidate is of particular significance, since IL-10 has been implicated in the establishment and maintenance of CD4+CD25+ T regulatory cells. The predicted functions of IL-10 in SLE, as well as the potential for the gene to be a susceptibility locus in IDDM, are of interest for future studies.
To assess the candidacy of the stein A-type inhibitors linked to Aod1b, we sequenced the C57BL/6J and A/J alleles of Sfa1, Sfa2, and Sfa3. Both Sfa1 (Fig. 3) and Sfa2 (Fig. 4) exhibited multiple nonconserved, amino acid sequence polymorphisms between C57BL/6J and A/J mice. No sequence polymorphisms were detected for Sfa3 (data not shown). Interestingly, the A/J alleles for both Sfa1 and Sfa2 were different from the reference sequences and C57BL/6J. Thus, either the combined effects of the A/J alleles at both loci or a single amino acid difference within only one of the genes are potential candidate polymorphisms for Aod1b. To date, the polymorphic residues of Sfa1 and Sfa2 have not been modeled with respect to their functionality as inhibitors of cathepsin activity, including cathepsin S. If the polymorphisms in Sfa1 and/or Sfa2 underlie Aod1b, then we would predict that the A/J alleles would be more potent inhibitors of cathepsin activity than the C57BL/6J alleles, since disease resistance is associated with an A/J allele at Aod1b. Importantly, these polymorphisms have the potential of functioning at both the selection phase of CD4+CD25+ suppressor T cells during thymopoesis and their activation and maintenance within the periphery (23–25, 59, 60) and at the effector or inflammatory phase of the disease mediated by CD4+ T effector cells (59, 60).

Additional congenic mapping studies may be useful for reducing the support interval for Aod1b, but are essential for Aod1a, before a classical positional candidate gene cloning approach can be undertaken. Notwithstanding, combining such studies with cellular immunology studies using the D3Tx-induced and depletion-transfer models of autoimmunity has the potential to provide fundamental information about the mechanisms governing the genesis and maintenance of peripheral tolerance mediated by CD4+CD25+ suppressor cells.

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References


