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Two Autoimmune Diabetes Loci Influencing T Cell Apoptosis Control Susceptibility to Experimental Autoimmune Myocarditis

Mehmet L. Guler,* Davinna L. Ligons,* Yan Wang,* Michael Bianco,* Karl W. Broman,† and Noel R. Rose2*‡

The pathogenesis of immune-mediated myocarditis depends on genetic and environmental factors. To study the genetic mechanisms, we have developed a model of experimental autoimmune myocarditis in the A.SW mouse. Here we provide evidence that loci on murine chromosome 6, and possibly chromosome 1, are involved in regulating susceptibility. Moreover, these loci overlap with loci implicated in other autoimmune diseases including diabetes in the NOD mouse. These two loci also regulate apoptosis in thymocytes as well as peripheral T cells in the NOD mouse, and we report further that A.SW mice demonstrate the same characteristics in apoptosis. These results suggest that common pathogenetic mechanisms involving apoptosis of both thymic and peripheral T cells are shared by multiple autoimmune diseases.

Materials and Methods

Mice

The H-2 congenic mice A.SW and B10.S, as well as NOD mice were purchased from The Jackson Laboratory and were bred and maintained in the conventional housing facilities at The Johns Hopkins University (Baltimore, MD). F1 mice were generated through a male A.SW × female B10.S cross. F2 animals were generated through an F1 × F1 intercross.

EAM: induction and phenotype quantification

EAM was induced in male and female, 8- to 10-wk-old, A.SW, B10.S, F1, and F2 mice by two axillary s.c. injections of 200 and 250 μg of purified murine cardiac myosin in PBS emulsified (1:1 ratio) in CFA (Sigma-Aldrich), in a total volume of 100 μl on days 0 and 7, respectively. On day 0, mice also received an i.p. injection of 500 ng of pertussis toxin (Sigma-Aldrich), in a total volume of 100 μl on days 0 and 7, respectively. On day 0, mice also received an i.p. injection of 500 ng of pertussis toxin (Sigma-Aldrich) in 100 μl of PBS to increase adjuvanticity. The myosin was prepared from an equal mixture of A.SW and B10.S hearts and has been described in detail before (10, 15). CFA was additionally supplemented with 1 mg of H37Ra extract (Difco). After 21 days, hearts were isolated, bisected coronally, and fixed in 10% formaldehyde. The hearts were examined grossly for lesions and were bisected along any major lesions that were identified. Coronal sections allowed visualization of all four chambers of the heart and intraventricular septum. The hearts were then sent to Associated Tissue Technologies where they were embedded in paraffin, and five coronal sections (5 μm thick each) at different levels throughout the heart were sent as slides to H&E according to standard protocol. The presence of myocarditis was assessed by examining the hearts histologically using a light microscope fitted with a digital camera (Olympus, magnification 2.1). The degree of myocarditis was quantified by measuring the percent area of myocardium infiltrated by chronic inflammatory cells in the most affected section using image analysis software from Scion Corporation (www.scioncorp.com). Digital photos of entire heart sections were obtained using a 4× objective. The area of infiltrated myocardium was manually outlined using the image analysis software and compared with the outlined area of the entire heart. Most hearts were assessed at least two times on separate occasions to determine intrameasurement variability which was no more than 6%.

Genotype analysis

Genomic DNA was prepared from tail tissue as described previously (16). Genomic analysis was performed on all F2 mice through determination of the inheritance pattern of A.SW and B10.S alleles of SSLP markers distributed throughout the murine genome. Eighty-one SSLP markers at ~20 cM intervals throughout the murine genome, listed to be polymorphic between A.SW and B10.S (see Table I for a list of markers). PCR primers were generated for each SSLP and genomic DNA from each F2 individual was amplified in a PCR with the following conditions: 1 time at 95°C for 2 min; 38 times at 94°C for 45 s, 54°C for 45 s, and 72°C for 30 s; and 1 time at 72°C for 7 min. PCR products were analyzed by gel electrophoresis on 2.5% high resolution agarose (Metaphore Agarose; Biowhittaker) containing ethidium bromide.

Statistical analysis for genetic linkage

All statistical analyses were performed with R/qtl version 0.97-22 (17), an add-on package to the statistical software, R (18). Our initial analysis was based on genome-wide genotype data from 144 individuals with extreme phenotypes (percent myocarditis <4.5 or >26.5%). For this analysis, the phenotype was treated as dichotomous, and linkage analysis was performed using the method of Xu and Atchley (19). Statistical significance was
briefly, cells were incubated with 50 μl of TdT enzyme and FITC-conjugated dUTP for 1 h at 37°C. Finally, cells were washed three times with PBS, and analyzed by flow cytometry on a FACScan (BD Biosciences). No prior drug treatment, and exclusion of TdT from the TUNEL reaction were included as negative and specificity controls, respectively.

**Statistical analysis of phenotypes**

All means and SDs were derived with standard methods, and p values for two-tailed unpaired comparisons between two phenotypes (e.g., percentage of myocarditis in A.SW vs B10.S, or percentage of apoptosis in A.SW thymocytes vs B10.S thymocytes, etc.) were calculated using a Student t test.

**Results**

**Susceptibility to EAM is a quantitative heritable trait**

We had previously shown that A and C57BL/6 mouse strains differ in their susceptibility to CB3 virus-induced and cardiac myosin-induced autoimmune myocarditis (10, 13). We initially observed that this trait was influenced by the H-2 locus but that non-H-2 background genes play a dominant role (24, 25). To focus on non-H-2 loci, we fixed the H-2 locus and compared susceptibility to cardiac myosin-induced EAM in the H-2^s congenic strains A.SW and B10.S.

After two injections, a week apart, with murine cardiac myosin in CFA, together with a single administration of pertussis toxin during the first immunization, A.SW mice developed a dense myocardial infiltrate composed mostly of mononuclear cells and a few scattered neutrophils. B10.S mice, in contrast, developed minimal inflammation. Quantitation of this trait through measurement of percent area of myocardium involved by inflammation revealed highly significant differences in susceptibility (Fig. 1A). In A.SW
determined by a permutation test, using 1000 permutation replicates (20). The analysis was also performed considering the female and male F1 mice separately.

Analysis of the quantitative phenotype for all individuals was performed using the multiple imputation approach of Sen and Churchill (21). The null distribution of the logarithm of the odds (LOD) score calculated by the multiple imputation approach was seen to vary according to proportion of genome-scan-adjusted p values, though still making proper adjustment for the genome-wide scan. Let P^c denote the chromosome-specific p value for chromosome c, estimated via a permutation test; that is, P^c is the proportion of the permutations for which the maximum LOD score on chromosome c was greater than the maximum LOD score on that chromosome in the observed data. We obtain a genome-scan-adjusted p value as P^c = 1 - (1 - P^c)^1/Lc where Lc is the genetic length of the chromosome. As considerably greater precision is required for the chromosome-specific p values due to this adjustment, 100,000 permutation replicates were used.

**Dexamethasone (Dxm)-induced thymocyte apoptosis**

The method was adapted from Bergman et al. (22). Male and female 6- to 8-wk-old A.SW, B10.S, and NOD mice were each injected i.p. with 200 μg of Dxm (Sigma-Aldrich) dissolved in 300 μl of PBS. Untreated mice served as controls. Mice were sacrificed 16 h later, and inguinal, axial cervical, and mesenteric lymph nodes were collected. Single-cell suspensions were prepared by gently dispersing the lymph nodes over 25-μm nylon-mesh filters. Apoptotic cells were then quantitated using the TUNEL reaction detailed below.

**Cyclophosphamide (Cy)-induced peripheral lymphocyte apoptosis**

The method was adapted from Colucci et al. (23). Male and female 6- to 8-wk-old A.SW and B10.S mice were each injected i.p. with 7.5 mg of Cy (Sigma-Aldrich) dissolved in 300 μl of H2O. Untreated mice served as controls. Twelve hours later, mice were sacrificed and thymuses were collected. Single cell thymocyte suspensions were prepared by gently dispersing thymuses through 25-μm nylon-mesh filters. The cells were then washed once with PBS and permeabilized in 200 μl of 4% paraformaldehyde in PBS for 30 min at room temperature with gentle agitation. The cells were then washed once with PBS and permeabilized in 200 μl of 0.1% saponin (Sigma-Aldrich) in PBS on ice for 3 min. After washing 2× with PBS, apoptotic cells were determined using the TUNEL reaction. We used the FITC In Situ Cell Death Detection kit from Roche Diagnostics and followed the manufacturer’s protocols.

**Table I. (Continued)**

<table>
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<tr>
<th>Marker</th>
<th>Position (cM)</th>
<th>A.SW (bp)</th>
<th>B10.S (bp)</th>
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mice, on average, 26.5 ± 9.2% of the myocardium was infiltrated by inflammation, whereas in B10.S mice, only 4.5 ± 7.5% of the myocardium was affected (p = 1.6 × 10^{-5}) after induction of EAM. Comparison of male and female mice among the two strains showed no sex-based differences (Fig. 1A).

To determine whether susceptibility to EAM was a dominant trait, we tested several cohorts of F1 (A.SW × B10.S) mice and found that susceptibility was intermediate and demonstrated substantial variability with 11.0 ± 13.4% of the myocardium infiltrated by chronic inflammatory cells after induction of EAM (Fig. 1B). This phenotype is significantly different from the phenotypes of both parents (p = 6.8 × 10^{-6} and 0.005 for comparisons of F1 with A.SW and B10.S parents, respectively). The variation in the genetically homogenous parents, as well as in the F1 strain, suggests substantial stochastic or environmental influence on susceptibility to EAM.

We next performed F1 × F1 intercrosses to generate several cohorts of F2 offspring and tested them by induction of EAM to identify loci that control susceptibility to disease by a standard Mendelian approach. F2 offspring (n = 296) displayed a wide range of susceptibilities, but were heavily skewed toward the resistant phenotype (Fig. 1B).

**Genetic crosses and linkage analysis reveal loci on Chr. 1 and 6 to be important in determining susceptibility to EAM.**

Because environmental factors seem to influence susceptibility to EAM, as displayed by the wide variation of parental A.SW, B10.S and especially F1 mice, we decided to first analyze the extremes in phenotypes among F2 animals for any linkage to chromosomal loci. F2 offspring (n = 144) displaying susceptibility greater than or equal to the mean A.SW phenotype (percentage of myocarditis, >26.5) and less than or equal to the mean B10.S phenotype (percentage of myocarditis, <4.5) were selected for genomic analysis through determination of the inheritance pattern of A.SW and B10.S alleles of 81 SSLP markers distributed throughout the murine genome. The phenotype was treated as a binary trait—resistant or susceptible. LOD scores achieved at each of the 81 loci representing the entire murine genome are shown for each chromosome, ordered from centromere (left) to telomere (right). Linkage to loci on Chr. 1 (LOD = 3.26; p = 0.08) and Chr. 6 (LOD = 3.26; p = 0.06) nearly reached the level of statistical significance (Fig. 2A). Loci on Chr. 4, 11, and 15 had LOD scores >2, but gave genome-wide p values of >0.25. Closer inspection of the strongest linkage, distal Chr. 6, revealed that there was a significant sex-related difference in inheritance of susceptibility. The locus on Chr. 6 appeared to affect only males. The LOD score for Chr. 6, for the analysis of the males only, was 4.99, with a genome-wide p value of 0.002.

![FIGURE 2. Genome-wide scan of F2 individuals with extreme phenotypes reveals two possible loci on Chrs. 1 and 6 controlling susceptibility to EAM. A, F2 offspring (n = 144) displaying susceptibility greater than or equal to the mean A.SW phenotype (percentage of myocarditis, >26.5) and less than or equal to the mean B10.S phenotype (percentage of myocarditis, <4.5) were selected for genomic analysis through determination of the inheritance pattern of A.SW and B10.S alleles of 81 SSLP markers distributed throughout the murine genome. The phenotype was treated as a binary trait—resistant or susceptible. LOD scores achieved at each of the 81 loci representing the entire murine genome are shown for each chromosome, ordered from centromere (left) to telomere (right). Linkage to loci on Chr. 1 (LOD = 3.26; p = 0.08) and Chr. 6 (LOD = 3.26; p = 0.06) were nearly statistically significant. Loci on Chr. 4, 11, and 15 had LOD scores >2, but gave genome-wide p values of >0.25. B, Closer inspection of the most significant linkage, distal Chr. 6, revealed that there was a significant gender difference in inheritance of susceptibility. LOD scores are displayed for each of the loci and determined separately for male and female mice. The locus on Chr. 6 appeared to have effect in only the males. Recalculation of the LOD score for Chr. 6, for the analysis of the males only, was 4.99, with a genome-wide p value of 0.002.](http://www.jimmunol.org/)

Next, we wanted to determine whether inclusion of all 296 F2 individuals in a QTL would provide additional evidence for the loci identified in the binary-trait analysis of 144 F2 individuals with extreme phenotypes (presented above). All individuals were genotyped for loci on Chrs. 1, 4, and 6 with the addition of more SSLP markers. In the analysis of the QTL in all 296 F2 mice, evidence for linkage was strongest for Chr. 6. The analysis of all mice gave a LOD score of 3.73 for Chr. 6 (genome-wide-adjusted p = 0.01; Fig. 3). The signal again came entirely from the male mice, with analysis in the male mice alone giving a LOD score of 5.70 (genome-wide-adjusted p < 0.001; Fig. 3B). The 1.5 LOD support interval covered ~9 cM. By this method, significant linkage was not observed for Chrs. 1 and 4. Controlling for the effect of the Chr. 6 locus did not reveal additional loci or affect the evidence for loci on Chrs. 1 and 4. No evidence for an interaction (epistasis) was observed.

The A.SW allele at the Chr. 6 locus had the effect of increasing myocarditis, and was seen to be recessive to the B10.S allele. Only male individuals bearing the homozygous A.SW (AA) genotype at distal Chr. 6 exhibited susceptibility to EAM (Fig. 3C). Despite the significant influence of the A.SW Chr. 6 locus on disease susceptibility in males, 29% of male F2 mice inheriting homozygous A.SW Chr. 6 alleles (AA) still exhibited intermediate susceptibility or resistance to autoimmune myocarditis similar to parental B10.S mice, suggesting that a combination of other loci (such as Chr. 1) and environmental factors influence susceptibility.

**Thymocytes from A.SW mice demonstrate diminished susceptibility to Dxm-induced apoptosis.**

The NOD Idd6 locus, which affects diabetes susceptibility, overlaps the A.SW EAM susceptibility locus on Chr. 6 identified here (22, 27, 28). In the NOD mouse, this locus has also been independently shown to modulate thymocyte susceptibility to Dxm-induced apoptosis, implicating defects in apoptotic pathways of autoreactive T cell precursors in the pathogenesis of diabetes (22, 29). After 12 h of Dxm treatment, female NOD thymocytes were shown to have a diminished degree of apoptosis compared with nondiabetic C57BL/6 controls (22, 29). We sought to determine whether A.SW thymocytes also demonstrate a similar phenotype because this locus is shared by two different strains demonstrating susceptibility to two different autoimmune diseases. In the original studies of NOD mice, information about male mice was not given.
Peripheral T cells from A.SW mice demonstrate diminished susceptibility to Cy-induced apoptosis

A second immunologic characteristic identified in NOD mice is the relative insensitivity of mature peripheral T lymphocytes to Cy-induced apoptosis compared with disease free C57BL/6 control mice (23). This trait, although similar to the thymic apoptosis trait described above, is associated with a different diabetes-susceptibility locus, Idd5, situated in the proximal portion of murine Chr. 1. This locus on Chr. 1 overlaps with the autoimmune myocarditis susceptibility locus identified here. Again, as reported in NOD mice, A.SW lymphocytes demonstrated diminished sensitivity to Cy-induced apoptosis as determined by TUNEL as described above. Lymphocytes from B10.S mice showed enhanced sensitivity to Cy compared with B10.S lymphocytes: 45.5 ± 16.3% of B10.S lymphocytes whereas 22.9 ± 13.1% of A.SW lymphocytes were TUNEL positive after Cy treatment (p = 1.23 × 10^-5) (Fig. 5). No sex-based differences were identified (data not shown).

Discussion

Susceptibility to EAM varies among different strains of mice and is influenced by both MHC and non-MHC genes (24, 25). However, unlike most models of autoimmune disease, non-MHC genes seem to have the greatest influence in EAM. For example, most A background mice such as A/J, A.SW, and A.CA, differing only at the MHC locus, develop severe myocarditis upon immunization with cardiac myosin, while most B strains of mice, such as C57BL/6 and C57BL/10J, are resistant to the induction of myocarditis. Therefore, it is an ideal system for the study of non-MHC genes.
genetic influences in autoimmune diseases. Thus MHC-congenic (H-2<sup>k</sup>) strains were compared to identify non-MHC loci which influence disease.

In this study, we have identified a recessive locus on distal Chr. 6 to be strongly associated with susceptibility to EAM (LOD score of 5.70, \( p < 0.001 \)), and will henceforth refer to this locus as Eam2. Uniquely, Eam2 seems to be a susceptibility factor only in male mice, suggesting that the alleles in this locus require interaction with sex-specific factors to influence susceptibility to autoimmune myocarditis. Female mice inheriting the Chr. 6 susceptibility locus do not acquire disease. This is an unexpected finding especially when considering that the relative susceptibility to EAM among male and female A.SW mice and male and female B10.S mice is identical. This finding strongly suggests that additional genetic loci, such as Chr. 1 explained below, which must also operate in females, are involved in influencing susceptibility. Thus when all genetic loci are integrated, there is no overall sex-based difference.

In addition to Chr. 6, we have also identified a locus on Chr. 1 which is likely to influence susceptibility. Marked stochastic and environmental effects on disease were made evident by a wide difference. When all genetic loci are integrated, there is no overall sex-based difference.

Both the Chr. 1 and Chr. 6 loci (Eam2) identified here as influencing autoimmune myocarditis, have been previously identified in other autoimmune diseases. The NOD model of diabetes and murine experimental autoimmune orchitis both share proximal Chr. 1 as a susceptibility locus (28). The human counterpart of the proximal murine Chr. 1 locus has also been implicated in diabetes and autoimmune thyroid disease. The murine diabetes locus Idd6 on distal Chr. 6 overlaps with Eam2. This strongly suggests that there are common mechanisms leading to autoimmune diseases, with other genetic and environmental influences determining tissue specificity of disease. Indeed, a recent survey of published linkage studies on autoimmune or immune-mediated diseases revealed several overlapping syntenic human and mouse chromosomal regions with a cluster of disease susceptibility loci (28). These studies provide support for the concept that autoimmune disorders in different species are controlled by a common set of susceptibility genes. Therefore, understanding the mechanisms that lead to the disease in animals may provide fresh insight into their human disease counterparts.

One of the next phases in autoimmunity research, stemming from mapping studies, will be the molecular identification of susceptibility alleles. Located in the proximal Chr. 1 locus identified here, CTLA-4 is an immunologically important regulatory molecule that has been implicated in several autoimmune diseases like autoimmune thyroid disease and in the murine model of type-1 diabetes, NOD (30, 31). Recently, it has been shown that polymorphisms within CTLA-4 genomic sequences influencing alternative splicing of CTLA-4 is most likely cause of linkage to this locus in human autoimmune thyroid disease and murine diabetes (32). It is currently not known how changes in the relative expression of these splice forms influences autoimmune disease, but it is thought that these particular alterations in the expression of CTLA-4 isoforms can diminish the total inhibitory signal that is delivered to activated self-reactive T cells, thus increasing the likelihood of autoimmunity. Consistent with this hypothesis, we have found that treatment with mAb to CTLA-4 intensifies EAM in moderately susceptible BALB/c mice and even renders resistant C57BL/6 mice susceptible (D. Cihakova and N. Rose, unpublished data).

Due to the overlapping autoimmune susceptibility loci in A.SW and NOD mice, we were interested to determine whether EAM-susceptible A.SW and diabetes-susceptible NOD mice shared phenotypic characteristics that may render both strains susceptible to autoimmune disorders. In addition to developing spontaneous diabetes, NOD mice display a multitude of immunologic peculiarities. For example, immature T cells in the thymus of female NOD mice are relatively insensitive to induction of apoptosis by the stressor, Dxm, compared with disease-free control mice (22, 33). Decreased potential for apoptosis in NOD thymocytes could potentially lead to retention of autoreactive T cells and susceptibility to an autoimmune disease like diabetes. Interestingly, this trait, differential sensitivity to Dxm-induced apoptosis, was independently mapped to the distal portion of murine Chr. 6—the same locus that already harbors a diabetes susceptibility locus, Idd6, and now, Eam2, the locus identified here as influencing susceptibility to autoimmune myocarditis (29). Due to the colocalization of autoimmune susceptibility and apoptosis-sensitivity in NOD mice, we asked whether A.SW mice, which are susceptible to EAM, also demonstrate diminished susceptibility to Dxm-induced thymocyte apoptosis. Indeed, like in NOD mice, A.SW thymocytes demonstrated diminished sensitivity to apoptosis compared with the autoimmune myocarditis resistant strain B10.S.

Recent NOD congenic lines have confirmed the role of Idd6 on Chr. 6 as a player in susceptibility to diabetes in the NOD mouse (22). Interestingly, it was discovered that this locus had a greater influence in male mice compared with females, paralleling our finding of the exclusive role of Eam2 in male mice. These two independent observations suggest that this autoimmune locus interacts with sex-specific factors in influencing susceptibility to autoimmune disease. Furthermore, this locus imparts differential thymocyte apoptosis in a sex-specific manner, where only female mice demonstrate phenotypic differences among the strains analyzed. Further investigation is needed to understand the discrepancy in the sex-based effects of this locus where differential susceptibility to autoimmune disease is apparent in males whereas differential susceptibility to Dxm-induced thymocyte apoptosis is only in females. It is not known whether the polymorphic genes responsible for differential susceptibility to autoimmune disease (diabetes in NOD, and EAM in A.SW) and differential sensitivity to apoptosis are identical, or just tightly linked. Discrepancy in the sex-bias between these two phenotypes could suggest that they are controlled by tightly linked but separate genes. Accordingly, this specific chromosomal location may be influenced in a gender-specific manner, imparting the effect on a multitude of genes in the region. Alternatively, the mere presence of a significant sex-based influence on both genetically linked phenotypes, autoimmunity
and apoptosis, could also suggest that there is a single gene controlling both phenotypes. According to this hypothesis, this gene would manifest opposite gender-bias depending on the phenotype analyzed. Finer mapping which may reveal two separate loci, or finally, identification of responsible polymorphisms controlling the two phenotypes will ultimately resolve this discrepancy in sex-bias.

A second immunologic feature of NOD mice is the relative insensitivity of mature peripheral T lymphocytes to Cy-induced apoptosis compared with disease free control mice. This trait, although similar to the thymic apoptosis trait described above, has been independently mapped in the NOD mouse, to the proximal portion of murine Chr. 1. This is the same area on Chr. 1 that is shared by the diabetes susceptibility locus, Idd5, and the autoimmune myocarditis susceptibility locus identified here with highly suggestive linkage. Due to this association, we asked whether A.SW mice demonstrated a phenotype similar to NOD mice. Again, like in NOD mice, A.SW lymphocytes demonstrated diminished sensitivity to Cy-induced apoptosis. Although the establishment of proximal Chr. 1 as a bona fide EAM susceptibility locus will require additional work, the highly suggestive linkage achieved with this study combined with the observation that A.SW and NOD mice share a similar apoptosis phenotype which has been firmly linked to this locus in NOD mice strengthens the evidence that Chr. 1 is an important player in susceptibility to autoimmune myocarditis.

In summary, NOD mice that spontaneously develop diabetes and A.SW mice which are susceptible to EAM not only share two susceptibility loci, but also demonstrate two functional abnormalities associated with apoptosis of T cells. These two loci affect apoptosis at different stages of T cell development: Chr. 6 influences immature thymocyte apoptosis and Chr. 1 affects apoptosis in mature peripheral T cells. Further work is required to establish that the genetic elements which control sensitivity to drug-induced apoptosis at either of these loci are the same genetic elements that control susceptibility to autoimmune disease. Finally, it will be important to determine how polymorphisms at these loci influence apoptosis and whether they control susceptibility to different autoimmune diseases.

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