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Multigenic Control of Disease Severity after Virulent *Mycobacterium tuberculosis* Infection in Mice

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Following challenge with virulent *Mycobacterium tuberculosis*, mice of the I/St inbred strain exhibit shorter survival time, more rapid body weight loss, higher mycobacterial loads in organs, and more severe lung histopathology than mice of the A/Sn strain. We previously performed a genome-wide scan for quantitative trait loci (QTLs) that control the severity of *M. tuberculosis*-triggered disease in [(A/Sn × I/St) F1 × I/St] backcross-1 (BC1) mice and described several QTLs that are significantly or suggestively linked to body weight loss. In the present study we expanded our analysis by including the survival time phenotype and by genotyping 406 (A/Sn × I/St) F2 mice for the previously identified chromosomal regions of interest. The previously identified 12-cM-wide QTL on distal mouse chromosome 3 was designated *tbs1* (tuberculosis severity 1); the location of the QTL on proximal chromosome 9 was narrowed to a 9-cM interval, and this QTL was designated *tbs2*. Allelic variants of the *tbs2* locus appeared to be involved in control of both body weight loss and survival time. Also, the data strongly suggested that a QTL located in the vicinity of the *H-2* complex on chromosome 17 is involved in control of tuberculosis in mice of both genders, whereas the *tbs1* locus seemed to have an effect on postinfection body weight loss in female mice. Interestingly, these loci appeared to interact with each other, which suggests that there might be a basic genetic network for the control of intracellular parasites. Overall, linkage data reported here for F2 mice are in agreement with, and add to, our previous findings concerning the control of *M. tuberculosis*-triggered disease in the BC1 segregation.

Identification of genes and their alleles that confer resistance and/or susceptibility to tuberculosis (TB) provides deep insight into basic mechanisms of immunity and pathology. Variations in *NRAMP1* (5) and/or *NRAMP1*-linked loci on human chromosome 2q35 (8), *VDR* (3, 27), and class II *HLA* genes (6, 7) were shown to be linked to or associated with susceptibility to TB in humans. However, linkage and association results vary between studies, which may be due to the genetic control being polygenic and ethnicity dependent and to the absence of clearly delineated phenotypes (4, 24).

Mouse models of TB have proved to be valuable for studies of antimycobacterial immunity and of the genetic control of susceptibility and resistance (16). For example, the *Nramp1* gene, which has provided great insight in our understanding of macrophage-mycobacterium relationships, was first discovered in a mouse model of susceptibility to *Mycobacterium bovis* BCG (9, 28). Numerous inbred mouse strains have been tested to determine their survival times after challenge with virulent *Mycobacterium tuberculosis* (1, 17, 20). Among these strains, I/StSnEgYCit (I/St) mice display the shortest survival time, while A/SnYCit (A/Sn) mice survive significantly longer. In addition, I/St mice display more severe and rapid disease progression than A/Sn mice in terms of body weight loss, mycobacterial loads in lungs and spleens, and lung histopathology (21). We previously reported the results of backcross-1 (BC1) ([A/Sn × I/St] F1 × I/St) analysis of the body weight loss in this strain combination after intravenous challenge with virulent *M. tuberculosis* H37Rv (15). In BC1 female mice, variations in this clinically relevant and easily measurable phenotype were linked to distal chromosome 3 (*D3Mit215* logarithm of the likelihood ratio [LOD] = 3.9) and proximal chromosome 9 (*D9Mit89* LOD = 6.8) and were suggestively linked to chromosome 8 (*D8Mit289* LOD = 3.0) and chromosome 17 (*D17Mit175* LOD = 3.0). In males, there was suggestive linkage to chromosome 5 (*D5Mit233* LOD = 3.0) and chromosome 10 (*D10Mit133* LOD = 2.3) (15). In the present study we more precisely defined the position of the quantitative trait locus (QTL) on chromosome 9 by using BC1 and performed an analysis of the linkage of the chromosomal regions listed above to body weight loss and to survival time postchallenge by using F2 segregation.

MATERIALS AND METHODS

Mycobacteria. *M. tuberculosis* H37Rv Pasteur (a kind gift from Gilles Marchale, Pasteur Institute, Paris, France) was grown and stored exactly as previously described (15, 21). Briefly, after two consecutive 1-week passages in Dubos broth (Difco, Detroit, Mich.) supplemented with 0.5% bovine serum albumin (Sigma, St. Louis, Mo.), mycobacteria were centrifuged for 20 min at 3,000 × *g* at 4°C, resuspended in saline containing 0.05% Tween 20 and 0.1% bovine serum albumin, and stored at −80°C. The concentration of bacteria was measured by plating serial dilutions of a suspension onto Dubos oleic agar (Difco), followed by 3 days of incubation and counting of the microcolonies with an inverted microscope. At the time of infection, bacteria were thawed and diluted, and bacterial aggregates were allowed to sediment for 1.5 h before the upper phase was used for challenge.

Mice and infection. Inbred mice belonging to strains I/St and A/Sn, as well as (A/Sn × I/St) F1 and (A/Sn × I/St) and (I/St × A/Sn) F2 hybrid mice and BC1

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mice ([A/Sn × I/St] F1 × I/St), were kept under conventional conditions in the animal facilities of the Central Institute for Tuberculosis, Moscow, Russia, in accordance with guidelines of the Russian Ministry of Health no. 755. F2 and BC1 mice that were 2 to 3 months old were infected intravenously via the lateral tail vein with 5×10^5 CFU of *M. tuberculosis* H37Rv. Survival time was monitored daily, and body weight was monitored weekly. The BC1 mice were the same animals used for a previous study in which the general location of several QTLs was detected (15).

Genotyping. Genotypes of the simple sequence length polymorphisms *D3Mit29*, *D3Mit215*, *D3Mit199*, *D5Mit182*, *D5Mit232*, *D5Mit233*, *D5Mit312*, *D5Mit366*, *D6Mit287*, *D6Mit129*, *D8Mit289*, *D8Mit291*, *D10Mit130*, *D10Mit133*, *D9Mit204*, *D9Mit89*, *D9Mit23*, *D9Mit94*, *D9Mit142*, *D9Mit207*, *D9Mit47*, *D9Mit269*, *D9Mit113*, *D17Mit101*, *D17Mit175*, *D17Mit28*, *D17Mit93*, and *DXMit170* (MapPairs; Research Genetics, Huntsville, Ala.) were determined by using PCR performed with isolated spleen DNA, followed by product separation on 6% polyacrylamide gels and detection by autoradiography. Female and male F2 mice were genotyped for 19 and 17 of the markers, respectively.

Statistical analysis. The markers were assigned to and mapped within the chromosomes by multipoint linkage analysis by using Mapmaker/Exp, version 3.0 (14). Analysis of multipoint linkage between genotype markers and the quantitative phenotypes square-root-transformed survival time and relative body weight loss ([body weight at zero time − body weight on day 20 postinfection]/body weight at zero time) for identification of QTLs was performed by using interval mapping and subsequent MQM mapping within the MapQTL software package (26). To compensate for gender effects when the sample containing both genders was analyzed, the male-female difference in the phenotype mean was subtracted from the male phenotype data. Interval mapping based on single QTL models was used for initial detection of putative QTLs. MQM mapping analyzes approximate multiple QTL models by assigning a marker near the QTL to a cofactor. This cofactor takes over the effect of the QTL on other loci and enhances the sensitivity for detection and exclusion of additional QTLs and artifactual QTLs, respectively. Cofactors were assigned to putative QTLs with LOD scores of >1.0 as detected by interval mapping. MQM mapping with forward selection of cofactors was performed; cofactors were added or dropped sequentially by using the threshold LOD score of 1.0 in each test. A free model of inheritance of the I/St allele was studied. The final cofactors were tested for representing real QTLs (rather than artifactual QTLs) by moving each cofactor along its linkage group to see whether the QTL remained at the original locus. Genome-wide significance for LOD scores was determined by permutation tests (10,000 runs of simulated data). The critical LOD values suggested by the tests were for suggestive linkage 1.9 (one QTL expected at random in a genome scan) and for significant linkage 2.8 (one QTL expected at random once every 20th genome scan; i.e., genome-wide P value of ~0.05) (13). Threshold correction for testing the hypothesis of linkage (i) to two modestly correlated phenotypes and (ii) within marker genotype subgroups gave LOD values of ≥3.0 (genome-wide P value of ~0.02) that were regarded as significant.

Distribution normality and variance equality were tested by using the normality test (test of skewness and kurtosis) and Bartlett's test, respectively. Relative body weight loss was normally distributed. However, survival time was not normally distributed, and therefore it was square root transformed in order to normalize its distribution. Statistical significance for inbred strains was determined by using the two-tailed t test. Correlations were calculated by using Pearson's correlation coefficient. The statistical significance of a difference in phenotype effect of loci genotypes was determined by using multivariate three-way analysis of variance (MANOVA) including the correlated outcome variables relative body weight loss and survival time, the predictor variable gender, and two of the factors genotype at *D9Mit89*, *D3Mit215*, *D17Mit175*, and *DXMit170* and followed by a two-way analysis of variance. For MANOVA, a P value of ≤0.01 was considered significant. These statistical tests were performed with STATA, version 6 (Stata Corporation, College Station, Tex.), and StatView, version 5 (SAS, Cary, N.C.).

RESULTS AND DISCUSSION

I/St mice lose weight and die much earlier after infection than A/Sn mice ($P < 0.0001$ for either gender). In addition, I/St females survive significantly shorter times than I/St males ($P < 0.05$) (15, 21). The QTL on distal chromosome 3, which previously was linked to body weight at day 20 postinfection in BC1 (15), was designated *tbs1* (tuberculosis severity 1) (Fig. 1D). This QTL maps to a 12-cM 90% confidence interval that encompasses *D3Mit215*. The interval was defined as the map locations corresponding to a 1-U decrease in the maximum LOD score observed.

The QTL on chromosome 9, previously identified in the same linkage analysis (15), was better defined in this study by using the BC1 mice and flanking markers on both sides. The 90% confidence interval was 9 cM long and spanned the marker *D9Mit89*. This QTL was designated *tbs2* (Fig. 1A). In the initial study it was observed that in relation to body weight loss after infection, there was support for linkage with a maximum LOD score of 6.7 at *D9Mit89* (in females), a valley at *D9Mit94*, and a secondary peak beyond this marker (LOD = 4.8). In the present study we genotyped *D9Mit23* and *D9Mit142*, which map between *D9Mit89* and *D9Mit94* and beyond *D9Mit94*, respectively, and the secondary peak was not detected, indicating that only one QTL was present around *D9Mit89*.

The linkage analysis was expanded by genotyping 406 (A/Sn × I/St) F2 infected mice (176 females and 230 males) for microsatellites that were previously significantly or suggestively linked to body weight loss in the BC1 mice (15). The survival times of the F2 mice ranged from 20 to 85 days for the males and from 18 to 71 days for the females (Fig. 2), whereas the relative body weight losses at day 20 postinfection ranged from −22.1 to 32.0% for the males and from −13.9 to 30.6% for the females. The MANOVA supported gender differences for both traits ($P = 0.013$). The values for both traits in individual F2 mice were distributed between the values for I/St and F1 hybrid mice. For relative body weight loss a few F2 mice had values slightly greater than those of the F1 mice. This supports the hypothesis that there is multigenic control of these traits, as suggested previously in the analysis of BC1 mice. The survival time correlated well with the relative body weight loss (for males, $r = 0.57$ [95% confidence interval, 0.48 to 0.65; $P < 0.0001$]; for females, $r = 0.62$ [95% confidence interval, 0.51 to 0.70; $P < 0.0001$]). The results of the linkage analysis for F2 mice are shown in Fig. 1B, C, and E. The intermarker distances observed were similar to those reported for the BC1 mice (15) and in public maps (Release 16 May 1999 of Whitehead Institute/MIT Center for Genome Research [http://www-genome.wi.mit.edu/cgi-bin/mouse/index, 2001 Chromosome Committee Reports, Mouse Genome Database, The Jackson Laboratory [http://www.informatics.jax.org/ccr/]). Because of the gender-specific linkages in the BC1 mice, we analyzed males and females separately.

As shown in Fig. 1B, relative body weight loss was suggestively linked to *tbs2* in males (LOD = 2.6; genome-wide $P = 0.085$) and in females (LOD = 1.9; genome-wide $P = 0.13$). Gender-adjusted data for males and females combined was also suggestively linked with relative body weight loss (LOD = 2.6; genome-wide $P = 0.044$).

Other peak LOD scores related to relative body weight loss and survival time were observed in males and in females at the chromosome 17 markers *D17Mit28* and *D17Mit175*, respectively (Fig. 1C). *D17Mit28* and *D17Mit175* are neighboring markers in the *H-2* complex region (intermarker distance, 6.6 cM), and their corresponding peaks, although shifted, might reflect linkage with the same putative QTL. Numerous recombination hot spots in the vicinity of and within the *H-2* complex on chromosome 17 (2, 29) are known to provide different

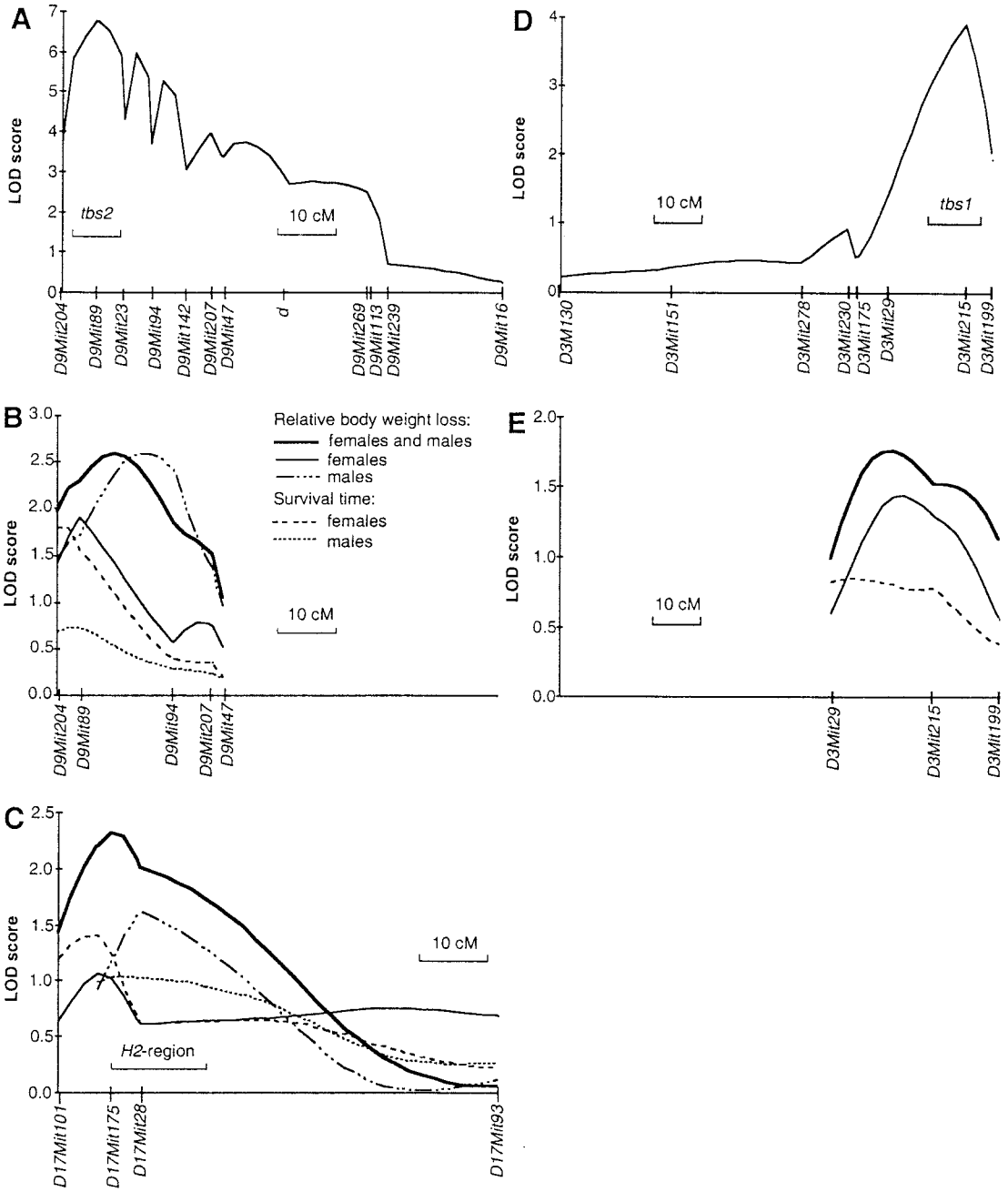

FIG. 1. QTL linkage analysis of TB severity following infection of mice with *M. tuberculosis* H37Rv: LOD score plots for gender-adjusted postinfection relative body weight loss and survival time from analyses of BC1 (A and D) and F2 (B, C, and E) animals. The LOD score peaks between *D9Mit23* and *D9M142* in panel A were not designated since they were not supported by a marker.

crossover frequencies in males and females, and this might explain the gender-related linkage differences. Importantly however, LOD score peaks for both traits and for both genders are present at this highly polymorphic chromosomal region. Gender-adjusted data for relative body weight loss indicated that there was suggestive linkage to *D17Mit175* (LOD = 2.3; genome-wide $P = 0.062$). This result was supported by the MANOVA ($P = 0.00093$). Suggestive linkage was also indicated with regard to the *tbs1* locus (LOD = 1.8; genome-wide $P = 0.15$) (Fig. 1E), which was supported by the MANOVA ($P = 0.016$). No linkage for relative body weight loss or survival time was found to the markers genotyped on chromosomes 5, 6, 8, and 10 (LOD scores, <1.0).

The means ± standard errors for the phenotypes corresponding to each genotype category at the *tbs2*, chromosome 17, and *tbs1* loci for the F2 mice of both genders are presented in Table 1. When the *tbs2* genotype was considered, heterozygous female mice (genotype *i*/*a*) had the lowest body weight loss and survived longer than females bearing the homozygous allelic combination *a*/*a* or *i*/*i*, thus following the pattern of F1

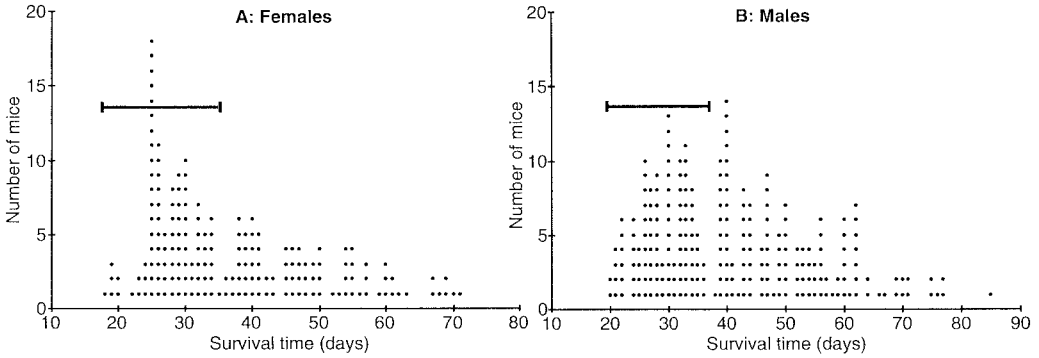

FIG. 2. Segregation of mortality in (A/Sn × I/St) F2 mice following *M. tuberculosis* H37Rv challenge. Animals that died early after challenge formed a rather homogeneous group (indicated by bars). Defining these animals as susceptible and the rest of the animals as resistant gave the following segregation results: 95 susceptible and 81 resistant female mice and 104 susceptible and 126 resistant male mice.

hybrids (15). In turn, *i*/*a* heterozygous males displayed an intermediate level of TB severity. Differences in control of disease progression between females and males could be due to sex hormones. This hypothesis is supported by reports of adrenal hyperplasia followed by adrenal atrophy and cytokine-mediated changes in the hypothalamic-pituitary-adrenal axis after *M. tuberculosis* H37Rv challenge (10) and of estrogen-induced differences in the development of pulmonary *Mycobacterium avium* infections in mice (25). F2 mice having the $tbs2^{a/a}$ homozygous genotype were characterized by the greatest relative body weight loss and the shortest survival time irrespective of gender (Table 1). The course of TB is less severe in A/Sn ($tbs2^{a/a}$) mice than in I/St mice ($tbs2^{i/i}$). However, $tbs2^{a/a}$ F2 mice control TB worse than their $tbs2^{i/i}$ counterparts (Table 1), suggesting that the effect of *tbs2* in parental strain A/Sn might be masked by epistasis. One likely participant in such nonallelic interactions is the chromosome 17 locus that confers better protection to the carriers of the *a*/*a* genotype (Table 1). In females, the *tbs1* locus behaves like *tbs2*, providing a heterozygous advantage. The *tbs1* locus also potentially participates in epistatic interactions.

To further assess gender differences in severity of TB in this model, the marker *DXMit170* was genotyped in all animals. This marker maps close to *lmr3*, a QTL that contributes to susceptibility to *Leishmania* in a BALB/c × C57BL/6 model (23). When data for the two genders combined and gender-specific data were examined, there was no indication of linkage between this marker and survival time or relative body weight loss after infection. However, it was observed that including only animals with genotype *a*/*a* at *DXMit170* resulted in an LOD score of 2.8 (genome-wide $P = 0.019$) at the chromosome 17 locus and an LOD score of 3.3 (genome-wide $P = 0.0080$) at *tbs2* for relative body weight loss. Figure 3 shows the distribution of gender-adjusted relative body weight loss according to genotype combinations between the *DXMit170*-chromosome 17 (*D17Mit175*) and *DXMit170-tbs2* loci. The graphs indicate that relative body weight loss is affected more by genotype at *D17Mit175* and *tbs2* if the genotype at *DXMit170* is *a*/*a*.

Due to an apparent influence of *DXMit170* on TB severity in this model, a separate linkage analysis of the 352 animals derived from F1 mice whose parents were A/Sn females and I/St males was performed. This analysis yielded LOD scores of 2.2 (genome-wide $P = 0.092$) for *tbs2*, 2.0 (genome-wide $P = 0.11$) for the chromosome 17 locus, and 1.3 (genome-wide $P = 0.60$) for *tbs1* for relative body weight loss. Roles for the chromosome 17 locus and the *tbs1* loci in TB severity in this mouse model were supported ($P = 0.0033$) and suggested ($P = 0.017$), respectively, by MANOVA.

Even though modeling of interactions between loci is difficult, it was possible to gauge the relationship between the QTLs reported here by examining the distribution of the phenotypes according to combinations of genotypes at different loci. It is clear from the data (Table 1) that the chromosome 17 locus is genotypically related to resistance and susceptibility in the same direction, as the parental strains I/St and A/Sn, while the *tbs1* and *tbs2* loci display heterozygous advantage, as the F1 hybrids, with the fairly common situation that when the F2 mice carry the genotype of one parent (here *a*/*a*), they behave like the other parent (here *i*/*i*) after infection. MANOVA of

TABLE 1. Phenotypes of F2 mice according to genotype

Gender	Locus	Relative body weight loss (%)			Survival time (days)		
		i/*i*	*i*/*a*	*a*/*a*	*i*/*i*	*i*/*a*	*a*/*a*
Females	*tbs1-D3Mit215*	8.68 ± 1.55 (59)[a]	4.49 ± 0.933 (82)	9.57 ± 1.61 (37)	32.9 ± 2.37	30.4 ± 1.34	29.2 ± 2.25
	tbs2-D9Mit89	7.27 ± 1.44 (52)	4.78 ± 1.04 (82)	10.2 ± 1.68 (40)	37.0 ± 1.89	41.5 ± 1.78	35.5 ± 2.01
	D17Mit175	10.8 ± 1.60 (49)	8.01 ± 1.04 (82)	6.61 ± 1.47 (39)	32.7 ± 1.54	35.2 ± 1.90	39.9 ± 2.13
Males	*tbs2-D9Mit94*	−2.29 ± 1.17 (63)	1.14 ± 1.04 (107)	5.11 ± 1.44 (50)	37.0 ± 2.21	34.2 ± 1.35	32.6 ± 1.73
	D17Mit28	3.99 ± 1.71 (51)	1.25 ± 0.933 (108)	−1.24 ± 1.29 (65)	38.5 ± 1.68	41.6 ± 1.38	44.1 ± 2.15

[a] Mean ± standard error. The numbers in parentheses are numbers of mice.

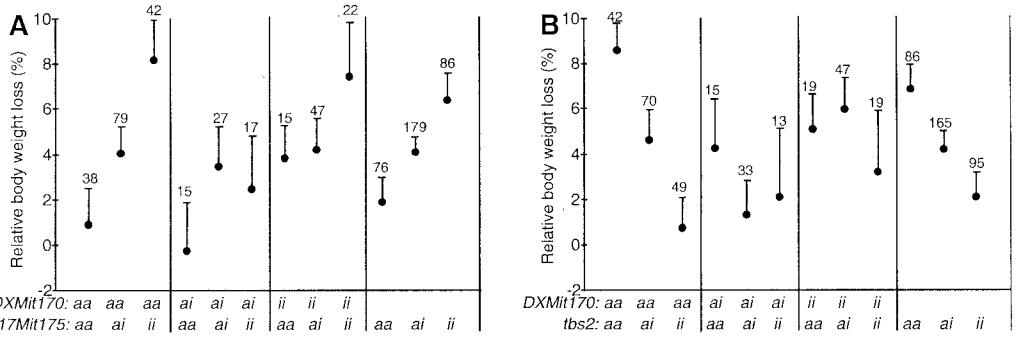

FIG. 3. Effect of the chromosome X locus on the distribution of TB severity according to genotype at the chromosome 17 locus (A) and the *tbs2* locus (B). The means and standard errors for gender-adjusted postinfection relative body weight loss in (A/Sn × I/St) F2 mice are shown. The numbers above the symbols are the numbers of mice.

the (A/Sn × I/St) F2 mice showed support for the gender-*tbs2*-*tbs1* interaction ($P = 0.0080$) and a trend for the gender-chromosome 17 locus-*tbs1* interaction ($P = 0.022$). Figure 4 shows the distribution of gender-adjusted relative body weight loss according to genotype combinations between the *tbs1*-chromosome 17 (*D17Mit175*) and *tbs2*-*tbs1* loci. The most interesting finding is that genotype *a*/*a* at *tbs1* enhances the effect of the *D17Mit175* genotype. Linkage analysis with data only for the mice having the $tbs1^{a/a}$ genotype increased the peak LOD score at *D17Mit175* from 2.0 to 4.3 (genome-wide $P < 0.00005$) for relative body weight loss. Similarly, including only animals heterozygous at *tbs2* resulted in an LOD score of 2.2 (genome-wide $P = 0.047$) at *tbs1*.

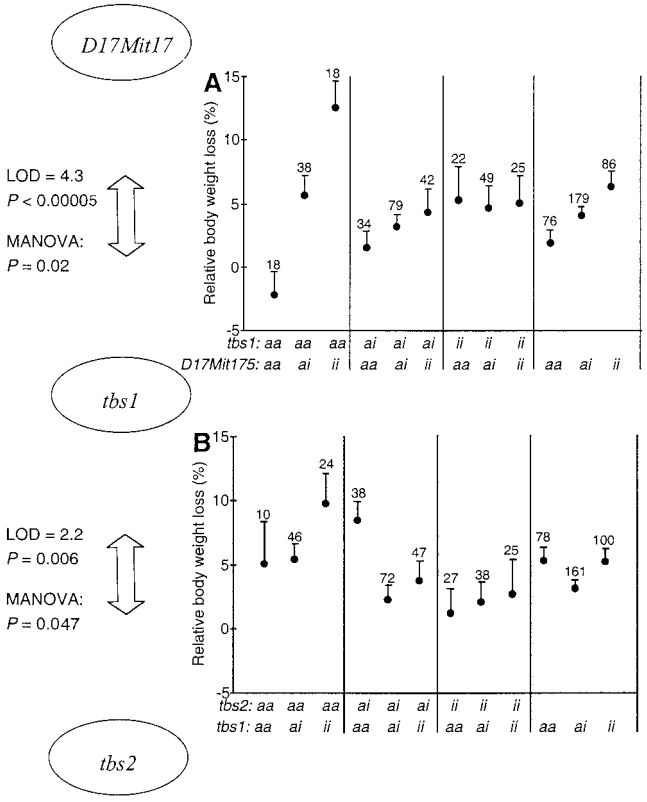

FIG. 4. Interaction among the *tbs1*, chromosome 17, and *tbs2* loci in control of TB severity in (A/Sn × I/St) F2 mice. The TB severity measurements used to determine the LOD scores and graphs were gender adjusted postinfection relative to body weight loss, and the TB severity measurements used for the MANOVA were adjusted postinfection relative to body weight loss and survival time. The means and standard errors for mice grouped on the basis of their genotypes at the three loci are shown. The numbers above the symbols are the numbers of mice. (A) LOD score for *D17Mit175*, including mice that were $tbs1^{a/a}$. MANOVA was performed for the gender-chromosome 17 locus-*tbs1* interaction. (B) LOD score for *D3Mit215*, including mice that were $tbs2^{i/a}$. MANOVA was performed for the gender-*tbs2*-*tbs1* interaction.

Overall, the linkage data reported here for F2 mice are in agreement with our previous findings for BC1 segregation, in that the *tbs2*, chromosome 17, and *tbs1* loci seem to be involved in TB control in the A/Sn-I/St strain combination. However, in BC1 mice linkage to these loci was found only in females, whereas in F2 mice the LOD score peaks were present for both genders. In addition, the F2 analysis resulted in LOD scores that were not significant, unlike the scores for the *tbs2* and *tbs1* loci in the BC1 segregation, although more mice were involved in the present study. The differences between the two trials may be explained by a difference in the modulation of the anti-TB responses in the F2 and BC1 generations. Epistatic effects are not necessarily identical in F2 and BC1 mice (which contain 50 and 25% A/Sn genetic material, respectively); in addition, F2 mice have one of three genotypes (*a*/*a*, *a*/*i*, and *i*/*i*) at each locus, whereas BC1 mice have one of two genotypes (*a*/*i* and *i*/*i*). Epistatic effects in BC1 mice were not examined because of the limited number of mice per group after stratification by genotype at several loci.

The genetic control of TB is generally assumed to be a complex phenomenon (reviewed in references 11 and 19), for which there is strong evidence of involvement of several QTLs. Kramnik et al. (12) mapped the QTL *sst1* (susceptibility to tuberculosis 1) gene to a 9-cM interval on mouse chromosome 1 clearly distal to the *Nramp1* gene by using the C3H-C57BL/6 strain combination. *sst1* controls several parameters of *M. tuberculosis* Erdman-triggered disease. The *sst1* locus can be considered a particularly important TB susceptibility locus, since it simultaneously influences survival time, bacterial loads in lungs and spleens, and the degree of lung pathology following TB challenge. Nevertheless, the authors clearly showed that TB control in their model is also multigenic and depends upon the presence of other, unknown loci outside the *sst1* region. Using a similar strain combination, DBA/2 and C57BL/6, Mitsos et al. identified three QTLs that control sur-

vival time after challenge with *M. tuberculosis* H37Rv, *Trl-1*, *Trl-2*, and *Trl-3* (tuberculosis resistance locus), at distal chromosome 1, proximal chromosome 3, and proximal chromosome 7, respectively (18). *Trl-1* and *sst1* are close to each other but not close enough to suggest that they reflect the same gene.

So far, different QTL analyses of TB have not revealed overlap in the genomic locations of the QTLs found. However, the *tbs2* and chromosome 17 loci overlap the two murine *Leishmania major* resistance loci, *lmr2* and *lmr1*, respectively, identified in the BALB/c-C57BL/6 strain combination (22). In addition, *lmr2* and *lmr1* were found to interact in the *L. major* model, like the *tbs2* and chromosome 17 loci in our model (23). This suggests the possibility that these two models might be influenced by similar genes and thus strengthens our suggestion that the *tbs2* and chromosome 17 loci reflect genes involved in TB control. Even though suggesting candidate genes for such QTLs might be premature, it is appealing to explore the human syntenic regions for the *tbs2*, chromosome 17, and *tbs1* loci for association with human TB. Additionally, the results support the derivation of congenic lines of mice that carry particular genotypic combinations that might improve the chances of isolating the candidate genes.

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