Severity of Tuberculosis in Mice is Linked to Distal Chromosome 3 and Proximal Chromosome 9

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Genetic factors play a role in host response to infection with *Mycobacterium tuberculosis*, the number one infectious killer worldwide. Mice of the inbred strains I/St and A/Sn show significant differences in disease severity after intravenous infection of a lethal dose of the virulent human isolate *M. tuberculosis* H37Rv. Following challenge with H37Rv, only I/St mice have rapid body weight loss and short survival times. A genome wide analysis for linkage with body weight after *M. tuberculosis* H37Rv infection was done in (A/Sn × I/St)F1 × I/St mice. Among females, quantitative trait loci (QTLs) on chromosomes 9 and 3 were significantly linked to postinfection body weight (logarithm of the odds ratio [LOD] scores of 6.68 and 3.92, respectively). Suggestive linkages were found for QTLs on chromosomes 8 and 17 (LOD scores of 3.01 and 2.95, respectively). For males, QTLs on chromosomes 5 and 10 showed suggestive linkages (LOD scores of 3.03 and 2.31, respectively). These linkages can be used to identify candidate regions for tuberculosis susceptibility loci in the human genome.

The incidence of tuberculosis (TB) is increasing worldwide, and multidrug-resistant strains of the tubercle bacterium are spreading [1, 2]. Although the mechanisms of host defense against this infection remain poorly understood, there is substantial evidence for the role of genetic factors in host susceptibility to TB. Studies have reported various degrees of susceptibility to TB among different ethnic groups [3], familial occurrence of TB [4], and an increased concordance of TB in monozygotic compared with dizygotic twins [5]. Some of the major limitations to identifying human TB susceptibility genes have been the oligogenic control of susceptibility [6], the absence of clearly delineated clinical phenotypes useful for stratified genetic analysis, and the lack of strong candidate TB susceptibility genes. Most human TB susceptibility genes identified to date were first identified in mouse models of infection with mycobacteria other than *Mycobacterium tuberculosis*. These include the *NRAMP1* gene (natural resistance-associated macrophage protein 1), which has been identified as a candidate susceptibility gene for TB in West Africa [7] and for leprosy in Vietnam [8], and the class II *HLA* genes [9]. However, the overall effect of *NRAMP1* and the *HLA* genes is acknowledged to be moderate, and other genes are certain to play a role in susceptibility to human TB.

In the present study, we applied a novel approach to localize candidate genomic regions that control the severity of disease triggered by the virulent human isolate *M. tuberculosis* H37Rv. To facilitate the mapping of loci controlling the severity, we used a mouse model and MQM (multiple quantitative trait loci [QTL] models) mapping, a multilocus method for QTL analysis, and performed a genome wide scan to reveal loci that influence disease severity. For this QTL analysis, we used mice of the inbred strains A/SnYCit (A/Sn) and I/StSnEgYCit (I/St), which are polar extremes with regard to survival time [10–12], mycobacterial load, lung histopathology, and weight loss [13] after intravenous injection with a lethal dose of *M. tuberculosis* H37Rv. In particular, the phenotype of severe loss of body weight, or wasting, is a common and well-described clinical symptom in TB patients [14]. Hence, we chose to investigate loss of body weight in *M. tuberculosis* H37Rv–infected I/St and A/Sn mice as a clinically relevant and easily measurable phenotype of *M. tuberculosis* H37Rv–triggered disease. We here present chromosomal loci linked to postinfection body weight loss in this mouse model.

**Materials and Methods**

*Mycobacteria. M. tuberculosis* H37Rv (Pasteur Institute, Paris) was grown on Loewenstein-Jensen medium (Difco, Detroit) at 37°C for 3 weeks. The bacterial mass was vigorously resuspended in physiologic saline, giving 10 mg of cells/mL. Fifty microliters of
this suspension was diluted in 5 mL Dubos broth (Difco) supplemented with 0.5% bovine serum albumin (BSA; Sigma, St. Louis) and cultured for 1 week at 37°C. For the second passage, 0.5 mL of this culture was diluted in 20 mL Dubos broth plus BSA and cultured for 1 additional week. Colony-forming units (cfu) of the bacterial suspensions were estimated as microcolonies that could be counted under an inverted microscope by plating of appropriate dilutions on Dubos oleic agar (Difco) followed by 3 days incubation at 37°C. The bulk culture was stored at 4°C. After cfu estimation, we centrifuged the bulk culture for 20 min at 3000 g at 4°C, and the bacteria were resuspended in saline (containing 0.05% Tween 20 and 0.1% BSA) to 7 × 10^7 cfu/mL and stored at −80°C. At the time of infection, 1 mL of frozen culture was thawed and diluted in 50 mL of saline, and bacterial aggregates were allowed to sediment with 0.5% bovine serum albumin (BSA; Sigma, St. Louis) for 1.5 h. The upper 40 mL of this suspension was removed and used for challenge. Parallel control plating experiments showed that 0.5 mL of this suspension consistently contains <5 × 10^4 cfu.

**Mice and infection.** Inbred mice of the strains A/Sn and I/St, their F1 hybrids (A/Sn × I/St)F1, and backcross (BC) mice (A/Sn × I/St)F1 × I/St were kept under conventional conditions in the animal facilities of the Central Institute for Tuberculosis, Moscow. Food and water was provided ad libitum. At 2.5 months of age, mice were infected intravenously with a lethal dose (5 × 10^6 cfu) of *M. tuberculosis* H37Rv. Littermates of the infected mice (A/Sn, I/St, and F1) were used as controls. They were kept uninected but otherwise under conditions identical to those for infected animals. Survival time and body weight were monitored. Statistical significance concerning inbred strains and F1 hybrids was determined by use of the two-tailed *t* test. Correlation was calculated by use of Pearson’s correlation coefficient.

**Genotyping and linkage analysis.** Genomic DNA was isolated from spleens of parental, F1, and BC mice and genotyped by use of polymerase chain reaction, as previously described [15], using 118 informative simple sequence length polymorphism markers (Research Genetics, Huntsville, AL) and 2 phenotypic markers (d and p) distributed over the entire genome. This corresponds to an average marker density of ~12 cM, with a maximum marker distance of ~30 cM.

The markers were assigned to and mapped within the chromosomes by multipoint linkage analysis, by use of MapMaker/Exp version 3.0 [16]. The order of markers on chromosomes was identical to those in published maps [17]. Genome-wide multipoint linkage analysis between the quantitative phenotype body weight (day 20 after infection) and genotype markers for the identification of QTLs was performed by use of interval mapping and subsequent MQM mapping within the MapQTL package [18]. Interval mapping is based on single QTL models and was used for initial detection of putative QTLs. MQM mapping analyzes multiple QTL models by assigning markers near the QTL to cofactors. These cofactors take over the effect of the QTL on other loci and enhance the sensitivity for detection and exclusion of additional QTLs and artificial QTLs, respectively. Cofactors were assigned to putative QTLs with a logarithm of the odds ratio (LOD) score >1.0, as detected by interval mapping.

MQM mapping with forward selection of cofactors was performed: cofactors were added or dropped sequentially, by use of the threshold LOD score, 1.0, in each test. The final cofactors were tested for representing real QTLs (rather than artefactual QTLs) by moving each cofactor along its respective linkage group to see if the QTL would remain at the original locus. The final cofactors were accepted only if they had LOD scores >2.2. All multipoint LOD scores presented here show the logarithm of the relative likelihood that an A/Sn allele on the locus increases body weight. For the genome-wide QTL analysis, an LOD score of 3.3 was used as the threshold for significant linkage (*P* = .0001; genome-wide significance level, *P* < .05), whereas an LOD score of 1.9 was used as the threshold for suggestive linkage (*P* = .0034)[19]. The significant and suggestive linkages detected are reported.

**DNA sequencing.** The genomic DNA sequence encoding a fragment of the *Nramp1* gene was determined in A/Sn and I/St mice. The sequenced region corresponds to the predicted transmembrane domain 4 of the gene product including an N-terminal extracellular segment [20, 21]. In brief, a *Nramp1* gene product fragment was amplified by polymerase chain reaction using oligonucleotides corresponding to nucleotide positions 521–540 and 651–631 as forward and reverse primers, respectively (nucleotide numbering according to GenBank accession no. L13732). The amplified fragments were subcloned into dT-tailed pBluescript vector, and inserts were sequenced, by use of T7 and T3 primers and the T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden).

**Results**

**Phenotypes of severity of *M. tuberculosis* H37Rv–triggered disease.** The mean survival time (MST) ± SE of I/St mice was 21.5 ± 1.2 days for females and 26.3 ± 1.6 days for males, and for A/Sn mice it was 45.4 ± 1.8 days for females and 45.2 ± 2.2 days for males. Thus, A/Sn and I/St inbred strains of mice that were stratified for sex differed significantly in MST (*P* < .0001). The intersex difference in MST was significant for I/St mice only, with males surviving longer (*P* < .05). All infected (A/Sn × I/St)F1 hybrids, irrespective of their sex, had a long survival time (>70 days), confirming a previous report of recessive inheritance of severe *M. tuberculosis*–triggered disease expressed by I/St mice [10].

Compared with their uninfected littermates, susceptible I/St mice had a severe body weight loss by 20 days after infection (*P* < .0001; figure 1A). Infected A/Sn mice did not show significant wasting until 30 days after infection (figure 1B). Furthermore, the body weight loss of up to 25% at day 20 for infected I/St mice did not occur in A/Sn mice until 40 days after infection. The infected F1 mice did not lose body weight during the 40 days of monitoring (figure 1C). Twenty days after infection, there was a significant difference in body weight, comparing infected I/St with A/Sn and F1 mice for each sex (*P* < .0001). Of importance, at day 0 (the day of infection) no difference in body weight was found between I/St and A/Sn mice (*P* > .05).

**Intersex difference in body weight was displayed by I/St mice 20 days after infection (**P** < .05) but not by infected or uninfected I/St or A/Sn mice at any other time of measurement (**P** > .05). An initial set of (A/Sn × I/St)F1 × I/St BC mice was generated and phenotyped for survival times and body weight...
Body weight in *Mycobacterium tuberculosis* H37Rv–infected and noninfected mice. Body weight of inbred mice of strains I/St (A), A/Sn (B), and F1 hybrid mice (A/Sn × I/St)F1 (C) was monitored immediately before injection of mycobacteria and then every 10th day after infection until day 40, unless mice died before then. Results are mean ± SD of body weight. □, infected females (nA/Sn = 12, nI/St = 7, nF1 = 4); △, infected males (nA/Sn = 11, nI/St = 7, nF1 = 4); ○, uninfected females (nA/Sn = 5, nI/St = 5, nF1 = 4); and ▲, uninfected males (nA/Sn = 5, nI/St = 6, nF1 = 4). Nos. of mice are valid for all respective data points because all mice lived for at least 20 days. For clarity, only positive SD is shown.

loss after infection day 20. For males and females, survival times ranged from 23 to 61 days and 21 to 55 days, respectively, whereas relative body weight loss ranged from −11.8% to 22.6% and −12.4% to 28.0%, respectively. Thus, values for both traits in individual BC mice were distributed between the values of the parental strains. Very good correlation between survival time and body weight loss was evident in the BC mice, for males (r = .78, n = 33, P < .0001) and females (r = .75, n = 31, P < .0001). The phenotype of body weight on day 20 after infection was thus selected as a parameter of severity of virulent *M. tuberculosis*–triggered disease to be studied by QTL analysis. Of note, in all tested parental mice the chosen phenotypes were fully penetrant, precluding the hypothetical effects of major environmental or nongenetic factors on phenotype expression.

**Genetic linkage analysis of body weight loss.** To identify chromosomal loci that prevent rapid body weight loss characteristic for I/St mice after *M. tuberculosis* H37Rv infection, we generated and infected 105 (A/Sn × I/St)F1 × I/St BC mice with *M. tuberculosis* H37Rv. A genome wide scan for QTLs controlling the body weight 20 days after infection was done on genomic DNA obtained from the BC mice. One locus on proximal chromosome 9, D9Mit89, and one locus on distal chromosome 3, D3Mit215, showed significant linkage with body weight in females, with LOD scores of 6.68 and 3.92, respectively (figures 2A, 2B). Furthermore, suggestive linkages to body weight in females were found on proximal chromosome 8, locus D8Mit289, and the mid-proximal one-third of chromosome 17, with LOD scores of 3.01 and 2.95, respectively (figures 2C, 2D). For males, the loci D5Mit233, proximal chromosome 5, and D10Mit133, distal chromosome 10, showed...
Figure 2. LOD (logarithm of odds ratio) score plots along chromosomes for which significant or suggestive quantitative trait loci controlling severity of *Mycobacterium tuberculosis*-triggered wasting were detected. A. Chromosome 3 in females. B. Chromosome 9 in females. C. Chromosome 8 in females. D. Chromosome 17 in females. E. Chromosome 5 in males. F. Chromosome 10 in males.
suggestive linkage, with LOD scores of 3.03 and 2.31, respectively (figures 2E, 2F). To verify that the QTLs identified in the analysis were related to *M. tuberculosis* infection and not to normal body weight differences, QTL analysis was performed on body weight immediately before infection (day 0). None of the loci identified in the postinfection phenotype analyses showed indication of linkage with body weight at day 0.

DNA sequencing of *Nramp1*. DNA sequencing of *Nramp1* was done to determine the *Nramp1* genotype of the 2 strains analyzed in this study, to verify whether *Nramp1* was a confounding genetic factor. The results showed that both I/St and A/Sn mice carry the resistant (G169) allele of *Nramp1*.

Discussion

The identification of clear, extreme phenotypes of susceptibility to *M. tuberculosis* is a valuable tool for genetic analysis in mouse models and human mycobacterial infections [22]. In this study, we performed a QTL analysis on body weight loss in I/St and A/Sn mice after infection with *M. tuberculosis*. These 2 mouse strains represent polar extremes in severity of *M. tuberculosis*-triggered disease, which, in addition to the rate of body weight loss, is manifested by several phenotypes. These phenotypes include (1) MST (comparing ~30 inbred strains of mice, I/St mice express the shortest MST and A/Sn mice express the longest MST [10–12]); (2) load of mycobacteria in lungs and spleens (I/St mice have a 20- to 100-fold higher load 1 and 2 months after infection with both high and low doses of *M. tuberculosis* H37Rv); and (3) lung histopathology (I/St mice have a more severe lung histopathology, compared with that for A/Sn mice) [13].

Although the abrogation of early-onset *M. tuberculosis*-induced wasting in BC mice by A/Sn alleles is the most attractive phenotype for analysis because of its ease of accurate determination and similarity to a known human TB phenotype, it remains to be seen if and to what extent the other phenotypes of extremely severe *M. tuberculosis*-triggered disease displayed by I/St mice are under the same genetic control.

Determination of body weight after *M. tuberculosis* H37Rv infection in (A/Sn × I/St)F1 × I/St mice revealed a continuous distribution of postinfection body weight between the values of the 2 parental strains, which suggested a polygenic control of the trait. Indeed, this was confirmed by genome scanning, since several QTLs were identified in the analysis. Among females, QTLs on proximal chromosome 9 and distal chromosome 3 were significantly linked to postinfection body weight, with LOD scores of 6.68 and 3.92, respectively. Furthermore, suggestive linkages were found for QTLs on chromosomes 8 and 17, with LOD scores of 3.01 and 2.95, respectively. For males, QTLs on chromosomes 5 and 10 were suggestively linked to postinfection body weight, with LOD scores of 3.03 and 2.31, respectively. Additional experiments are needed to determine if the suggestive linkages identified by the present study will become significant by increasing the number of mice.

In terms of candidate genes for severity of *M. tuberculosis*-triggered disease, the regions surrounding the identified QTLs contain several genes that regulate the function of macrophages and other immune cells known to be important for antimycobacterial responses. The D3Mit215 region contains the peroxisomal membrane protein 1 gene (*Pxmp1*) [23]. Peroxisomes participate generally in the pathway of reactive oxygen metabolites, which potentially are important for antimicrobial function of macrophages. Possible candidates for the D9Mit89 QTL are the macrophage metalloelastase gene (*Mme1*) [24] and the α-chain interleukin (IL)-10 receptor gene (*IL10ra*) [25]. Metalloproteinases degrade extracellular matrix proteins and are involved in remodeling of the connective tissue framework, an important feature in chronic inflammation. Likewise, the importance of IL-10 for the regulation of acquired immune responses is well established, and variants of its receptor are attractive candidates for disease-predisposing alleles. Among the chromosomal segments that are suggestively linked to *M. tuberculosis* H37Rv-induced wasting is the D5Mit223 region carrying the genes *IL6* and extracellular superoxide dismutase 3 (*Sod3*), which may be involved in protection against *M. tuberculosis* [26, 27] and the generation of reactive oxygen metabolites, respectively. The suggestive hit on chromosome 17 is consistent with an involvement of *H-2* loci or tumor necrosis factor–α in the control of *M. tuberculosis*-triggered cachexia [28, 29]. Finally, candidates for the chromosome 8 suggestive QTL are the β-defensin 1 (*Defb1*) [30] and lysosomal membrane glycoprotein 1 (*Lamp1*) genes. The β-defensins are broad-spectrum bactericidal agents expressed in monocytes/macrophages, granulocytes, and epithelial cells [31]. *Lamp1* [32] may be involved in the regulation of phagosome-lysosome fusion, which is an essential step of phagocyte antimycobacterial activity with which intracellular *M. tuberculosis* bacilli interfere [33].

An interesting aspect of our results is the localization of QTLs influencing severity of *M. tuberculosis*-triggered disease to proximal chromosome 9 and chromosome 17. In a previous QTL analysis of resistance to *Leishmania major*-triggered disease, loci had been detected on chromosome 9 (*lmr1*) and chromosome 17 (*lmr2*) [34]. The chromosome 9 locus detected in our experiments directly overlies the *L. major*-resistance locus. Moreover, the suggestive linkage to chromosome 17 in our cross coincides with the *H-2* region localization of *lmr* and the well-established fact that *H-2* loci influence TB susceptibility of inbred mice. These observations raise the intriguing possibility that loci controlling basic aspects of resistance or susceptibility to intracellular macrophage parasites are localized to homologous regions in the murine genome; hence, by exploiting cross-species chromosome homologies, they identify prime candidate regions for the genetic study of TB susceptibility in humans.
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References


