Malaria Liver Stage Susceptibility Locus Identified on Mouse Chromosome 17 by Congenic Mapping

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Abstract

Host genetic variants are known to confer resistance to Plasmodium blood stage infection and to control malaria severity both in humans and mice. This work describes the genetic mapping of a locus for resistance to liver stage parasite in the mouse. First, we show that decreased susceptibility to the liver stage of Plasmodium berghei in the BALB/c mouse strain is attributable to intra-hepatic factors and impacts on the initial phase of blood stage infection. We used QTL mapping techniques to identify a locus controlling this susceptibility phenotype (LOD score 4.2) on mouse chromosome 17 (belr1 locus). Furthermore, analysis of congenic mouse strains delimited the belr1 locus boundaries distally to the H2 region. Quantification of parasites in the liver of infected congenic mice strongly suggested that the belr1 locus represents a genetic factor controlling the expansion of P. berghei in the hepatic tissue. The mapping of belr1 locus raises the hypothesis that host gene variation is able to control the progression of Plasmodium liver stage infection and opens the possibility that the human genomic region orthologous to belr1 may contain genes that confer resistance to the human malaria liver stage.

Introduction

Malaria is caused by the hematozoan of the genus Plasmodium and provides one of the best examples of how positive selective pressure upon host genetic variants may confer resistance to disease. For example, the mutation underlying sickle cell anemia conferred resistance to malaria (cited in [1]) other erythrocyte traits such as G6PD deficiency, alpha-thalassemia and hemoglobin C are thought to have beneficial effects by reducing parasite invasion or growth in the erythrocytes or by facilitating the elimination of infected erythrocytes (reviewed in [2]). More recently, genome-wide analysis using inbred mouse strains has revealed that a considerable number of chromosomal regions that enhance control of infection with different Plasmodium species (reviewed in [3]).

Genetic analysis of infection with P. chabaudi has identified nine loci that contribute to control of the parasitemia (char1–9) [3,4]. Analysis of recombinant mouse strains allowed the dissection of multivariate loci in char2 [5,6], char3 [7] and char4 [8] regions and to the identification of positional candidate genes for the char9 locus [9]. In particular, a mutation in the pyruvate kinase gene has been identified as mediating a protective mechanism to P.chabaudi infection involving increased splenic clearance of erythrocytes through hemolysis [10,11].

Similarly, resistance to cerebral malaria caused by P. berghei infection was attributed to a combinatorial effect of berr1 and berr3 loci, illustrating that resistance to infection may arise from genetic interaction among host resistance factors [15,16].

Many malaria-related traits have been analyzed but the role of genetic variants in controlling liver infection has not been addressed. During the initial liver stage of infection, individual sporozoites infect hepatocytes and grow to large schizonts that finally differentiate into 10,000–40,000 merozoites that are released in the bloodstream and infect the red blood cells. A major obstacle to genetic studies in man is the fact that the liver stage infection is largely asymptomatic. Genetic studies in the mouse were initially focused on evaluating the role of MHC alleles in conferring protection induced by irradiated sporozoites [17]. More recently, analysis of knockout mouse models has proved the involvement of host genes like CD81 in the malaria liver stage [18]. As early observations suggested that natural genetic variance between mouse strains may underlie differences in susceptibility to infection with Plasmodium sporozoites [19] we have conducted a genetic mapping study to identify genetic factors controlling the liver stage of infection. We report that a quantitative trait locus (belr1) mapping to mouse chromosome 17 partially controls expansion of P.berghei sporozoites during liver infection.

Results

Course of liver stage infection

Early reports suggest that infection of BALB/c mice with P. berghei sporozoites resulted in low level of hepaticocyte infection at the end of the liver stage (44 h post-infection) [19]. We quantified P.berghei 18S rRNA in the liver during the course of infection in
BALB/c and C57BL/6 mice, after intravenous sporozoite injection. No difference was observed between the two mouse strains during the initial phase of infection but from 24 h up to 40 h post-infection the parasite burden in BALB/c mice was significantly lower (Figure 1). Given that sporozoites invade the liver in less than 1 h, this result suggested that the low parasite expansion in BALB/c mice was controlled within the liver. Next, we quantified the parasite burden in liver of BALB/c and C57BL/6 mice infected by intra-hepatic injection (Figure 2). Once again the parasite burden was lower in BALB/c mice confirming that the poor expansion of \textit{P. berghei} in BALB/c mice was attributable to intra-hepatic factors.

**Liver stage susceptibility phenotype**

Blood smear analysis of parasitemia rising after sporozoite injection showed that BALB/c mice displayed lower parasitemia than C57BL/6 mice on days 4 and 5 after infection (Figure 3), suggesting that the decreased parasite expansion in the BALB/c delays the development of blood stage infection. Thus, we used parasitemia at day 5 post-infection as an assay to assess the previously occurring parasite expansion in the liver. We studied the segregation of the day-5 parasitemia phenotype in genetic crosses of BALB/c and C57BL/6 mice and we observed that the first generation crosses showed an intermediate phenotype (Figure 3) and that the phenotypic spectrum of the second generation progeny indicated that alleles controlling the phenotype were segregating in the (C57BL/6 X BALB/c) F2 cross. The phenotype in the 115 F2 progeny approached a normal distribution suggesting that could be analyzed as a quantitative trait (Figure 4).
Genetic mapping of liver susceptibility phenotype

In order to map the gene(s) controlling the parasite expansion in the liver, we used 93 microsatellite markers across the whole genome and scanned for quantitative trait loci (QTL) in the 115 (C57BL/6 X BALB/c) F2 progeny. Using QTL statistical analysis, significant linkage was found to the medial region of chromosome 17, where the highest linked marker D17Mit20 reached a LOD score of 4.2 (Figure 5). No other region in the genome reached the genome-wide level of significance, but markers on distal chromosome 1 have shown suggestive linkage (LOD score 2.73), raising the possibility that a second locus mapping in this region may also contribute to the phenotype. The mapped locus in chromosome 17 was named belr1 locus (berghei liver resistance) and the genetic effect of the highest linked marker (D17Mit20) was estimated to explain 15.4% of phenotypic variance observed in the F2 progeny.

Congenic mapping of belr1 locus

To confirm the genetic mapping of the belr1 locus we analyzed the decreased susceptibility to liver infection in congenic strains containing defined chromosome 17 segments of one parental strain introgressed in the background of the other parental strain. The B6.C-H2d congenic strain carries a 38 Mb segment of BALB/c chromosome 17 encompassing the H2 locus in the genetic background of the C57BL/6 strain. Conversely, the C.B10-H2b strain carries the H2b haplotype in the BALB/c genetic background. We found that the B6.C-H2d strain showed decreased parasite burden in the liver, indicating that the genetic factor mediating the belr1 effect is contained in this congenic region (Figure 6A). In contrast, C.B10-H2b mice showed liver-stage resistance similar to BALB/c mice (Figure 6B). These results demonstrate that the H2b haplotype per se is not controlling the liver burden phenotype, and strongly suggest that the belr1 locus is mapping distally to the H2 locus. The combined analysis of the congenic strains confines the belr1 locus to a region of 28 Mb on chromosome 17 distal to the H2 locus (Figure 7). As an alternative approach, we performed a conditional analysis in the F2 progeny that fixed the H2 genetic effect by using the D17MIT233 marker as a co-variate in the QTL analysis. Under this analysis the LOD score curve still persisted on the belr1 locus albeit with lower values. These results suggest that the belr1 genetic effect is independent of the H2 locus, but do raise the possibility that genes in the H2d haplotype present in the B6.C-H2d mouse strain may also contribute to the malaria liver-stage resistance.

Collectively, the results indicate that a genetic factor(s) controlling susceptibility to liver infection by P. berghei is located in the belr1 locus and acts at the intra-hepatic level to control parasite liver stage expansion.

Discussion

The key observation in this work is the demonstration that the belr1 locus controls the expansion of P. berghei in the liver, and that impacts on the level of subsequent blood parasitemia. Using mouse congenic strains the locus controlling parasite expansion in the liver was delimited within an interval of 28 Mb on mouse chromosome 17 close to the MHC locus previously linked to malaria resistance in both man and mouse. Although this analysis indicated that the major genetic effect on chromosome 17 maps outside the H2 region it is still possible that the H2 locus could contribute to the observed phenotype. In addition, genetic markers in distal chromosome 1 suggest that a second locus could also be involved in the genetic control of this phenotype. Incidentally, this region coincides with a locus (ber1), that we have previously shown to control cerebral malaria and parasitemia clearance following P. berghei ANKA infection [12,13]. To further dissect such multiple genetic effects will include the generation and analysis of subcongenic strains carrying different congenic intervals derived from the B6.C-H2d mouse strain.

The possible involvement of the MHC region in the genetic control of resistance to malaria resistance in humans has stimulated a search for the contribution of the MHC and TNF molecules to the pathogenesis of malaria. For example, a significant number of reports claim genetic linkage and allelic association of these genes with severe forms of disease and re-

Figure 5. Genome-wide mapping of liver stage susceptibility trait. LOD score curves representing the likelihood for linkage of parasite burden trait. Genome-wide linkage is significant for LOD score above 3.3. (A) Shows LOD score curves for 19 mouse autosomes. (B) Shows LOD score curve for chromosome 17 where the X-axis ticks represent the relative position of microsatellite markers. Markers from left to right: D17Mit62; D17Mit103; D17Mit233; D17Igc2; D17Mit11; D17Igc3; D17Mit139; D17Mit20; D17mit152; D17Mit193; D17Mit187; D17Mit221. doi:10.1371/journal.pone.0001874.g005
infection risk [20–24]. At present, it is difficult to determine whether the genetic effects presented in these studies could result from genetic factors mapping just outside the MHC region like the belr1 locus. It is of immediate interest to evaluate the human orthologue to belr1 region to malaria genetic resistance in humans through unbiased association mapping studies.

Previous genetic analysis determined that mouse chromosome 17 contains loci exerting weak control of resistance to P. chabaudi parasitemia [25], in particular the H2-linked locus, char3, which mediates delayed parasitemia kinetics [26]. Further work suggested that these genetic effects were possibly contributed by an additional locus, char7, mapping distally to the H2 in a region that co-localizes with belr1 locus [7]. Thus, it remains to be determined whether the locus described in this report coincides with char7 locus, thereby representing a genetic factor controlling malaria infection at both liver and blood stages and having a relevant role in the disease caused by different murine Plasmodium species.

The belr1 region contains 384 genes and includes known genes, predicted genes and other ORFs. Certainly, some genes are readily excluded from the liver resistance phenotype, as is the case of 49 genes coding for olfactory receptors. However, the presence of at least 80 unknown genes precludes the identification of plausible candidates. Subcongenic mapping will be performed to narrow down the number of credible positional candidate genes which will then be subjected to functional and structural analysis. The identification of candidate genes for the belr1 locus may lead to the characterization of similar human genetic factors playing a role in malaria resistance by though their impact on asymptomatic liver infection.

It is likely that cell and molecular components that are involved in the observed host resistance exert their effect by limiting the expansion of the parasite liver stages in the hepatic tissue, raising the question of the underlying resistance mechanism. One possibility is suggested by work with P. berghei [19] where the blockage of liver-stage parasite development in BALB/c liver has been correlated with the immediate liver inflammatory response [27]. However, the relevant effectors and target cells have not been defined. One attractive hypothesis is that, liver resident cell types, such as Kupfer cells or NK and NKT cells act on the parasite, in either extracellular or intracellular forms.

Alternatively, the observed resistance to Plasmodium expansion could be mediated by a hepatocyte factor that controls the survival of infected hepatocytes, for example sporozoite mediated inhibition of hepatocyte apoptosis. In fact, release of hepatocyte growth factor (HGF) [28] and signaling through the HGF/c-Met pathway [29] are considered as part of a chain of events that leads to apoptosis resistance of P. berghei-infected hepatocytes [30]. It has
been shown that the initial protection of the host cell from spontaneous apoptosis mediated by HGF/c-Met signaling is extended in time possibly by induction of additional apoptosis resistance mechanisms [31]. We are currently testing this possibility by studying the induction of hepatocyte apoptosis in the presence of P. berghei sporozoites.

Searching for such cellular phenotypes in relevant congenic strains is part of a strategy to identify the cell type(s) mediating the belb1 effect. If successful, this approach will accelerate the identification of the belb1 gene(s).

Materials and Methods

Mice

Parental mouse strains used in this study were C57BL/6, BALB/c, (BALB/c X C57BL/6) F1 and (C57BL/6 X BALB/c) F1. (C57BL/6 X BALB/c) F1 mice were used to generate the (C57BL/6 X BALB/c) F2 progeny of 115 males. Two H2 congenic mouse strains were obtained from the Jackson Laboratory (Maine, USA) and maintained at IGC. B6.C-H2b/H2d carries a BALB/c-derived congenic segment of 37.9 Mb on chromosome 17, flanked by markers D17Mit198 and D17Mit152, on C57BL/6 genetic background and is here referred to as C.B10-H2d. C.B10-H2d/LilMcdJ carries a C57Bl/10-derived segment of 10.4 Mb on chromosome 17, flanked by markers D17Mit60 and D17Mit232, on BALB/c genetic background and is here referred to as C.B10-H2b. The haplotype b of H2 is common to strains C57BL/6 and C57BL/10. All mice were bred and maintained in conventional housing facilities at the Institute Gulbenkian de Ciência. All experiments used male mice with 8 to 15 weeks of age. All procedures were in accordance with national regulations on animal experimentation and welfare.

Infection

Plasmodium berghei ANKA sporozoites were obtained from dissection of salivary glands from infected female Anopheles stephensi mosquitoes. Sporozoites suspensions in RPMI medium were injected i.v. in 100 μl inocula of 10⁵ sporozoites per mouse.

To perform the intra-hepatic injection, the mice were anesthetized with Ketamine by i.p. injection. The abdominal cavity was surgically accessed through the medial line, partially exposing the left liver lobe. Sporozoites were injected under the liver capsule in a 50 μl inoculum. Then, the abdominal wall muscles and skin were sutured, and the animals were maintained at IGC. B6.C-H2d and maintained at IGC. B6.C-H2d/H2d carries a BALB/c-derived congenic segment of 37.9 Mb on chromosome 17, flanked by markers D17Mit198 and D17Mit152, on C57BL/6 genetic background and is here referred to as C.B10-H2d.

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Parasite quantification

Parasitemia progression was measured at day 5 post-infection by blood smears with Giemsa. Liver parasite 18S rRNA was quantified by real-time PCR. Livers were collected at 40 h post-infection, immediately homogenized in denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl and 0.7% β-mercaptoethanol in DEPC treated water) and total RNA was obtained using RNeasy Mini Kit (Qiagen). One microgram of total RNA was converted to cDNA (Transcriptor First Strand cDNA Synthesis Kit, Roche) and cDNA specific to P. berghei 18S rRNA was amplified with primers NYU-Pb1 5′-AAG CAT TAA ATA AAG GGA ATA CAT CCT TAC-3′ and NYU-Pb2 5′-GGA GAT TGG GTT TTT TGA CCT TTA TGT-3′. The real-time PCR reactions were performed in ABI Prism 2900HT system using ABI Power SYBR Green PCR Master Mix. Absolute P.berghei 18S rRNA estimates were normalised for mRNA of Hypoxanthine Guanine Phosphoribosyl-Transferase (HPRT), a mouse housekeeping gene.

Genotyping

Genomic DNA was prepared from mouse tails before infection, using standard techniques. The 115 (C57BL/6 X BALB/c) F2 progeny mice were genotyped by using conventional PCR protocols for 93 microsatellite markers obtained from the Whitehead/MIT Center for Genome Research collection (www.genome.wi.mit.edu/cgi-bin/mouse/index). Primers for Igk markers were: D17Igc1 For GGG AGT GGG AAT TGT TCT TTT ATT TTA and Rev TGC TTT TTT CTG GTG TCT CGT AA; D17Igc2 For GCT CAC TTT TTG CTA GGA TCA TC and Rev GCC ATG GGA AGA AGT TAT ATG TC; D17Igc3 For GAT AGG TTT GTA GTC AGG CCT AA and Rev ACT TAT TCA CTC CTG AGC CCT GT. Individual mouse genotypes were scored using established agarose gel electrophoresis protocols.

Statistical Analysis

Quantitative trait locus analysis was performed using the R/QTL software [32]. This program calculates logarithm of odds (LOD) scores over intervals between linked markers, generating LOD score curves representing the likelihood of genetic linkage of quantitative phenotypes with markers along the chromosome. The level of statistical significance was empirically determined by permutation tests (5,000) and genome-wide significant linkage was considered when LOD ≥3.3 (p<0.05).

Comparisons between groups of animals were considered statistically significant when the p-value of the unpaired t-test was <0.05.

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Author Contributions

Conceived and designed the experiments: MM LG CP. Performed the experiments: LG PA. Contributed reagents/materials/analysis tools: MM. Wrote the paper: LG CP.

References

1. Lederberg J (1999) J. B. S. Haldane (1949) on infectious disease and evolution. Genetics 153: 1–3.
2. Fortin A, Stevenson MM, Gros P (2002) Susceptibility to malaria as a complex trait: big pressure from a tiny creature. Hum Mol Genet 11: 2469–2478.
3. Hernandez-Valladares M, Naessens J, Iraqi FA (2005) Genetic resistance to malaria in mouse models. Trends Parasitol 21: 352–355.
4. Lin E, Pappenfuss T, Tan RB, Senychyn D, Bahlo M, et al. (2006) Mapping of the Plasmodium chabaudi resistance locus char2. Infect Immun 74: 5814–5819.
5. Hernandez-Valladares M, Naessens J, Gibson JP, Musoke AJ, Nagda S, et al. (2004) Confirmation and dissection of QTL controlling resistance to malaria in mice. Mamm Genome 15: 390–398.
6. Fortin A, Cardon LR, Tam M, Skamene E, Stevenson MM, et al. (2001) Identification of a new malaria susceptibility locus (Char) in recombinant congenic strains of mice. Proc Natl Acad Sci U S A 98: 10793–10798.
7. Min-Oo G, Fortin A, Pitari G, Tam M, Stevenson MM, et al. (2007) Complex trait: big pressure from a tiny creature. Hum Mol Genet 11: 2469–2478.
8. Lin E, Pappenfuss T, Tan RB, Senychyn D, Bahlo M, et al. (2006) Mapping of the Plasmodium chabaudi resistance locus char2. Infect Immun 74: 5814–5819.
9. Hernandez-Valladares M, Naessens J, Gibson JP, Musoke AJ, Nagda S, et al. (2004) Confirmation and dissection of QTL controlling resistance to malaria in mice. Mamm Genome 15: 390–398.
10. Min-Oo G, Fortin A, Tam MF, Nantel A, Stevenson MM, et al. (2003) Pyruvate kinase deficiency in mice protects against malaria. Nat Genet 35: 357–362.

11. Min-Oo G, Tam M, Stevenson MM, Gros P (2007) Pyruvate kinase deficiency: correlation between enzyme activity, extent of hemolytic anemia and protection against malaria in independent mouse mutants. Blood Cells Mol Dis 39: 63–69.

12. Bagot S, Campino S, Penha-Goncalves C, Ped S, Cazenave PA, et al. (2002) Identification of two cerebral malaria resistance loci using an inbred wild-derived mouse strain. Proc Natl Acad Sci U S A 99: 9919–9923.

13. Nagayasu E, Nagakura K, Akiki M, Taniya G, Makino S, et al. (2002) Association of a determinant on mouse chromosome 18 with experimental severe Plasmodium berghei malaria. Infect Immun 70: 512–516.

14. Ohno T, Nishimura M (2004) Detection of a new cerebral malaria susceptibility locus, using CBA mice. Immunogenetics 56: 675–678.

15. Campino S, Bagot S, Bergman ML, Almeida P, Sepulveda N, et al. (2005) Genetic control of parasite clearance leads to resistance to Plasmodium berghei ANKA infection and confers immunity. Genes Immun 6: 416–421.

16. Sepulveda N, Paulino CD, Carneiro J, Penha-Goncalves C (2007) Allelic penetrance approach as a tool to model two-locus interaction in complex binary traits. Heredity 99: 173–184.

17. Weiss WR, Good MF, Hollingdale MR, Miller LH, Berzofsky JA (1989) Genetic control of immunity to Plasmodium yoelii sporozoites. J Immunol 143: 4263–4266.

18. Silvie O, Rubinstein E, Franetich JF, Premant M, Belnoue E, et al. (2003) Hepatocyte growth factor and malaria morbidity in a Gambian community. Trans R Soc Trop Med Hyg 87: 286–287.

19. Flori L, Delahaye NF, Izquierdo-Valladares M, Fumoux F, et al. (2003) TNF as a malaria candidate gene: polymorphism-screening and family-based association analysis of mild malaria attack and parasitemia in Burkina Faso. Genes Immun 4: 472–480.

20. McGuire W, Knight JC, Hill AV, Allopp CE, Greenwood BM, et al. (1999) Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. J Infect Dis 179: 287–290.

21. Bennett S, Allen SJ, Olerup O, Jackson DJ, Wheeler JG, et al. (1993) Human leucocyte antigen (HLA) and malaria morbidity in a Gambian community. Trans R Soc Trop Med Hyg 87: 286–287.

22. Carrolo M, Giordano S, Cabrita-Santos L, Corso S, Vigario AM, et al. (2003) Hepatocyte growth factor and its receptor are required for malaria infection. Nat Med 9: 270–275.

23. Leiriao P, Albuquerque SS, Corso S, van Gemert GJ, Sauerwein RW, et al. (2005) HGF/MET signalling protects Plasmodium-infected host cells from apoptosis. Cell Microbiol 7: 603–609.

24. Broman KW, Wu H, Sen S, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics 19: 889–890.