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# Mapping a Quantitative Trait Locus Involved in Melanotic Encapsulation of Foreign Bodies in the Malaria Vector, *Anopheles gambiae*

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ABSTRACT

A Plasmodium-refractory strain of *Anopheles gambiae* melanotically encapsulates many species of Plasmodium, whereas wild-type mosquitoes are usually susceptible. This encapsulation trait can also be observed by studying the response of refractory and susceptible strains to intrathoracically injected CM-Sephadex beads. We report the results of broad-scale quantitative trait locus (QTL) mapping of the encapsulation trait using the bead model system. Interval mapping using the method of maximum likelihood identified one major QTL, *Pen1*. The 13.7-cM interval containing *Pen1* was defined by marker AGH157 at 8E and AGH46 at 7A on 2R. *Pen1* was associated with a maximum LOD score of 9.0 and accounted for 44% of the phenotypic variance in the distribution of phenotypes in the backcross. To test if this QTL is important for encapsulation of *Plasmodium berghei*, $F_2$ progeny were infected with *P. berghei* and evaluated for degree of parasite encapsulation. For each of the two markers that define the interval containing *Pen1*, a significant difference of encapsulation was seen in progeny with at least one refractory allele in contrast with homozygous susceptible progeny. These results suggest that *Pen1* is important for melanotic encapsulation of Plasmodium as well as beads.

THE mosquito, *Anopheles gambiae*, is a principal vector of human malaria, a disease caused by parasites of the genus Plasmodium. Malaria is a serious health problem in many parts of the world, causing an estimated 300–500 million clinical cases of the disease and killing ≥1–3 million people annually (NUSSENZWEIG and LONG 1994). Malaria-control strategies that have met with some success include treatment of infected individuals with drugs, application of insecticides to reduce mosquito populations, and reduction of human contact with infected mosquitoes via bed nets. Unfortunately, the lack of an efficacious malaria vaccine and the development of drug resistance in the parasites and insecticide resistance in mosquitoes have hampered large-scale efforts at malaria control. The development of vector-based control measures may play an important role in future malaria control efforts, and such strategies depend on a thorough understanding of the process of malaria transmission by the mosquito (COLLINS and PASKEWITZ 1995).

The malaria cycle in the mosquito vector begins when the female takes an infective blood meal from an infected host. Within the mosquito midgut the parasites undergo gametogenesis, fertilization, and zygote formation. Zygotes develop into ookinetes, which pass through the midgut epithelium and settle between the midgut epithelium and the basal lamina where they develop into oocysts. After an oocyst matures, sporozoites emerge into the hemocoel and travel to the salivary glands, their final location within the mosquito vector.

Genetic variations that block Plasmodium development within vector mosquitoes have been discovered, but they are rare (CURTIS and GRAVES 1983). The most extensively studied example of refractoriness to malaria transmission is a melanotic encapsulation phenotype originally identified in some individuals of the G3 colony of *A. gambiae* (COLLINS *et al.* 1986). Genetic selection with the simian malaria, *Plasmodium cynomolgi*, generated a strain that is completely refractory to *P. cynomolgi* and several other animal malarias and highly refractory to most of the human malaria strains that have been tested (COLLINS *et al.* 1986). Genetically selected susceptible strains rarely encapsulate any malaria parasites (COLLINS *et al.* 1986; F. H. COLLINS, unpublished data). In the refractory-strain mosquitoes, Plasmodium development is blocked at the ookinete or early oocyst stage when the parasites are encapsulated with a layer of melanin (COLLINS *et al.* 1986; PASKEWITZ *et al.* 1988, 1989). Genetic analyses have suggested that this acellular melanotic encapsulation trait is quantitative but controlled by a small number of genes (COLLINS *et al.* 1986; VERNICK and COLLINS 1989; VERNICK *et al.* 1989).

During a study of the biochemical nature of the melanization process, the refractory and susceptible strains were found to differ in their ability to melanize CM-

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Sephadex beads (PASKEWITZ and RIEHLE 1994). After intrathoracic injection, these beads become melanotically encapsulated in the refractory mosquitoes but are rarely and only weakly melanized in the susceptibles (PASKEWITZ and RIEHLE 1994; CHUN *et al.* 1995). Several lines of evidence suggest that the bead encapsulation trait and *P. cynomolgi* (B strain) encapsulation trait share a common genetic mechanism. A strong tendency of the bead and parasite encapsulation phenotypes to cosegregate in backcross progeny demonstrates that the same chromosomal regions are involved in both processes (GORMAN *et al.* 1996). In addition, both traits appear to have the same mode of inheritance: for both traits, the melanizing phenotype is dominant to the nonmelanizing; they are autosomal; and they are controlled mainly by one major gene (VERNICK and COLLINS 1989; CREWS-OYEN *et al.* 1993; GORMAN and PASKEWITZ 1997).

Studies of the melanotic encapsulation trait have provided few clues about the molecular nature of the gene products that cause it. For this reason, map-based cloning may be the most effective strategy for identifying the loci that control this form of refractoriness. In this report, we describe the results of the first step of this approach. We used broad-scale quantitative trait loci (QTL) mapping to locate the principal QTL for melanotic encapsulation. Because all data collected to date suggest that bead and *P. cynomolgi* B encapsulation have a common genetic basis and because the bead model system has many technical advantages (see DISCUSSION), we chose to use beads rather than parasites for this mapping experiment.

## MATERIALS AND METHODS

**Mosquito strains and culture techniques:** Two strains of *A. gambiae* were used: a Plasmodium-refractory strain (L35) and a Plasmodium-susceptible strain (4arr) (F. H. COLLINS, unpublished data). The L35 strain was reselected from the original G3-derived refractory strain (COLLINS *et al.* 1986). The 4arr strain was selected from an unrelated parent colony. The 4arr chromosomes are homosequential with those of the L35 strain, except both strains are polymorphic for the 2Rbc inversions, which rearrange DNA between 11B and 14A (COLUZZI *et al.* 1979; F. H. COLLINS, unpublished data).

Mosquitoes were maintained at 26–29° and ~70% humidity. Eggs were washed with 0.1% bleach. Larvae were grown at a density of approximately 300 per liter of water and fed ground TetraMin, dog food, and yeast.

**Genetic crosses:** The crosses and progeny used for mapping the bead encapsulation trait are the same as some of those used in the initial genetic analysis of the bead melanotic encapsulation trait (GORMAN and PASKEWITZ 1997). Virgin L35 females were mated to 4arr males, then virgin $F_1$ females were backcrossed to susceptible males. The bead melanization phenotype in a sample of backcross progeny was determined, and mosquito carcasses were frozen at −80° for later DNA isolation and genotype determination.

To generate segregating progeny for infection with *P. berghei*, virgin L35 females were mated to 4arr males, and $F_1$ progeny were crossed to generate $F_2$ progeny. The parasite encapsulation phenotype was assayed in a sample of $F_2$ progeny, and mosquito carcasses without abdomens were kept for genotype determination.

**Bead encapsulation assay:** As described in GORMAN and PASKEWITZ (1997), CM-Sephadex (C25) beads were incubated in mosquito saline (13 mM NaCl, 0.5 mM KCl, 0.01 mM $CaCl_2$) with 0.001% methyl green. Stained beads of ~50 μm in diameter were aspirated into a pulled glass needle and then injected (one per mosquito) into the thoraxes of 2-day-old females. Injected mosquitoes were incubated in a humid chamber at ~27° for 24–26 hr. Healthy mosquitoes (those able to fly) were dissected, and the bead melanization phenotype was evaluated on a subjective scale of 1–10. Beads that had less than ~5% of their surface covered with melanin were scored as level 1, 10% as level 2, 20% as level 3, and so forth, ascending to 90–100% as level 10.

**Parasite encapsulation assay:** A Balb/C mouse was infected with *P. berghei* ANKA by intraperitoneal injection of stabilate. $F_2$ females were fed on the infected mouse when the parasitemia was 5%. Refractory and susceptible control mosquitoes were also fed. Fully engorged females were maintained at 21° for 7–9 days to allow the parasites to develop. Midguts were dissected, stained with mercurochrome, and analyzed by light microscopy for the presence of encapsulated and nonencapsulated parasites. The parasite encapsulation phenotype was determined as the percentage of parasites that were encapsulated (rounded to the nearest 10%).

**Genomic DNA isolation:** DNA was isolated from pooled mosquitoes by the method of SEVERSON *et al.* (1993). DNA was isolated from single mosquitoes by the following method. Single mosquitoes or carcasses were homogenized in 200 μl of grinding buffer (0.5% SDS, 0.2 M NaCl, 25 mM EDTA, 10 mM Tris, pH 8.0), digested with 30 μg RNase A at 37° for 1 hr, and then digested with 110 μg proteinase K at 50° for 1 hr. After adding 300 μl of 10 mM Tris pH 8.0/ 0.2 M NaCl, standard phenol/chloroform extractions and ethanol precipitations were done.

**PCR amplification and gel electrophoresis of microsatellites:** The primer sequences used for PCR amplification of microsatellites are published in ZHENG *et al.* (1996). PCR reactions were done as described by ZHENG *et al.* (1993) with some modifications. One primer for each reaction was end-labeled with $^{32}P$. Reactions, using ~0.5% of the DNA from single mosquitoes, were done with the Gene Amp Kit (Perkin Elmer) using 1× PCR buffer (with 1.5 mM $MgCl_2$), 0.4 μM primers, 0.25 units AmpliTaq, 200 μM dNTPs, and 0.1 μg/μl BSA. Amplification was done in a PTC-100 96V thermocycler from MJ Research (1 min at 94°; 30 cycles of 10 sec at 94°, 20 sec at 55°, 30 sec at 72°; and 5 min at 72°). Samples were run on a 5% Long Ranger (FMC) modified polyacrylamide sequencing gel with 7 M urea, and gels were exposed to autoradiography film without fixing or drying. Labeled pBR322/*Msp*I was included on gels as a size standard (LANZARO *et al.* 1995). To identify informative microsatellite markers, microsatellite DNA from 20 refractory and 20 susceptible mosquitoes was amplified using a total of 37 primer pairs.

**Southern blotting and hybridization:** Genomic DNA was digested by restriction enzymes as described by SEVERSON *et al.* (1994b), size-fractionated on a 0.7% agarose gel, and blotted to GeneScreen Plus (NEN) by alkaline transfer (BROWN 1993). Lambda DNA digested with *Hin*dIII was included as a size standard. The prehybridization, hybridization, and washing methods were those recommended by NEN. Blots were stripped in 0.4 M NaOH at 45° for 30 min followed by two 10-min, room-temperature washes (200 mM Tris, pH 7.0, 0.1× SSC, 0.1% SDS). DNA probes were made from cloned *A. gambiae* DNA that had been excised and purified with the GENECLEAN Kit (Bio101). Radiolabeling was done with a random primer labeling kit (Boehringer Mannheim). La-

beled lambda DNA probes were made and used as described by SEVERSON *et al.* (1994a).

**DNA clones and *in situ* mapping to polytene chromosomes:** A total of 32 DNA clones (mostly cDNA clones) were used to probe Southern blots in a search for restriction fragment length polymorphisms (RFLPs) between DNA from refractory and susceptible mosquitoes. DNA clones that were not previously mapped were cytologically mapped by *in situ* hybridization to polytene chromosomes by the methods of KUMAR and COLLINS (1994).

**Linkage analysis and QTL mapping:** Approximate locations for microsatellite and RFLP markers were known before linkage analysis. A combined genetic linkage map (for both microsatellite and RFLP markers) was made with the use of MAPMAKER/EXP (version 3.0) computer program (LANDER *et al.* 1987; LINCOLN *et al.* 1992b). Map distances in centimorgans were calculated using the Kosambi function. QTL mapping was done by the interval mapping method of LANDER and BOTSTEIN (1989) with the use of MAPMAKER/QTL (version 1.1 for parametic and 1.9 for nonparametric methods) (PATERSON *et al.* 1988; LINCOLN *et al.* 1992a; KRUGLYAK and LANDER 1995).

**Statistical analyses:** Statistical tests were done with the use of the computer software SigmaStat (Jandel).

## RESULTS

**Marker identification and linkage map construction:** The first step in QTL mapping of the melanotic encapsulation trait was to find genetic markers that distinguished the strains of *A. gambiae* used for this experiment. Because the most complete genetic linkage map for *A. gambiae* is a microsatellite map (ZHENG *et al.* 1996), this marker type was chosen. To find microsatellite markers, each candidate marker locus was amplified from the DNA of 20 individuals of each strain, and then band patterns were compared. Of 37 candidates, 15 fit the criterion that all refractory strain alleles were different from all susceptible strain alleles. We also identified some RFLP markers to narrow a gap in the microsatellite map and link the genetic map to the cytological map (COLUZZI and SABATINI 1967; COLUZZI *et al.* 1997). To find useful RFLP markers, DNA clones with known cytological locations were needed. Most clones used were gifts from other labs and have published cytological locations; the rest were mapped by *in situ* hybridization to polytene chromosomes (data not shown). Thirty-two DNA clones were used to probe Southern blots of genomic DNA from both pooled and individual samples of refractory and susceptible mosquitoes. Using *Xho*I digestion, five suitable RFLP markers were identified: Anchym1 at 25D (H. M. MUELLER, personal communication), SP24D at 24D, c256 at 16B, c42 at 11B, and c110 at 7A (data not shown).

With data from 90 backcross progeny, a genetic linkage map of the 20 identified markers was constructed using the MAPMAKER/EXP computer program (LANDER *et al.* 1987; LINCOLN *et al.* 1992b). Using multipoint analysis with a LOD score threshold of 2.0, a maximum likelihood map of each of the three *A. gambiae* chromosomes was generated (Figure 1). All locations and distances (calculated with the Kosambi function) are consistent with previous maps, with three exceptions. (1) Microsatellite marker AGH802 maps to polytene band region 26D but was positioned between Chym1 at 25D and SP24D at 24D in this experiment. (2) The order of markers AGH125 and AGH95 was switched. (3) The distance between markers AGH125 and AGH802 is much greater in this experiment than in the original mapping study (53 *vs.* 16.6 cM). These last two discrepancies can be explained by the presence of inversion polymorphisms in the multiple crosses used to generate mapping progeny for the original map (ZHENG *et al.* 1996). The 20 markers were distributed throughout the genome, except for a 44.7-cM interval

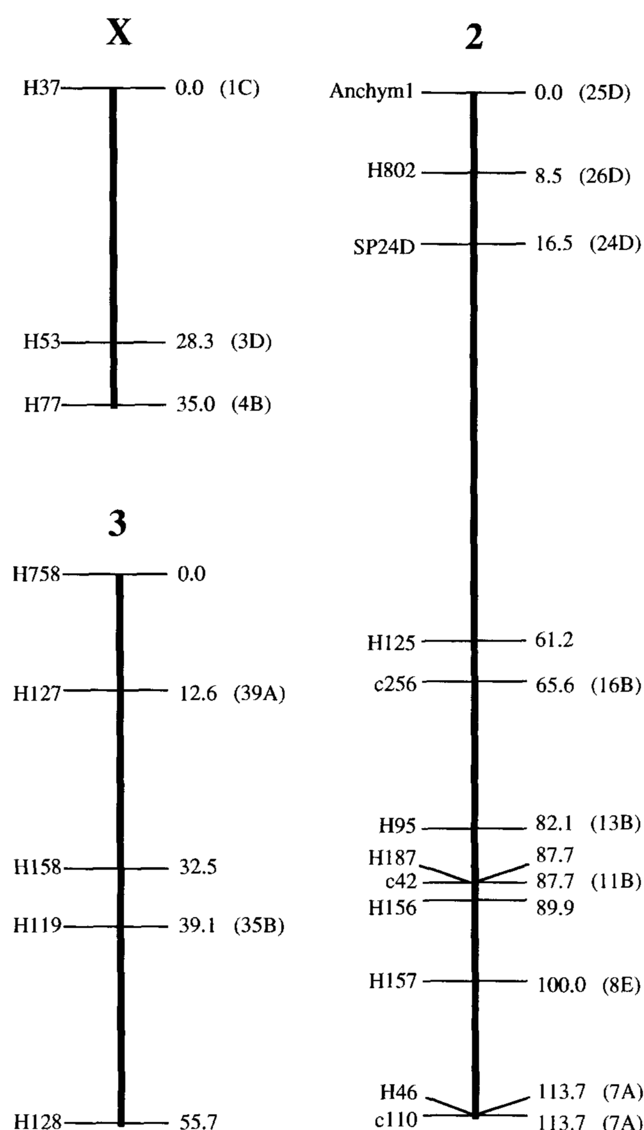


FIGURE 1.—Composite genetic linkage map. Diagrams of the three *A. gambiae* chromosomes are shown. To the left of each chromosome are the marker names (those prefixed with H are microsatellite markers, and the rest are RFLP markers). Markers were mapped by multipoint analysis, and the resulting genetic locations are shown on the right. Cytological locations, if known, are shown in parentheses.

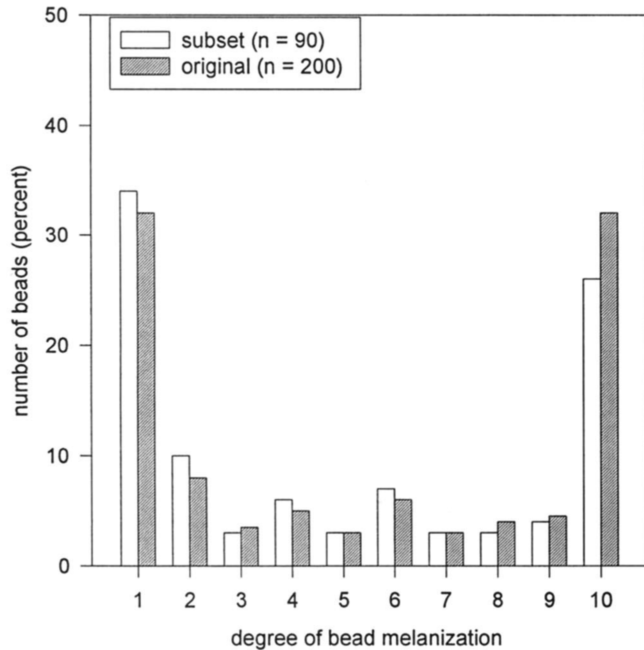


FIGURE 2.—Distribution of bead encapsulation phenotypes of backcross progeny. Bimodal distributions of the phenotypes of the original backcross population (shaded bars) (GORMAN and PASKEWITZ 1997) and a subset of the population (unshaded bars) are evident. These distributions are not significantly different ($T = 12486$, $P = 0.36$).

between markers SP24D and AGH125. Although 23 candidates were tested, no informative markers were found in this interval.

**QTL mapping:** The bead encapsulation trait was analyzed in progeny from the backcross described above. The distribution of phenotypes of the progeny used for this experiment ($n = 90$) was similar to that of the total population of female backcross progeny ($n = 200$) described previously (GORMAN and PASKEWITZ 1997). Like the total population distribution, the phenotypic distribution of the subset population was bimodal (Figure 2). The two distributions were not significantly different from each other as determined by a Mann-Whitney rank sum test ($T = 12{,}486$, $P = 0.36$).

QTL analysis was done with the MAPMAKER/QTL computer program (PATERSON *et al.* 1988; LINCOLN *et al.* 1992a; KRUGLYAK and LANDER 1995). Because the melanotic encapsulation trait does not follow a normal distribution, the nonparametric as well as the parametric mapping functions were used. One QTL was identified on chromosome *2* by the parametric method (Figure 3). It mapped within a 13.7-cM region defined by markers AGH157 at 8E and AGH46 at 7A. This interval is estimated to comprise ~6% of the genome [based on a genome size of 215 cM (ZHENG *et al.* 1996)]. The maximum LOD score associated with the interval was 9.0, well above standard LOD score thresholds (which are usually set ≤3.0). Backcross progeny that were homozygous susceptible for both AGH157 and AGH46 had a median bead encapsulation score of 2.0 ($n = 36$), whereas those that were heterozygous at both marker loci had a median score of 8.0 ($n = 42$). Forty-four percent of the observed phenotypic variance of the backcross population was explained by the QTL. No QTLs were detected on the *X* or third chromosome, which had maximum LOD scores of 0.1 and 0.3, respectively. Fixing for the major QTL and rescanning for QTLs of smaller effect did not uncover any additional QTLs. QTL analysis using the nonparametric mapping function identified the same, unique interval on chromosome *2*. The maximum *z* score for this interval was 5.3, which is above the *z* score threshold of 4 (calculated for a false positive rate of 0.05 and a genome size of 200 cM) (KRUGLYAK and LANDER 1995).

Previous genetic studies of the bead and *P. cynomolgi* B encapsulation traits suggested that they share a common genetic mechanism (GORMAN *et al.* 1996; GORMAN and PASKEWITZ 1997); therefore, it seemed likely that the QTL was involved in encapsulation of Plasmodium as well as beads. To test this hypothesis, we looked to see if the interval was linked to the ability to encapsulate the rodent malaria, *P. berghei*. The parasite encapsulation phenotype was observed in $F_2$ progeny, and the genotypes of four maker loci on 2R were determined for each individual ($n = 50$). Markers analyzed were AGH157 and AGH46, which define the QTL interval, and two adjacent markers, AGH156 and AGH187. The distribution of $F_2$ progeny was bimodal, with most individuals having completely susceptible or completely refractory phenotypes (data not shown). Mosquitoes that were homozygous for susceptible alleles at either AGH157 or AGH46 had lower parasite encapsulation scores than those with at least one refractory allele (Table 1, Figure 4). A Mann-Whitney rank sum test was used to compare mosquitoes with two susceptible alleles to those with at least one refractory allele. For both AGH157 and AGH46, the homozygous susceptible mosquitoes had a significantly lower encapsulation score (for AGH157, $T = 72$, $P = 0.02$; for AGH46, $T = 112.5$, $P < 0.01$). No significant difference between the two groups was seen for markers AGH156 ($P = 0.07$) and AGH187 ($P = 0.31$). In addition, of the three intervals defined by the four markers, only the AGH157-AGH46 interval was associated with a LOD score >2.0. Maximum LOD scores for the intervals AGH157-AGH46, AGH156-AGH157, and AGH187-AGH156 were 4.1, 1.8, and 0.8, respectively. These results suggest that the QTL contributes to the *P. berghei* encapsulation trait.

## DISCUSSION

We have identified one QTL that affects a mosquito immune response, the melanotic encapsulation of CM-Sephadex beads. On the basis of previous studies that indicated that bead and *P. cynomolgi* B encapsulation phenotypes are controlled by the same principal locus (GORMAN *et al.* 1996; GORMAN and PASKEWITZ 1997),

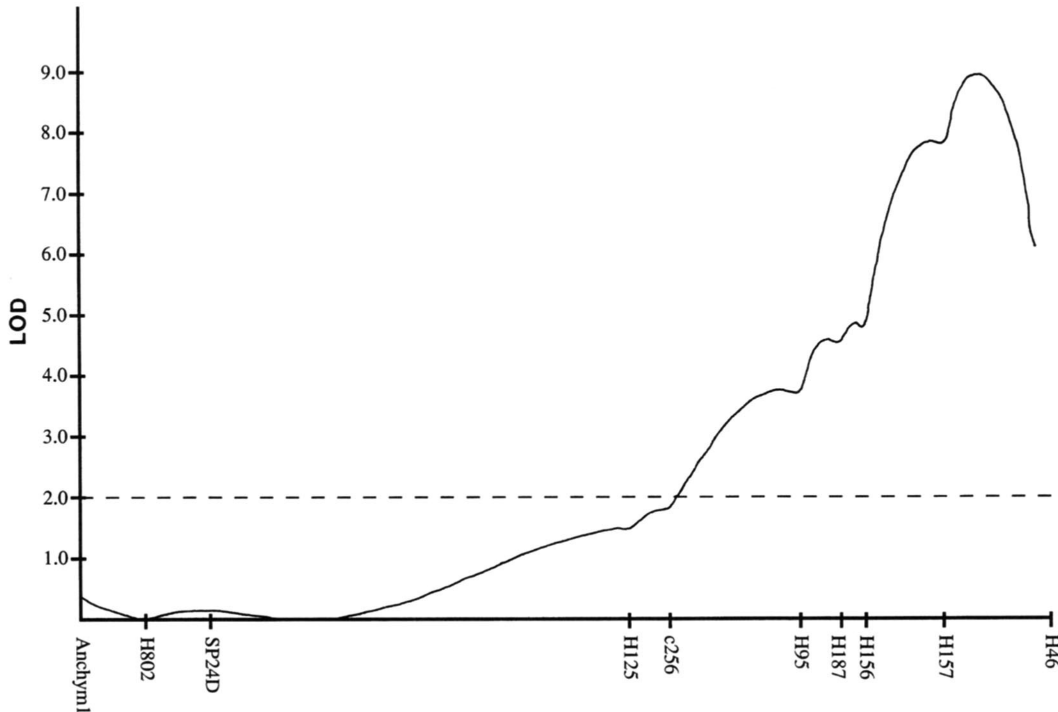


FIGURE 3.—LOD score plot of chromosome *2* for the melanotic encapsulation trait. A single QTL with a maximum LOD score of 9.0 was detected on chromosome *2* between markers AGH157 and AGH46.

we suggest that this QTL is associated with *P. cynomolgi* B encapsulation as well. Furthermore, linkage of the QTL to the *P. berghei* encapsulation trait suggests that it plays an important role in broad-spectrum refractoriness to other Plasmodium species.

The identification of a single QTL with a large effect on the encapsulation phenotype is consistent with previous studies of the mode of inheritance of the bead melanizing trait and the *P. cynomolgi* B encapsulation trait (VERNICK and COLLINS 1989; GORMAN *et al.* 1996; GORMAN and PASKEWITZ 1997). The location of the QTL is also consistent with work by CREWS-OYEN *et al.* (1993) that demonstrated a genetic association of the *P. cynomolgi* B encapsulation phenotype with chromosome *2*. However, this study suggested that the *P. cynomolgi* B encapsulation trait was associated with the left arm of chromosome *2* rather than the right arm. Several conditions of the original studies could have led to an artificially strong association with the left arm. First, the genetic association was a secondary one, that is, the encapsulation phenotype was associated with an esterase phenotype, and the esterase phenotype was associated with an inversion on 2L (VERNICK and COLLINS 1989; CREWS-OYEN *et al.* 1993). Second, the principal experiment demonstrating the association of the encapsulation phenotype with the esterase phenotype used a

**TABLE 1**

**Plasmodium encapsulation phenotypes of $F_2$ progeny**

| Marker locus | Score[a] | Median |
|---|---|---|
| AGH157 | | |
| S/S | 17 ± 17 (6) | 0 |
| S/R | 70 ± 7 (34) | 100 |
| R/R | 63 ± 15 (9) | 100 |
| AGH46 | | |
| S/S | 14 ± 11 (10) | 0 |
| S/R | 78 ± 7 (30) | 100 |
| R/R | 66 ± 14 (10) | 100 |

[a] Values are means ± SE with sample size in parentheses.

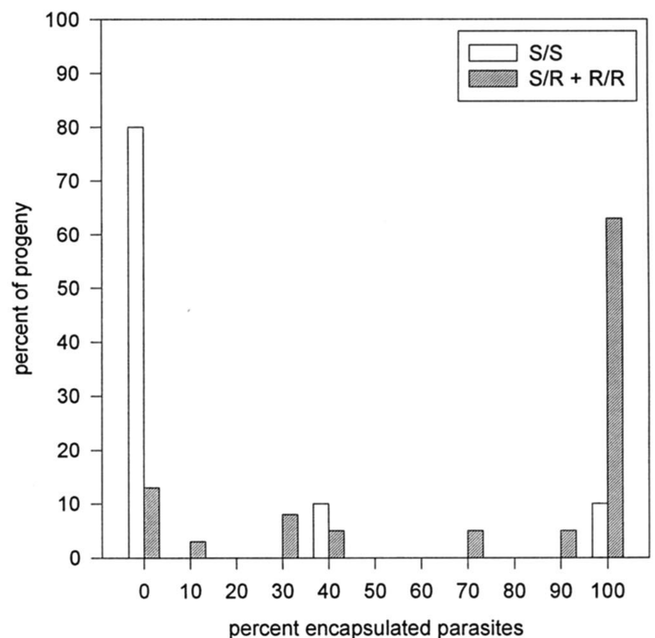


FIGURE 4.—Effect of genotype at AGH46 on the *P. berghei* encapsulation phenotypes of $F_2$ progeny. Phenotypes of homozygous susceptible mosquitoes ($n$ = 10, unshaded bars) and those with at least one refractory allele ($n$ = 40, shaded bars) are shown.

small data set (n = 38) (Vernick and Collins 1989), although Crews-Oyen et al. (1993) showed that lines reselected for the esterase phenotype were fixed for the same combination of inversion orientation and encapsulation phenotype. Third, the presence of inversion polymorphisms on both arms of the second chromosome would tend to inhibit genetic recombination and thereby increase the strength of the genetic association. Finally, in both sets of experiments there was significant under representation of one or more of the esterase phenotypes, suggesting that unidentified deleterious factors might have biased the samples. Therefore, although the original data linking a refractory locus to 2L were convincing, they are not inconsistent with a map location on 2R.

A recent QTL mapping study of the *P. cynomolgi* B encapsulation phenotype has implicated the same interval on 2R as that identified in this study (Zheng et al. 1997). A QTL accounting for ~50% of the phenotypic variance is linked to marker AGH175, which is located within the AGH157-AGH46 interval. Thus, chromosome region 7/8 is associated with melanotic encapsulation of CM-Sephadex beads and two species of malaria parasites. Because it is likely that the same QTL is responsible for all three encapsulation traits, we have tentatively labeled the QTL in the AGH157-AGH46 interval *Pen1* (*Plasmodium encapsulation 1*) in keeping with the terminology used by Zheng et al. 1997.

Because the molecular basis for the melanotic encapsulation trait is unknown, map-based cloning is a reasonable strategy for identifying the major genes involved in this interesting and potentially important trait. The results reported here describe the first step of this process and suggest that this is a feasible undertaking. One QTL was identified that exhibits a large effect on the phenotype; therefore, it is likely that fine-scale QTL mapping techniques can be used to map this locus to within ≤3 cM (Paterson 1995). The use of beads as a substitute for parasites will be advantageous at this stage of the endeavor for several reasons: they are technically much easier to work with, they can be used to score males as well as females (effectively increasing family sizes by a factor of two), and they reduce the experiment to experiment variability associated with different parasite infections.

In addition to generating information that may be useful in the control of malaria transmission, the identification of genes involved in melanotic encapsulation should also lead to a better understanding of insect immunity. The melanotic encapsulation trait is an example of a specialized type of humoral immune response in insects. Although vector mosquitoes normally do not melanize malaria parasites, acellular melanotic encapsulation is a common response to foreign objects in mosquitoes and other dipteran flies that have low numbers of hemocytes (for review, see Vey 1993). Melanization is mediated by the enzyme phenoloxidase, which acts on tyrosine and dopa to produce melanin. In mosquitoes, as in other arthropods, phenoloxidase exists in a zymogen form that can be activated by a serine protease (Ashida et al. 1990). Other biochemical factors responsible for activating and regulating phenoloxidase in mosquitoes are unknown, as is the relative importance of other enzymes that may be involved in formation of the melanin capsule. Because the melanization process must be activated or controlled differently in the refractory and susceptible strains, identifying the genetic differences between the strains should lead to the discovery of factors involved in the melanization pathway.

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