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The Role of Immune-Mediated Apparent Competition in Genetically Diverse Malaria Infections

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abstract: Competitive interactions between coinfecting genotypes of the same pathogen can impose selection on virulence, but the direction of this selection depends on the mechanisms behind the interactions. Here, we investigate how host immune responses contribute to competition between clones in mixed infections of the rodent malaria parasite *Plasmodium chabaudi*. We studied single and mixed infections of a virulent and an avirulent clone and compared the extent of competition in immunodeficient and immunocompetent mice (nude mice and T cell–reconstituted nude mice, respectively). In immunocompetent mice, the avirulent clone suffered more from competition than did the virulent clone. The competitive suppression of the avirulent clone was alleviated in immunodeficient mice. Moreover, the relative density of the avirulent clone in mixed infections was higher in immunodeficient than in immunocompetent mice. We conclude that immune-mediated interactions contributed to competitive suppression of the avirulent clone, although other mechanisms, presumably competition for resources such as red blood cells, must also be important. Because only the avirulent clone suffered from immune-mediated competition, this mechanism should contribute to selection for increased virulence in mixed infections in this host-parasite system. As far as we are aware, this is the first direct experimental evidence of immune-mediated apparent competition in any host-parasite system.

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Infections by pathogenic microorganisms are often genetically diverse, with hosts infected by more than one genotype of the same pathogen species (Thompson 2000). Interactions between coinfecting genotypes in such mixed infections have been identified as a potentially important factor in the evolution of medically relevant pathogen traits, including drug resistance and virulence (Read and Taylor 2001). Here, we are primarily concerned with how within-host interactions influence the evolution of virulence (the degree to which a pathogen reduces its host's fitness).

The research field of virulence evolution has a solid theoretical foundation (reviewed in Frank 1996; for recent development of the theory, see, e.g., Day and Proulx 2004; Alizon and van Baalen 2005). Briefly, faster exploitation of hosts and higher within-host density are assumed to increase a pathogen's between-host transmission rate. However, higher within-host density should also lead to increased virulence, which will shorten the time span for transmission because of host death. As a result of these conflicting selection pressures, there should be an intermediate optimum for virulence that maximizes a pathogen's fitness (between-host transmission success). However, this scenario concerns genetically uniform infections. When hosts are infected by multiple genotypes of the same pathogen species, there may be an additional selection pressure on virulence as a result of interactions between coinfecting genotypes. For example, it is often assumed that coinfecting genotypes compete over limited host resources. Such competitive interactions have traditionally been expected to select for faster exploitation of hosts and thereby higher virulence because a "prudent" genotype that exploits hosts relatively slowly should be outcompeted by more virulent genotypes (Frank 1996). However, other forms of within-host interactions may also occur, and recently it has been realized that the direction of selection on virulence imposed by such interactions depends on their mechanistic details (Taylor et al. 1997; Chao et al.

2000; Read and Taylor 2000; Brown et al. 2002; West and Buckling 2003). There are several different types of potential interactions, including resource competition, interference competition, and immune-mediated interactions. Here, we report an experimental analysis of the latter.

Interactions between two (or more) coinfecting pathogen genotypes mediated by host immune responses are analogous to interactions between two prey species mediated by a shared predator species. Such indirect interactions have received much attention in community ecology and are considered to play an important role in structuring ecological assemblages (Holt and Lawton 1994; Menge 1995). Interestingly, indirect effects mediated by a shared predator can have both negative and positive effects on the prey species, depending on the behavioral and numerical response of the predator to a change in the abundance of its prey. If an increased abundance of one prey species increases the abundance of the predator (a numerical response), it can have a negative effect on the other prey species, an effect termed "apparent competition" by Holt (1977). However, if an increased abundance of one prey species leads the predator to divert its attention to this species (a behavioral response), it can have a positive effect on the other prey species, that is, "apparent commensalism" (Abrams and Matsuda 1996). The relative importance of numerical and behavioral responses will determine whether the net effect is a positive or negative interaction between prey species (Abrams and Matsuda 1996).

Applying this community ecology theory to immune-mediated interactions between coinfecting pathogen genotypes yields the following two scenarios for selection on virulence in mixed infections. First, if the immune response is genotype transcending and the strength of the response increases with the density of parasites, so that a genotype that finds itself in a mixed infection encounters a stronger immune response than it would have induced on its own, this could result in immune-mediated apparent competition. Given that a more virulent genotype induces a stronger response (because it has higher density), it seems likely that immune-mediated competition will be asymmetrical, so that an avirulent genotype suffers more from the presence of a virulent genotype than vice versa. If so, this kind of apparent competition should reduce the relative fitness of the avirulent genotype in mixed infections and thereby contribute to selection for increased virulence.

Second, if the immune response is genotype specific and the immune system focuses primarily on the most virulent genotype (because it is initially most abundant), it is possible that a genetically diverse infection can allow a genotype with a relatively low exploitation rate to partly elude the attention of the immune system (Taylor et al. 1997; Read and Taylor 2000; Almogy et al. 2002), especially since the immune system has a tendency to retain its focus on the antigenic variant that stimulated the response originally, a phenomenon known as "original antigenic sin" (Janeway and Travers 1996). This can result in an avirulent genotype doing better in genetically diverse infections than it does alone (immune-mediated facilitation, analogous to apparent commensalism between prey species). This kind of interaction should increase the relative fitness of the avirulent genotype and therefore has the potential to cause selection for reduced virulence in genetically diverse infections.

Results from empirical studies of the relationship between the virulence of a genotype and its performance in genetically diverse infections are mixed. In some cases, the most virulent genotype is also the most competitive, but several studies have found the opposite (reviewed in Read and Taylor 2000, 2001; see also Gower and Webster 2005; de Roode et al. 2005*b*). One explanation for these contrasting results is that the relative importance of different mechanisms of interaction differs between host-pathogen systems. To understand how genetically diverse infections affect the evolution of virulence, we therefore need to elucidate the mechanistic basis of interactions between coinfecting genotypes. Here, we investigate how host immune defense contributes to interactions between clones in mixed infections of the rodent malaria parasite *Plasmodium chabaudi*. In laboratory mice, *P. chabaudi* is commonly used as a model system for human malaria (e.g., Langhorne et al. 2002). Understanding the role of the immune system in mediating interactions between coinfecting clones is particularly interesting in this model of an important human disease because the extent of such interactions, and hence the direction and strength of selection on virulence in mixed infections, could be influenced by vaccination (Mackinnon and Read 2004*a*).

Previous experiments with *P. chabaudi* have shown that there is strong within-host competition between clones (Snounou et al. 1992; Taylor et al. 1997; de Roode et al. 2003, 2004*b*) and that relatively avirulent clones suffer most (de Roode et al. 2005*b*; Bell et al., forthcoming). How does host immune defense contribute to this pattern? *Plasmodium chabaudi* infections induce immune responses that are at least partially cross-reactive between clones (Jarra and Brown 1989; Buckling and Read 2001; Mackinnon and Read 2003), so immune-mediated competition could be the proximate cause of the suppression of avirulent clones. However, there is some specificity of the response (Jarra and Brown 1989; Buckling and Read 2001; Martinelli et al. 2005). Thus, it is also possible that host immune defense alleviates competition caused by, for example, lack of resources. To investigate whether host immune responses enhance or alleviate competition, we per-

formed an experiment with immunodeficient mice. The immune response to *P. chabaudi* is initially cell mediated, then followed by the production of antibodies (Langhorne et al. 2002; Stevenson and Riley 2004). Both these responses are dependent on helper T cells. We therefore used nude mice (which are athymic and therefore lack T cells) to investigate whether and how host immune defense contributes to interactions between coinfecting clones. Specifically, we compared the extent of competition between a virulent and an avirulent *P. chabaudi* clone in immunodeficient nude mice and immunocompetent mice (nude mice reconstituted with T cells). If the competition observed in previous studies is at least partly a result of apparent competition through T cell–mediated immunity, we expect that competition should be reduced in nude mice. In contrast, if clone-specific immunity alleviates other forms of competition, we expect competition to be more intense in nude mice than in reconstituted mice.

Methods

Plasmodium chabaudi

Like other *Plasmodium* species, *Plasmodium chabaudi* replicates asexually in red blood cells (RBCs). Infected RBCs rupture synchronously, each releasing six to eight parasites, which infect new RBCs (Carter and Diggs 1977). In *P. chabaudi,* this asexual replication cycle is repeated every 24 h. The density of asexual parasites reaches a peak 4–11 days postinfection (depending on the inoculation dose), followed by a rapid decrease over the next few days (Timms et al. 2001). This acute phase of the infection is followed by a chronic phase with recurrent lower peaks. A small proportion (≈1%) of the asexuals differentiate into gametocytes, the stage responsible for transmission to new hosts. Gametocytes are produced mainly toward the end of the acute phase of the infection (Buckling et al. 1997). Asexual parasitemia is positively correlated with gametocytemia, which in turn predicts transmission success (Taylor and Read 1998; Mackinnon and Read 2004*b*).

We used two clones of *P. chabaudi,* denoted AS and AJ. Both clones were originally isolated from thicket rats (*Thamnomys rutilans*) in the Central African Republic (Beale et al. 1978). The clones are maintained as frozen stabilates. We use subscript codes to identify their position in the clonal history; the codes of the clones used here are AS_{11930} (derived from AS by selection for pyrimethamine and subsequently passaged several times through mice for maintenance purposes) and AJ_{4777}. The AS clone has lower peak parasitemia and is less virulent (causing less anemia and weight loss in mice) than the AJ clone (de Roode et al. 2004*b*).

Mice

We used female BALB/c-*nu/nu* mice ("nude mice"; Harlan UK). The mutation *nu* is a recessive mutation that blocks the development of the thymus so that nude mice have no mature T cells, whereas heterozygotes (*nu/+*) have a normal immune system (Pantelouris 1968). The nude mutation also has some pleiotropic effects. Most importantly, nudes are hairless and smaller than normal mice. These pleiotropic effects could potentially affect resistance to infections. Consequently, it would be difficult to ascertain that a difference in within-host interactions between *nu/+* and *nu/nu* mice was due to the presence/absence of T cell–dependent immunity. We therefore used *nu/nu* mice reconstituted with T cells as controls, rather than using *nu/+* mice. Reconstituted nude mice are nude mice that have received T cells from *nu/+* mice. Thus, nude mice and reconstituted nude mice differ only with respect to whether they have T cells or not.

Mice were kept in individually ventilated cages in a 12L : 12D cycle. They were fed on 41B maintenance diet (Harlan UK), and their drinking water was supplemented with 0.05% para-aminobenzoic acid to enhance parasite growth (Jacobs 1964).

Reconstitution of Nude Mice by Adoptive Transfer of Lymphocytes

Pooled lymphocytes purified from spleen and lymph nodes of *nu/+* mice were depleted of B cells using CD19 magnetic beads (AUTOMACS, Miltenyi Biotech) according to the manufacturer's protocol. When mice were 10–12 wk old, 15×10^6 B cell–depleted lymphocytes were transferred intravenously into nude recipients.

Setup and Sampling

Mice of each phenotype were infected with 10^5 AS, 10^5 AJ, or 10^5 AS and 10^5 AJ parasites. We used the same dose of each clone in single and mixed infections (rather than the same total dose in single and mixed infections) because the aim of the study was to compare the performance of a clone when it is on its own with its performance when it is in a mixed infection. Thus, type of infection (single or mixed) and total infective dose are confounded. However, a twofold difference in infective dose has negligible effects on the population dynamics of the parasite (even a 10-fold difference is barely detectable; Timms et al. 2001).

Among nude mice, there were seven mice in each treatment group, and among reconstituted mice, there were six in each treatment. Inoculations were done contem-

poraneously, as described by de Roode et al. (2004*b*). Mice were 12–14 wk old at infection.

We measured RBC density (using flow cytometry; Beckman Coulter) and took blood samples from the tail for polymerase chain reaction (PCR) analysis and thin blood smears on days 0, 2, and 4 and then daily up to day 18 postinoculation, when the experiment was terminated. Blood smears were fixed with methanol and stained with Giemsa.

We quantified parasites in three ways. First, parasitemia (the proportion of infected RBCs) was determined by counting the number of infected cells per 500 RBCs in at least four microscopic fields under 1,000 × magnification. Immunological and parasitological studies of rodent malaria have generally used this measure of infection intensity to estimate host resistance (i.e., the ability to control the intensity of the infection). To allow for comparison with previous studies, we therefore used parasitemia as the dependent variable when testing whether the mouse phenotypes in our experiment differed in resistance. Second, parasite density (parasites/μL blood) was determined by clone-specific real-time quantitative PCR (qPCR) assays, which allow us to estimate the density of each of the morphologically indistinguishable clones in mixed infections. We used this measure of infection intensity when investigating how the experimental treatment affected the performance of the different clones because it is a measure of the absolute number of parasites in a mouse, as opposed to parasitemia, which is a ratio of parasites to RBCs. Finally, parasite density was also determined by multiplying parasitemia and RBC density values. Because different clones cannot be distinguished morphologically, this method cannot be used to estimate the density of each clone in mixed infections. However, we used estimates of parasite density in single infections obtained by this method to calibrate the qPCR assays. Parasitemia and parasite density give slightly different patterns of parasite dynamics (because parasite density depends on RBC density).

Quantitative PCR

In the morning, when most parasites in the peripheral blood were in the ring or early trophozoites stages, 5 μL of tail blood was taken from each mouse and added to 100 μL of citrate saline on ice. Samples were subsequently pelleted by centrifugation, the citrate saline was removed, and the blood was stored at −80°C until required. DNA extraction was performed using the BloodPrep kit (Applied Biosystems) on the ABI Prism 6100 Nucleic Acid PrepStation according to manufacturer's instructions. DNA was eluted in a total volume of 200 μL and stored at −80°C until quantification. Clone-specific PCR primers and a common minor groove–binder (MGB) probe, targeting the *P. chabaudi ama1* gene, were designed using Primer Express (Applied Biosystems) software. The amplicon lengths are 127 and 129 bp for clones AJ and AS, respectively. Clones AS and AJ were quantified in separate assays, using the appropriate clone-specific primers. A 25-μL PCR reaction was run including 2 μL of DNA and the following components: 1.5 μL each of the forward (AS: 5′ GGA AAA GGT ATA ACT ATT CAA AAT TCT AAG GT 3′; AJ: 5′ GGA AAA GGT ATA ACT AAT CAA AAA TCT ACT AAA 3′) and reverse primers (AS: 5′ AAT TGT TAT AGG AGA AAT GTT TAC ATC TGT TTG 3′; AJ: 5′ GTG TTA TAG GAG AAA TGT GTA CAT CTG TTT T 3′), both at a final concentration of 300 nM; 12.5 μL of TaqMan Universal PCR Master Mix (hot start); 1 μL of MGB probe (5′ 6-FAM-ATC CTC CTT CTC TTA CTT TC-MGB 3′) at a final concentration of 200 nM; and 6.5 μL of sterile water. Amplification was performed on an ABI Prism 7000 real-time thermal cycler with an initial denaturation of 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Absolute quantification of experimental samples was performed by comparison of threshold cycle numbers against a standard curve covering six orders of magnitude. Standard samples were added in triplicate. We calibrated the standard curve by regressing the values obtained from the qPCR against the densities obtained from smear counts (i.e., proportion of infected RBCs × RBC density) for all single infections over days 5–12. Densities obtained from qPCR and smear counts were highly correlated (AS, $r = 0.93$; AJ, $r = 0.94$). Each qPCR run included DNA samples of the mouse host and the nontarget clone as negative controls. The real-time qPCR assays used here do not discriminate between asexual parasites and gametocytes. Consequently, parasite numbers obtained also include gametocytes (when present), although gametocytes are always two to three orders of magnitude less numerous than asexual parasites (Buckling et al. 1997; Buckling and Read 2001; Mackinnon and Read 2003) and hence contribute minimally to overall quantifications.

To compare the repeatability (Lessells and Boag 1987) of parasite densities obtained from qPCR and smear counts (percentage of infected RBCs × RBCs/μL), we performed a separate experiment where 10 mice were infected with AJ or AS. On days 5, 7, 10, 12, and 14 postinoculation, we took two blood samples for qPCR analysis, two blood smears, and two measures of RBC density from each mouse. The two blood samples for qPCR analysis were extracted on different plates and quantified in different qPCR runs. The repeatability of parasite densities obtained by qPCR was 0.98, compared with a repeatability of 0.92 obtained from smear counts. We conclude that the qPCR technique is at least as repeatable as conventional methods.

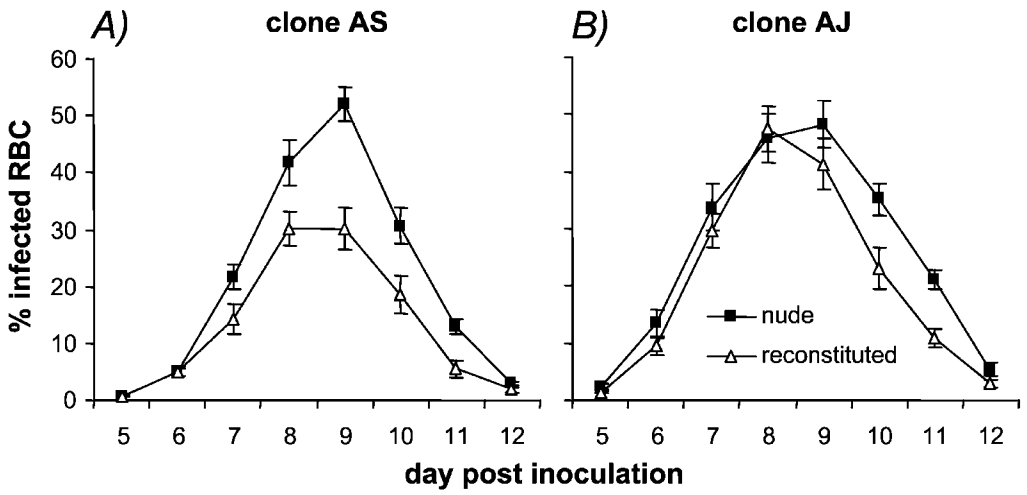

Figure 1: Parasitemia (percentage of infected red blood cells; mean ± SE) over time in nude and reconstituted mice. *A*, Mice infected with parasite clone AS. *B*, Mice infected with parasite clone AJ.

Statistical Analyses

The main analyses to investigate whether and how T cell–dependent immune responses influenced parasite and RBC dynamics were performed as repeated-measures analyses, using data from the part of the acute phase when the immune response was effective (i.e., when there was a significant difference in parasitemia between nude and reconstituted mice). These analyses were performed with PROC MIXED in SAS 8.2 (SAS Institute 1999) using the REPEATED statement (subject = mouse) and the Satterthwaite approximation of the denominator degrees of freedom. Of the three covariance structures proposed for repeated-measures analysis by Littell et al. (1996), the autoregressive covariance structure AR(1) generally gave the best fit, as assessed by the Bayesian information criterion, and we therefore used this for all analyses. All other analyses were performed with PROC GLM using type 3 sums of squares. Parasite density values were log transformed because we are interested in testing for a proportionate difference in competitive suppression between different treatments. Analyses of clonal proportions in mixed infections were performed with angularly transformed values.

Results

Mortality

Of the nude mice with mixed infections, one died (after sampling) on day 10, three on day 11, one on day 12, and the last two on day 13. During this period (days 0–13), four nude mice infected with AJ died on day 11, and one nude mouse infected with AS died on day 12. None of the reconstituted mice died during this period. To avoid having too many missing values, we restricted analyses to days 0–12 unless otherwise stated.

Parasitemia of AS and AJ in Different Mouse Phenotypes

To test whether nude and reconstituted mice differed in resistance to *Plasmodium chabaudi*, we compared their parasitemia values (percentage of infected RBCs) in single infections (fig. 1). In mice infected with AS, nude mice had higher parasitemia than reconstituted mice from 2 days before the peak and onward ($P < .05$, as assessed by one-way ANOVA, from day 7 postinoculation). In mice infected with AJ, nude and reconstituted mice did not differ in peak parasitemia, but reconstituted mice cleared parasites faster than nude mice (significant difference from day 10). We thus conclude that reconstituted mice were indeed more resistant than nude mice.

As in previous studies (e.g., de Roode et al. 2004*b*), AJ had higher peak parasitemia than AS, although this difference was found only in reconstituted mice (reconstituted: $F = 6.1$, df $= 1, 10$, $P = .033$; nude: $F = 0.05$, df $= 1, 12$, $P = .83$).

Competition in Immunocompetent Mice

We first tested whether there was competition within the reconstituted mice. To allow comparison with previous studies that used C57 and CBA mice (de Roode et al. 2004*b*, 2005*b*), we used the same statistical approach; that is, for each clone, we compared its total parasite density

during the acute phase (obtained by summing the daily densities during days 0–12) in single and mixed infections. The AS clone was suppressed by 68% and the AJ clone by 26% (AS: $F = 108.2$, df $= 1, 10$, $P < .001$; AJ: $F = 29.5$, df $= 1, 10$, $P < .001$). Thus, as in previous studies, competition between AS and AJ was asymmetrical, so that the avirulent clone AS suffered most.

Immune-Mediated Interactions

To investigate how T cell–dependent immunity contributed to the competition observed in reconstituted mice, we tested for an interaction between mouse phenotype and type of infection (single or mixed). If competition is at least partly immune mediated, there should be a significant interaction between mouse phenotype and type of infection such that competitive suppression (the difference in parasite density a clone achieves in single and mixed infections) is reduced in nude mice. Alternatively, if immune-mediated facilitation alleviates other forms of competition, there should be a significant interaction such that the suppression is stronger in nude mice than in reconstituted mice.

We first ran a repeated-measures analysis with parasite densities on days 7–12 as dependent variables. The analysis was restricted to day 7 and onward because immune-mediated competition is likely to occur only during the later stage of the infection, when the immune response is effective, and day 7 was the first day when reconstituted mice had lower parasitemia than nude mice (fig. 1). We then tested whether the total parasite density during the acute phase of the infection was affected by immune-mediated competition. We analyzed each clone separately.

Parasite clone AS. The density of AS over time in the different types of infections and mouse phenotypes is shown in figure 2*A*. A repeated-measures analysis of days 7–12 (table 1) showed that there were significant main effects of mouse phenotype and type of infection (single or mixed) such that AS densities were higher in nude mice than in reconstituted mice and higher in single infections than in mixed infections. There was also a significant three-way interaction between phenotype, infection, and day, indicating that T cell–dependent immunity indeed had an effect on the extent of competitive suppression of AS in mixed infections but that this effect varied over time. To investigate this further, we divided the data set into two parts—days 7–9 and days 10–12—and repeated the analysis with each of these (table 1). As in the analysis of days 7–12, there were significant main effects of phenotype and infection during both days 7–9 and days 10–12 such that AS densities were higher in nude mice than in reconstituted mice and higher in single infections than in mixed infections. However, the phenotype-by-infection interaction was significant only during days 10–12. Inspection of figure 2*A* and 2*C* shows that during days 10–12, the suppression of AS in mixed infections was greater in reconstituted mice than in nude mice. Thus, there was immune-mediated competition.

Could the immune-mediated competition that occurred toward the end of the acute phase account for the reduction in the total number of AS parasites present during the acute phase observed in reconstituted mice? A two-way ANOVA revealed main effects of mouse phenotype ($F = 7.1$, df $= 1, 21$, $P = .014$) and type of infection ($F = 353.6$, df $= 1, 21$, $P < .001$), but there was no evidence of a phenotype-by-infection interaction ($F = 1.6$, df $= 1, 21$, $P = .22$). As detailed above, mice started to die on day 10, so the calculation of total density was restricted to days 0–11 to maintain statistical power. An analysis including only the mice that survived to day 12 gave the same result. Thus, there was no evidence that the overall competitive suppression of AS was caused by immune-mediated competition.

Parasite clone AJ. The density of AJ over time in the different types of infections and mouse phenotypes is shown in figure 2*D*. In the repeated-measures analysis of days 7–12 (table 2), there were significant main effects of mouse phenotype and type of infection such that AJ densities were higher in nude mice than in reconstituted mice and higher in single infections than in mixed infections, but neither the phenotype-by-infection nor the three-way interaction between phenotype, infection, and day was significant. Although the three-way interaction was not significant, for comparison with the analysis of AS, we performed analyses with days 7–9 and days 10–12 separately, but in neither case was the interaction between mouse phenotype and type of infection significant ($P \geq .3$; fig. 2*E*, 2*F*). In the analysis of total AJ numbers, single infections contained more AJ parasites than did mixed infections ($F = 75.2$, df $= 1, 21$, $P < .001$), but the difference between mouse phenotypes was not statistically significant ($F = 3.4$, df $= 1, 21$, $P = .079$). There was no phenotype-by-infection interaction ($F = 0.1$, df $= 1, 21$, $P = .73$). Thus, there was no evidence that the extent of suppression of AJ in mixed infections was T cell dependent.

Relative Densities of Clones in Mixed Infections

To test whether the two clones were differently affected by the presence/absence of T cell–dependent immunity when in a mixed infection, we compared the relative densities of the clones in nude and reconstituted mice. The densities of AS and AJ over time in mixed infections in the different mouse phenotypes are shown in figure 3*A*. The analysis was performed as a repeated-measures analysis with proportion of AS against mouse phenotype, day, and their

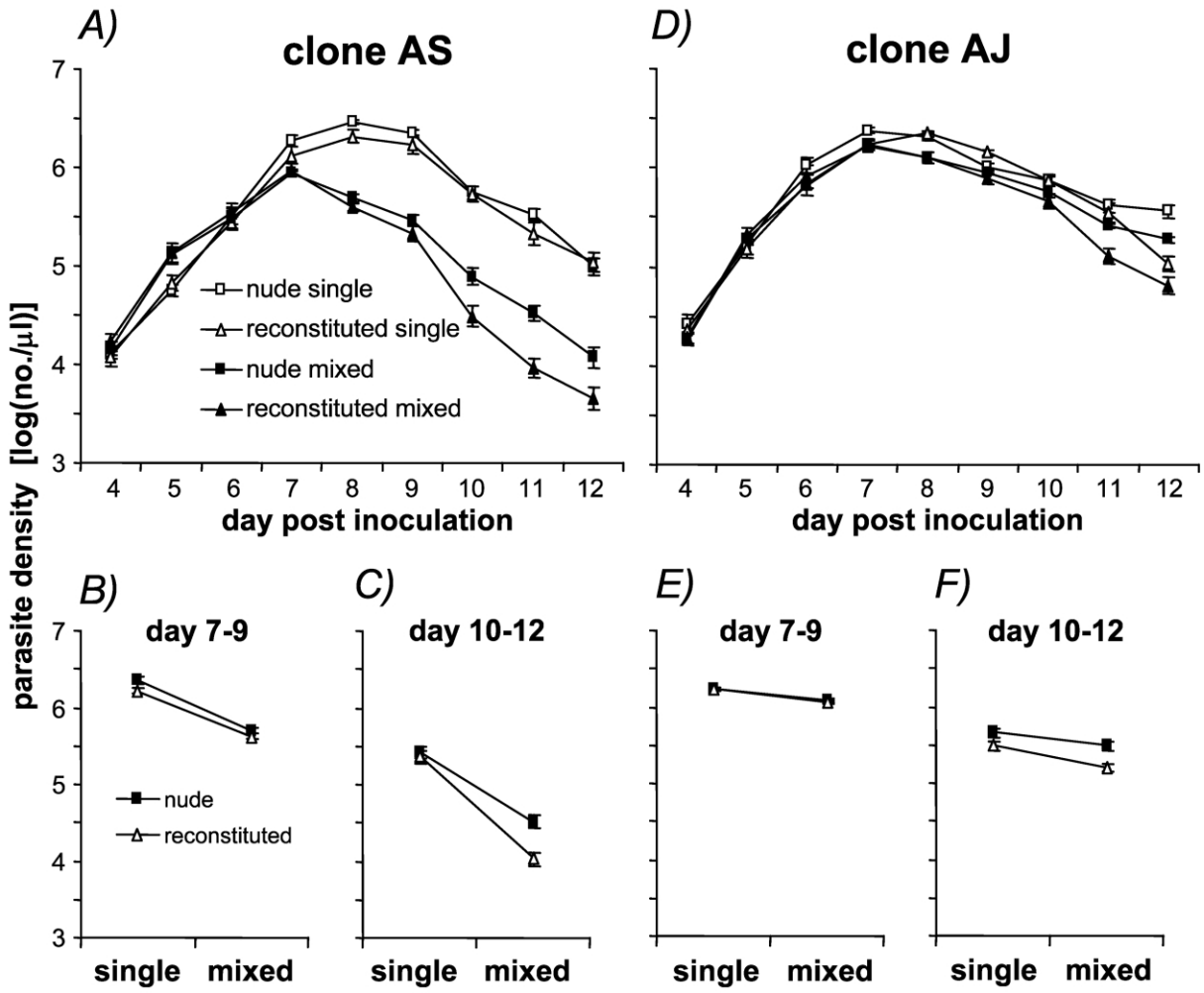

Figure 2: Densities of parasite clones AS and AJ in single and mixed infections in nude and reconstituted mice. *A*, Density (mean ± SE) of AS over time. *B*, Average density (least-squares mean ± SE from repeated-measures analysis) of AS on days 7–9. *C*, Average density of AS on days 10–12. *D*, Density of AJ over time. *E*, Average density of AJ on days 7–9. *F*, Average density of AJ on days 10–12.

interaction. As previously, the analysis was restricted to the period when host immune defense was effective, that is, days 7–12. There was a significant effect of day ($F = 78.0$, df $= 5, 44.2$, $P < .0001$), with proportion of AS decreasing over time, and a significant effect of mouse phenotype ($F = 5.9$, df $= 1, 18.1$, $P = .026$), with reconstituted mice having a lower proportion of AS than nude mice. However, there was also a day-by-phenotype interaction ($F = 3.0$, df $= 5, 44.2$, $P = .021$), indicating that this effect varied over time. To investigate this further, we again divided the data set into two parts—days 7–9 and days 10–12—and repeated the analysis with each of these. During days 7–9, there was no difference in proportion of AS between phenotypes (phenotype: $F = 1.4$, df $= 1, 15.4$, $P = .26$; day: $F = 20.6$, df $= 2, 24.4$, $P < .0001$; phenotype × day: $F = 1.7$, df $= 1, 24.4$, $P = .20$; fig. 3*B*). During days 10–12, reconstituted mice had a lower proportion of AS than nude mice (phenotype: $F = 5.6$, df $= 1, 11.4$, $P = .037$; day: $F = 3.4$, df $= 1, 17.8$, $P = .054$; phenotype × day: $F = 3.9$, df $= 1, 17.8$, $P = .039$; fig. 3*B*). Thus, T cell–dependent immunity reduced the relative density of AS in mixed infections, but this effect was significant only during days 10–12.

RBC Density

Uninfected RBCs form an important resource for malaria parasites. To assess whether the potential for competition over this resource differed between mouse phenotypes, we compared the RBC densities of nude and reconstituted

Table 1: Repeated-measures analyses of the density of parasite clone AS in mixed and single infections in nude and reconstituted mice

	Days 7–12			Days 7–9			Days 10–12		
Effect	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Mouse			<.0001			.10			<.0001
Phenotype	1, 25.7	13.2	.0012	1, 21.3	7.6	.012	1, 22.6	12.1	.0020
Infection	1, 25.7	293.5	<.0001	1, 21.3	286.6	<.0001	1, 22.6	209.0	<.0001
Day	5, 93.6	249.0	<.0001	2, 40.4	20.8	<.0001	2, 39.4	138.9	<.0001
Phenotype × infection	1, 25.7	3.0	.096	1, 21.3	.9	.36	1, 22.6	7.5	.012
Phenotype × day	5, 93.6	2.7	.026	2, 40.4	.5	.62	2, 39.4	3.6	.036
Infection × day	5, 93.6	23.8	<.0001	2, 40.4	43.2	<.0001	2, 39.4	2.5	.095
Phenotype × infection × day	5, 93.6	3.4	.0070	2, 40.4	.6	.57	2, 39.4	.9	.41

Note: The factors mouse phenotype (nude or reconstituted), type of infection (single or mixed), and day of infection were treated as fixed effects, while mouse individual was treated as a random effect. For the effect of mouse individual, $Z = 8.8$ for days 7–12, 1.6 for days 7–9, and 8.59 for days 10–12.

mice with mixed infections (fig. 4). The statistical analysis was performed in the same way as the analyses of parasite densities above, that is, as a repeated-measures analysis of days 7–12. There were significant effects of day ($F = 129.5$, df $= 5, 47.2$, $P < .0001$) and phenotype ($F = 7.4$, df $= 1, 10.1$, $P = .022$), with nude mice having lower RBC density than reconstituted mice. There was also a significant interaction between day and phenotype ($F = 4.5$, df $= 5, 47.2$, $P = .002$). We therefore repeated the analysis with days 7–9 and days 10–12 separately. The difference in RBC density between phenotypes was significant during both these periods (days 7–9: $F = 5.5$, df $= 1, 11.8$, $P = .038$; days 10–12: $F = 12.4$, df $= 1, 11.1$, $P = .0048$), although the effect was apparently stronger during the latter period. Thus, although nude mice lost more RBCs than reconstituted mice, AS suffered more from competition in reconstituted mice than in nude mice, implying that the immune response played a large part in causing this competition.

Discussion

We found that the competitive suppression of the avirulent clone AS was alleviated toward the end of the acute phase of the infection (days 10–12 postinfection) in immunodeficient nude mice as compared to immunocompetent reconstituted mice. Thus, AS suffered from immune-mediated apparent competition. In contrast, there was no significant effect of mouse phenotype on the suppression of the virulent clone AJ. Apparent competition has previously been demonstrated in several different types of ecological assemblages—between prey species sharing a predator, between plants sharing an herbivore, and between hosts sharing a parasite or parasitoid (reviewed in Chaneton and Bonsall 2000)—but this study is to our knowledge the first to experimentally demonstrate apparent competition between coinfecting pathogen clones mediated by host immune responses. Apparent competition between prey, plant, or host species is typically asymmetrical, so that only one of the victim species is affected by the presence of the other (Chaneton and Bonsall 2000), which we also found.

Relative Importance of Immune-Mediated Competition

The immune-mediated competitive suppression of AS occurred only toward the end of the acute phase of the infection. Because the parasite density during this period is low compared to the peak density, this immune-mediated competition did not have a measurable effect on the total number of parasites present during an infection. The competition that occurred around the peak of the acute phase (days 7–9), which accounts for most of the reduction in total parasite density, must instead have been caused by mechanisms other than T cell–dependent immunity. Furthermore, even during days 10–12, there was also strong competition in the absence of T cell–dependent

Table 2: Repeated-measures analysis of the density of parasite clone AJ in mixed and single infections in nude and reconstituted mice

Effect	df	*F*	*P*
Mouse			<.0001
Phenotype	1, 34.2	14.2	.0006
Infection	1, 34.2	38.4	<.0001
Day	5, 86.3	163.5	<.0001
Phenotype × infection	1, 34.2	1.3	.27
Phenotype × day	5, 86.3	7.9	<.0001
Infection × day	5, 86.3	2.8	.023
Phenotype × infection × day	5, 86.3	1.6	.16

Note: Analyses are for days 7–12 postinfection. The factors mouse phenotype (nude or reconstituted), type of infection (single or mixed), and day of infection were treated as fixed effects, while mouse individual was treated as a random effect. For the effect of mouse individual, $Z = 4.2$.

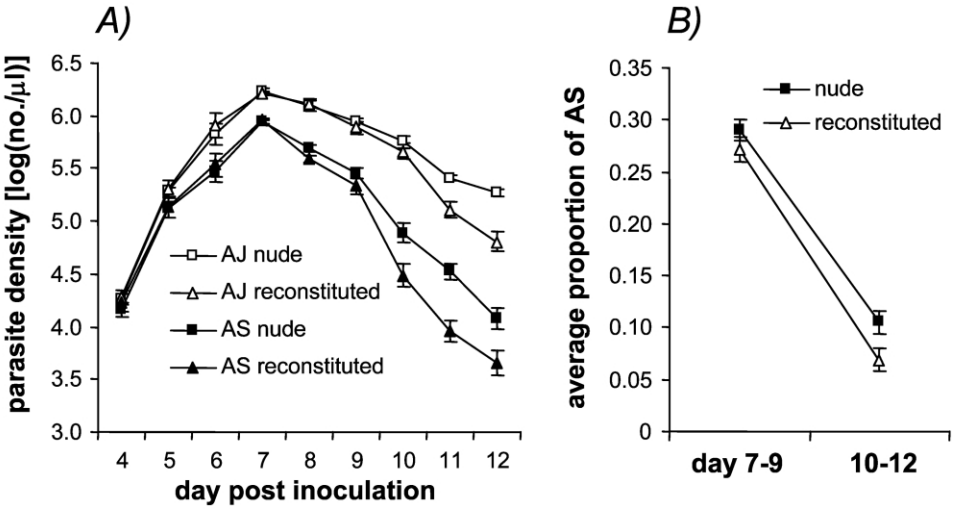

Figure 3: Relative success of parasite clones AS and AJ in mixed infections in nude and reconstituted mice. *A*, Densities of AS and AJ over time in mixed infections in nude and reconstituted mice. *B*, Average proportion (least-squares mean ± SE from repeated-measures analysis) of AS in mixed infections during days 7–9 and days 10–12 in nude and reconstituted mice.

immunity (fig. 2). Thus, other mechanisms are clearly also important during this period. What are these other mechanisms?

First, they could be T cell–independent innate immune responses. Innate and adaptive immunity to malaria are intimately linked. For example, $CD4^+$ T cells produce IFN-γ, which stimulates macrophages to produce antiparasite molecules such as nitrous oxide (Stevenson and Riley 2004). It is nonetheless possible that parts of the innate immune system could contribute to resistance in the absence of T cells. Further, a common finding is that mutant mice that lack specific components of the adaptive immune system compensate for their lack of adaptive immunity by a higher activity of innate immunity (e.g., Kaufmann and Ladel 1994). Thus, even though T cell–dependent immunity is considered to be by far the most important component of defense against malaria (Langhorne et al. 2002; Stevenson and Riley 2004), the redundancy of the immune system should make our estimate of the significance of immune-mediated competition conservative.

Second, competition could be caused by lack of resources, such as RBCs or glucose. The RBC density decreases dramatically during the infection, and lack of uninfected RBCs is known to affect parasite population growth in single infections (Yap and Stevenson 1994). It is therefore plausible that red cell availability plays an important role also in competition (Hellriegel 1992). The minimum RBC density was lower in nude mice than in reconstituted mice. Hence, the potential for competition over this resource was higher in nude mice. It is possible that increased competition for resources in nude mice partly obscured the release from immune-mediated competition. Again, this should make our estimate of the importance of immune-mediated competition conservative.

Third, an interesting possibility is that competition is due to direct interference between coinfecting genotypes, although such allelopathic mechanisms have so far been

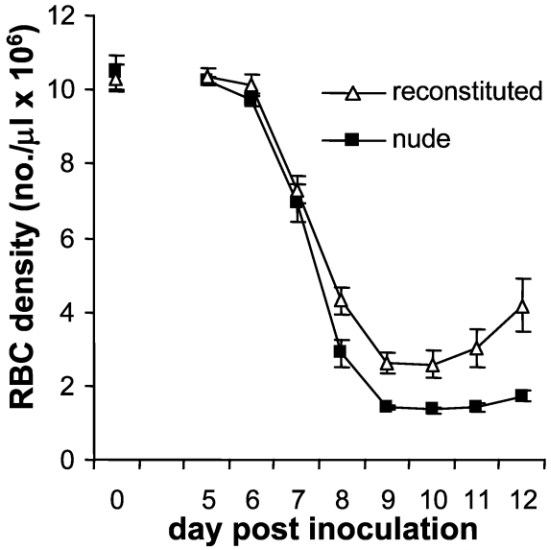

Figure 4: Red blood cell density (mean ± SE) over time in nude and reconstituted mice with mixed infections. Note that days 1–4 (when there is no change in red blood cell density) are omitted to enhance resolution.

described only in bacteria and viruses (Hart and Cloyd 1990; Riley and Wertz 2002).

How General Are Our Results?

Can we expect the outcome of immune-mediated competition observed in this study to be typical for *Plasmodium chabaudi*? One factor that can affect the extent of immune-mediated competition is the antigenic similarity of coinfecting clones. The more antigenically similar coinfecting clones are, the more immune-mediated competition should be expected. Experiments where mice were immunized with one clone and then challenged with mixtures of these clones showed that the responses to AJ and AS had a relatively high degree of cross-reactivity (K. Grech, B. Chan, and A. F. Read, unpublished data). It therefore seems unlikely that another clone combination would have shown markedly stronger immune-mediated competition. Another factor that can affect the extent of immune-mediated competition is the timing of infections. In our experiment, infections were simultaneous, but in nature, infections are sometimes sequential. Experiments with *P. chabaudi* have shown that competition is stronger when infections are sequential (de Roode et al. 2005*a*). This could be because the second clone encounters an environment with fewer resources (e.g., RBCs) or a stronger immune response. That immune-mediated competition is potentially important when infections are sequential is demonstrated by studies where mice have been immunized with one clone, cured and allowed to recover, and then inoculated with a heterologous clone. Such experiments have without exception found that immunity to one clone suppresses heterologous clones (e.g., Buckling and Read 2001; Mackinnon and Read 2003). Still, the relative importance of immune-mediated and resource competition when infections are sequential remains to be determined.

Is the extent of immune-mediated competition in this rodent malaria system representative for human malaria? *Plasmodium chabaudi* shares many features with the most virulent of the *Plasmodium* species infecting humans, *Plasmodium falciparum*. However, there is also an important difference that could affect competition: the peak parasite density is about an order of magnitude lower in *P. falciparum* than in *P. chabaudi* (Mackinnon and Read 2004*b*). It seems likely that a lower parasite density will reduce competition for resources, for example, uninfected RBCs. If anything, the relative importance of immune-mediated competition could therefore be expected to be higher in *P. falciparum* than in *P. chabaudi* infections.

Evolutionary Consequences of Apparent Competition

The evolutionary consequences of immune-mediated competition depend on how it affects the relative fitness of the coinfecting clones, which in turn depends on how it affects the production of transmission stages (gametocytes) and subsequent transmission to mosquitoes. In this study, we did not measure gametocyte density and transmission, but previous studies have shown that the frequency of a clone among the asexual parasites in mixed infections predicts its transmission success (Taylor and Read 1998; de Roode et al. 2005*b*). We found that only the avirulent clone was affected by immune-mediated competition. Moreover, in mixed infections, the avirulent clone obtained a lower share of the overall parasite density in immunocompetent as compared to immunodeficient hosts. Even though these effects were quite weak and occurred only toward the end of the acute phase of the infection, they could still have had a significant effect on the relative fitness of coinfecting clones because gametocytes are generally produced mainly toward the end of the acute phase (e.g., Buckling et al. 1997). Taken together, these facts imply that the relative fitness of the avirulent clone in mixed infections was lower in immunocompetent than in immunodeficient mice. By using just two clones to investigate how virulence influences sensitivity to immune-mediated competition, we cannot formally establish a general relationship between these traits; there is a possibility that sensitivity to immune-mediated competition is determined by another unknown trait that is unrelated to virulence across a wider range of clones. However, it is difficult to see what kind of trait this could be that would not also affect virulence, and we therefore tentatively conclude that the link between virulence and sensitivity found here represents a general pattern. If so, T cell–dependent immunity should contribute to selection for increased virulence in mixed infections.

Large-scale vaccination campaigns against malaria could have the undesirable consequence of prompting evolution of parasites that are more virulent to unvaccinated hosts (Gandon et al. 2001; Mackinnon and Read 2004*a*). In several mathematical models, evolution of higher virulence occurs because vaccines that reduce parasite growth rate or toxicity also reduce the cost of virulence for the parasite (i.e., host death), which in turn causes between-host selection for more virulent parasites (Gandon et al. 2001, 2002, 2003). Vaccination could also change the fitness function for the parasite by affecting the extent of within-host selection in mixed-genotype infections. For example, serial passage of malaria parasites increases their virulence, but parasites passaged through immunized hosts become virulent more rapidly (Mackinnon and Read 2004*a*). Mixed-genotype infections are common in human ma-

laria, exceeding 50% of all infections (Babiker et al. 1999), so it is important to also assess how immune-imposed selection will affect within-host selection on virulence. The malaria vaccine candidates currently considered induce T cell–dependent immunity (Good 2005), and our study showed that T cell–dependent immune responses contribute to competition. Hence, it seems likely that vaccination will increase the strength of competition. We are currently directly testing this possibility experimentally. For the moment we note that the result of this study—that the relatively avirulent genotype suffered most from immune-mediated competition—could explain why experimental virulence evolution was more rapid in immunized animals (Mackinnon and Read 2004*a*). To the extent that we can generalize the experimental results we report here, we expect many types of vaccine to exacerbate immune-mediated competition, thus imposing selection for increased virulence.

However, a beneficial flip side of vaccine-enhanced immune-mediated competition is that it could retard the spread of drug resistance. Resistant mutants often compete with wild-type parasites in mixed infections when they first arise and then compete with unrelated susceptible strains in mixed infections as they spread through a population. It seems highly likely that resistant strains will be less competitively successful (or they would have spread anyway), so that in-host competition in untreated hosts will act to slow the spread of drug resistance (Hastings and D'Alessandro 2000; de Roode et al. 2004*a*). If vaccination enhances immune-mediated competitive suppression, drug-resistant mutants will have an enhanced disadvantage in untreated vaccinated patients who have not received chemotherapy and thus will spread more slowly. The extent to which competitive suppression can be used to affect the spread of drug resistance has been little considered, but the possibility that vaccination could slow resistance evolution, thus enhancing the useful life of chemotherapeutic agents, seems to us an idea worthy of theoretical and empirical consideration.

Finally, we note that when considering potential evolutionary consequences of competition between coinfecting pathogen genotypes, evolutionary ecologists have focused mainly on selection for competitiveness (and ensuing evolution of virulence). However, within-host competition can also lead to selection on traits other than competitiveness. Indeed, in other areas of evolutionary ecology, competition has received attention mainly as a potential factor selecting for phenotypic diversification (e.g., Schluter 2000). There is now strong empirical evidence that resource competition promotes phenotypic divergence (in traits affecting resource utilization; Schluter 2000). It has also been proposed that apparent competition can promote divergence (in antipredator traits), although empirical evidence for this process is scarce (Abrams 2000). Like competition in other ecological assemblages, competition between coinfecting pathogen genotypes could potentially select for phenotypic divergence. For example, resource competition could select for divergence in tissue tropism (Frank 2002), while immune-mediated apparent competition could select for divergence in antigenic profile (Gupta and Maiden 2001). One would expect that such divergence should reduce competition between coinfecting pathogens and thus reduce selection on competitiveness. Selection for competitiveness and selection for phenotypic divergence therefore represent two alternative outcomes of within-host competition. Importantly, if within-host competition selects for divergence rather than competitiveness, it will not necessarily influence the evolution of pathogen virulence. Given that mixed-genotype infections of pathogens are common (Read and Taylor 2001), it would be of great biomedical interest to investigate what determines when within-host competition results in selection for phenotypic divergence or enhanced competitiveness.

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