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Host heterogeneity is a determinant of competitive exclusion or coexistence in genetically diverse malaria infections

Jacobus C. de Roode*, Richard Culleton, Sandra J. Cheesman, Richard Carter and Andrew F. Read

Institute of Cell, Animal and Population Biology, University of Edinburgh, Kings Buildings, West Mains Road, Edinburgh EH9 3JT, UK

During an infection, malaria parasites compete for limited amounts of food and enemy-free space. Competition affects parasite growth rate, transmission and virulence, and is thus important for parasite evolution. Much evolutionary theory assumes that virulent clones outgrow avirulent ones, favouring the evolution of higher virulence. We infected laboratory mice with a mixture of two *Plasmodium chabaudi* clones: one virulent, the other avirulent. Using real-time quantitative PCR to track the two parasite clones over the course of the infection, we found that the virulent clone overgrew the avirulent clone. However, host genotype had a major effect on the outcome of competition. In a relatively resistant mouse genotype (C57Bl/6J), the avirulent clone was suppressed below detectable levels after 10 days, and apparently lost from the infection. By contrast, in more susceptible mice (CBA/Ca), the avirulent clone was initially suppressed, but it persisted, and during the chronic phase of infection it did better than it did in single infections. Thus, the qualitative outcome of competition depended on host genotype. We suggest that these differences may be explained by different immune responses in the two mouse strains. Host genotype and resistance could therefore play a key role in the outcome of within-host competition between parasite clones and in the evolution of parasite virulence.

Keywords: *Plasmodium chabaudi*; malaria; virulence; within-host competition; evolution; host genotype

1. INTRODUCTION

According to a large body of theory, competition within hosts generates selection for pathogens that do more damage to their host (i.e. are more virulent; Levin & Pimentel 1981; Bremermann & Pickering 1983; Frank 1992, 1996; Van Baalen & Sabelis 1995; Gandon 1998; Mosquera & Adler 1998; Ebert 1999; Read *et al.* 2002). Parasite fitness in singly infected hosts is assumed to be maximized when the benefits of host exploitation (increased transmission rate) balance the costs (increased host mortality) (Levin & Pimentel 1981; Anderson & May 1982; Bremermann & Pickering 1983; May & Anderson 1983; Sasaki & Iwasa 1991; Frank 1992, 1996; Antia *et al.* 1994; Van Baalen & Sabelis 1995; Antia & Lipsitch 1997). But, in genetically diverse infections, parasites that slowly exploit hosts will be outcompeted by those that exploit hosts more rapidly. Even if host life expectancy is reduced so that all parasites do worse, prudent parasites do disproportionately worse and are thus eliminated by natural selection.

The majority of mixed-infection models of virulence assume that more virulent strains have a competitive advantage. Logical as this may seem, there is hardly any experimental evidence to suggest that this is indeed the case, and some evidence that the opposite is true (Read & Taylor 2001). Within-host competition is certainly widespread and is mediated by limited resources, strain-transcending immune responses (apparent competition) or direct interference between competing genotypes (Read & Taylor 2001). In rodent malaria, for example, both resource and apparent competition probably play a role (Snounou *et al.* 1989, 1992; Hellriegel 1992; Taylor *et al.* 1997*a*; Taylor & Read 1998; Read & Taylor 2001; De Roode *et al.* 2003). But, while strains with a higher growth rate probably will do better in resource competition, they could also become a preferred target of strain-specific immune responses, giving less-abundant genotypes an advantage (Bruce *et al.* 2000; Read *et al.* 2002).

If there is a lack of experimental evidence to suggest that virulent clones are competitively superior to avirulent clones, there is even less evidence that any superiority is consistent across genetically different hosts. Numerous studies have shown that disease virulence varies with host genotype, with some host types being more susceptible to severe disease than others (e.g. Stevenson *et al.* 1982; Ebert & Hamilton 1996; Ebert *et al.* 1998; Imhoof & Schmid-Hempel 1998; Carius *et al.* 2001; Mackinnon *et al.* 2002). This variation in resistance is likely to be reflected in variation in the strengths of resource- or immune-mediated competition between parasites within hosts. Indeed, one study has now shown that this is the case: an experiment on the endophyte *Epichloë bromicola* parasitic on the grass *Bromus erectus* showed that parasite strains that competitively excluded some strains on one host genotype were excluded themselves on another (Wille *et al.* 2002).

Here, we ask whether host genotype is also an important determinant of competitive outcome in a model of human disease. We studied mixed infections of virulent and

*Author for correspondence (j.de.roode@ed.ac.uk).

avirulent clones of the rodent malaria parasite *Plasmodium chabaudi* in two different strains of laboratory mice. Based on the theory outlined above, we predicted that the virulent parasite clone would rapidly outgrow the avirulent clone in both mouse strains. This did occur in one mouse strain, but in the other competitive suppression was transient, with the avirulent clone persisting to do better in the chronic phase of the infection than it would have done on its own.

2. MATERIAL AND METHODS

(a) *Parasites and hosts*

We used two genetically distinct *P. chabaudi chabaudi* clones, denoted AS(pyr1A) and AJ. AS(pyr1A) was derived through pyrimethamine selection from clone AS (Walliker *et al.* 1975). Both AS and AJ were originally isolated from thicket rats (Beale *et al.* 1978). For simplicity AS(pyr1A) will be referred to as AS from here on. We chose AS and AJ clones because they differ in their growth rates and virulences (Mackinnon & Read 1999), with AS producing fewer parasites and causing less virulence than AJ.

Hosts were eight-week-old C57Bl/6J and CBA/Ca inbred female mice (Ann Walker, University of Edinburgh). They were fed on 41B maintenance diet (Harlan, UK) and their drinking water was supplemented with 0.05% para-amino benzoic acid to enhance parasite growth (Jacobs 1964). They were kept in a 12 L : 12 D cycle. We will refer to these mouse strains as C57 and CBA from here on.

(b) *Experimental design and inoculation of mice with parasites*

The experiment consisted of three treatments for both C57 and CBA mice: infections with AS alone infections with AJ alone and mixed AS+AJ infections. Each treatment group had five mice, resulting in 30 mice in total. Mice infected with just AS or AJ received 10^6 parasites, whereas mice infected with both clones received 2×10^6 parasites, made up of 10^6 AS and 10^6 AJ parasites. The latter quantity was chosen because in our analysis we wanted to compare the performance of a clone on its own with its performance in a mixed infection, requiring equal numbers of each parasite clone at inoculation. Although mice that were infected with both clones received a double dose of parasites, we know from previous work that a twofold difference in parasite numbers has a negligible effect on parasite dynamics and virulence (Timms *et al.* 2001).

We prepared inoculations from donor mice by diluting blood in 0.1 ml of calf serum solution (50% heat-inactivated calf serum, 50% Ringer's solution (27 mM of KCl, 27 mM of $CaCl_2$ and 0.15 M of NaCl) and 20 units of heparin ml^{-1} mouse blood), and injected them via the intraperitoneum. All procedures were carried out under Home Office guidelines.

(c) *Monitoring of virulence and infections*

To monitor virulence, we recorded mouse live weights and densities of red blood cells (using flow cytometry; Beckman Coulter). Both body mass and red blood cell density decrease dramatically during infection, and these decreases correlate with host mortality and thus virulence (Mackinnon *et al.* 2002).

To monitor production of asexual parasites and gametocytes (the sexual transmission stages) we took thin blood smears from tail blood. These were fixed with methanol and stained with Giemsa to determine levels of asexual parasitaemia and gametocytaemia using 1000× microscopy. When asexual parasitaemia was high, we counted 500 red blood cells in at least four microscopic fields. With lower parasitaemias and gametocytaemias we counted at least 20 microscopic fields (corresponding to at least 10 000 red blood cells), and calculated the average number of cells per field. Gametocyte numbers were counted using polarized light. We calculated parasite densities and gametocyte densities as the products of parasitaemias and gametocytaemias, respectively, and red blood cell densities taken on the same day.

We took measurements every day from day 0 to day 23 post-infection (PI), three times a week from day 23 to day 48 PI, once a week from day 48 to day 69 PI and on day 83 PI, when the experiment was terminated. Measurements were taken between 09.00 and 10.30, when peripheral blood almost exclusively harboured haploid ring stages of the parasites.

(d) *Monitoring of individual clones in mixed infections*

We collected 5 µl samples of tail blood from mice harbouring mixed infections in citrate saline. After 1 min of centrifugation at 13 000 r.p.m., we removed the supernatant and stored the pelleted blood at −70 °C for subsequent DNA extraction using Instagene Matrix (BioRad). We used real-time quantitative PCR to measure the DNA concentrations of both AS and AJ in these samples (Cheesman *et al.* 2003) and calculated the proportions of AS and AJ in the total parasite population. Absolute numbers of AS and AJ were then calculated by multiplying these proportions by the overall parasite density on the same day.

We took blood samples on the same days that we took our other measurements, but performed PCRs only when parasitaemias were higher than 0.1%, which is the lowest level at which we can accurately estimate AS and AJ proportions (Cheesman *et al.* 2003). Our real-time quantitative PCR protocols cannot distinguish between asexual parasites and gametocytes, and so they estimate the densities of all parasites. In our data analyses, we treated these as estimates of asexual density. Gametocyte densities were two to three orders of magnitude lower than asexual-parasite densities, and gametocytes are thus a negligible component of overall parasite numbers.

(e) *Trait definition*

Prior to statistical analysis we defined and constructed the following traits that described part of or all of the infection. For measures of virulence we determined the 'minimum weight' and 'minimum density of red blood cells' that mice reached. Mice that died were included in our analysis of minimum weights and red blood cell densities, using weight and red blood cell density on the day of death as the minima they reached. For all other traits, these mice were excluded from the analyses.

Parasite levels in mice rose and fell several times, with the first peak being substantially higher and lasting for longer than the other peaks. For each mouse, we calculated the period until the end of the first wave, and defined this as the acute phase of the infection. The subsequent period, the chronic phase, was defined as starting on the day that parasite numbers began to recover after the collapse of the first wave (day 15.6 ± 0.55 PI; mean ± 1 s.e.). To calculate the numbers of parasites present during the whole infection and during the acute and chronic phases, we calculated the areas under the relevant parasite density by day PI curves. As our limit of detecting parasites was *ca.* 100 μl^{-1}, we treated observations of zero parasites as being 100 parasites μl^{-1}. We could accurately quantify AS and AJ

proportions of 0.01 at levels of 0.1% parasitaemia or higher (Cheesman *et al.* 2003); below that we set the clone densities at our limit of detection (100 μl^{-1}). Parasite densities in single infections were set to the same level when parasitaemias were lower than 0.001%.

For gametocytes, we chose slightly different ways of calculating densities, as they showed different dynamics from asexual parasites, having a first wave before day 9 and one or several thereafter. We therefore calculated the total gametocyte densities over days 0–9 and days 10–83 PI, as well as the total density over the whole infection. We were able to analyse overall numbers of gametocytes only, as our real-time quantitative PCR protocol does not distinguish between AS and AJ gametocytes (see § 2d).

(f) *Statistical analysis*

We analysed all traits mentioned in § 2e using ANOVAs and ANCOVAs in MINITAB (v. 13.30, Minitab Inc.). The explanatory variables used were mouse 'strain' and infection 'treatment'. Strain had two factor levels (C57 and CBA); treatment had up to three factor levels (AS, AJ and AS+AJ), depending on the analysis. For all our models we first fitted the maximal model including covariate (when relevant), treatment, strain and an interaction between treatment and strain. We then minimized the models by removing non-significant terms ($p > 0.05$), beginning with the interaction.

We log-transformed initial and minimum densities of red blood cells as well as all parasite and gametocyte densities prior to analysis, to meet the necessary normality and homogeneity-of-variance assumptions.

(g) *Follow-up experiment*

To confirm the most important findings of this study, we infected groups of five C57 and five CBA mice with AS+AJ, exactly as described in § 2b, and took blood samples from them on days 6 and 13 PI. We then extracted DNA and analysed these DNA samples with real-time quantitative PCR, to determine whether both clones were present, and in what proportions.

3. RESULTS

One C57 mouse infected with AJ died on day 12 and one infected with AS+AJ died on day 7; two CBA mice infected with AS+AJ died on days 10 and 11 PI. AJ infections induced greater weight loss and lower minimum red blood cell densities than AS infections (treatment: $F_{1,17} = 25.4$, $p < 0.001$; and treatment: $F_{1,18} = 25.3$, $p < 0.001$, respectively), regardless of mouse genotype (strain and treatment × strain n.s.). Thus, as found previously, AS was the less virulent clone.

(a) *AS and AJ parasite densities*

In mixed infections in both C57 and CBA mice, there were substantially more AJ than AS parasites during the first 10–14 days (figure 1*a*,*b*). After day 10, AS disappeared below detectable levels in C57 mice and never reappeared. It persisted, however, in the three CBA mice that survived the first two weeks. One of these experienced separate AS and AJ parasite waves around days 35 and 55 PI (figure 2*a*). In the other two mice, AS started to overgrow AJ around days 22 and 18, respectively (figure 2*b*,*c*). Later in the infection AJ overgrew AS again in one of these (figure 2*c*).

Formal analysis confirmed this picture. During the acute phase in both mouse strains, AS was competitively suppressed by AJ, producing far fewer parasites in mixed infections than it did alone (figures 1*c*,*d* and 3*a*; treatment: $F_{1,13} = 155$, $p < 0.001$). This suppression was greater in CBA than in C57 mice (treatment × strain: $F_{1,13} = 9.1$, $p = 0.01$). In C57 mice, AS disappeared below detectable levels before the end of the acute phase, showing competitive exclusion.

In CBA mice, AS was also competitively suppressed during the acute phase, but it was not excluded from the infection. During the chronic phase, it produced more parasites than it would have done on its own (figures 1*d* and 3*b*; treatment: $F_{1,13} = 6.8$, $p = 0.022$; treatment × strain: $F_{1,13} = 34$, $p < 0.001$), thus showing that, after the chronic phase, AS actually benefited from the presence of AJ (that is, facilitation, not competition). During both the acute and chronic phases, AS produced more parasites in CBA mice than in C57 mice, whether it was alone or in a mixture (figure 3*a*,*b*; strain: $F_{1,13} = 125$, $p < 0.001$; $F_{1,13} = 52$, $p < 0.001$, respectively).

During the acute phase, AJ produced roughly the same numbers of parasites in mixed and single infections, in both C57 and CBA mice (figures 1*e*,*f* and 3*c*; treatment: $F_{1,12} = 1.42$, $p = 0.25$), thus showing that, unlike AS, AJ did not suffer from competition. Like AS, it produced more parasites in CBA than in C57 mice (strain: $F_{1,14} = 27$, $p < 0.001$). During the chronic phase, AJ produced slightly fewer parasites in C57 mice, but more in CBA mice, than it did on its own (figure 3*d*; treatment × strain: $F_{1,12} = 8.1$, $p = 0.015$). When analysing these numbers for CBA mice only, however, there was no difference between single and mixed infections (treatment: $F_{1,6} = 2.7$, $p = 0.15$), thus showing that, unlike AS, AJ did not experience facilitation during this phase.

(b) *Follow-up experiment*

In the follow-up experiment, we infected another five C57 and five CBA mice with AS+AJ, and sampled these on day 6 and day 13 PI. In both mouse strains AS was present on day 6. By day 13, however, AS had disappeared below detectable levels in the peripheral blood of C57 mice (figure 4*a*), but was still present in CBA mice (figure 4*b*), actually overgrowing AJ at this time. These results thus confirmed the qualitatively different dynamics of mixed infections in C57 and CBA mice observed in the main experiment.

(c) *Overall parasite densities*

During the acute phase, infections in C57 mice consisted of fewer parasites than those in CBA mice (figure 3*e*; strain: $F_{1,20} = 110$, $p < 0.001$). In both mouse strains, AJ and AS+AJ infections produced more parasites during the acute phase than did AS infections (treatment: $F_{2,20} = 74$, $p < 0.001$).

During the chronic phase, AJ and AS+AJ infections produced more parasites than did AS infections (figure 3*f*; treatment: $F_{2,20} = 28$, $p < 0.001$). AJ and AS+AJ infections also differed from each other, but differently in the two mouse genotypes: in C57 mice AS+AJ infections produced slightly fewer parasites than AJ infections, whereas

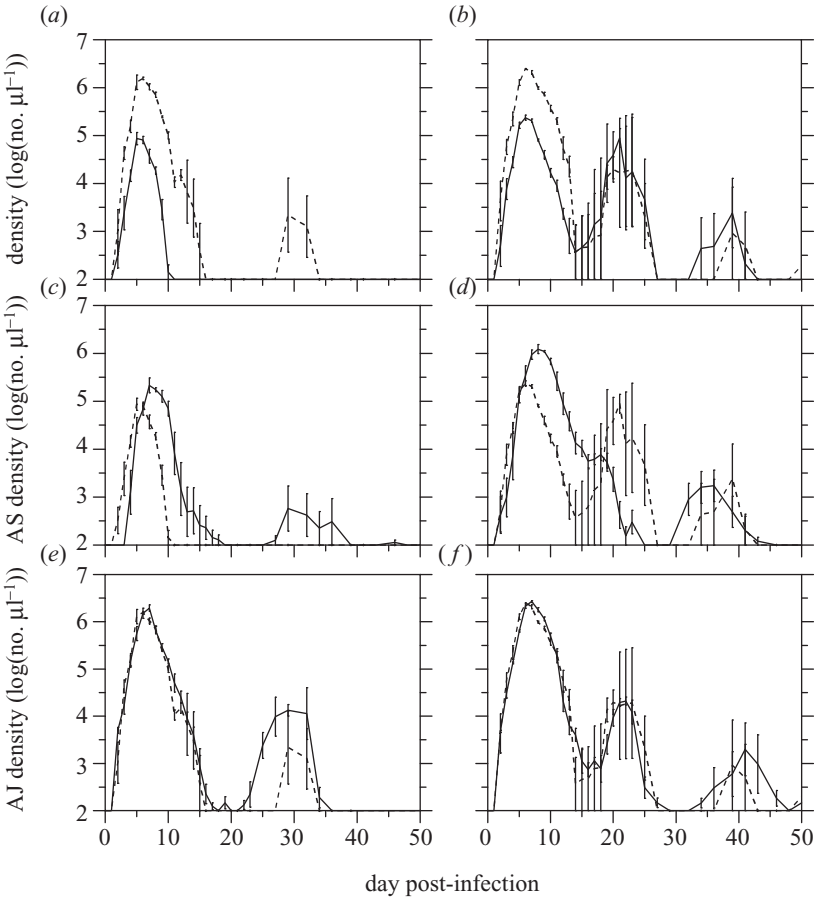

Figure 1. Log parasite densities over time (mean ± 1 s.e.) for (*a*,*c*,*e*) C57 mice and (*b*,*d*,*f*) CBA mice; (*a*) and (*b*) show AS (solid line) and AJ (dashed line) parasite densities in mixed AS+AJ infections; (*c*) and (*d*) compare AS parasite densities in single AS (solid line) and mixed AS+AJ (dashed line) infections; (*e*) and (*f*) compare AJ parasite densities in single AJ (solid line) and mixed AS+AJ (dashed line) infections. All data points are based on five replicate mice, except for single AJ infections in C57 mice (four mice after day 12), mixed AS+AJ infections in C57 mice (four mice after day 7) and mixed AS+AJ infections in CBA mice (four mice on day 11 and three mice from day 12 onwards). As the limit of detection was 100 parasites µl^{-1} blood, *y*-axes start at 2.

in CBA mice they produced more (figure 3*f*; treatment × strain: $F_{2,20} = 11$, $p = 0.001$). Owing to these higher numbers in CBA mice during the chronic phase, AS+AJ infections produced more parasites in CBA mice over the whole of the infection than did AJ infections (treatment × strain: $F_{2,24} = 26$, $p < 0.001$).

(**d**) *Gametocyte densities*

In C57 mice, most gametocytes were produced during the first 9 days of the infection, with AS having lower densities than AJ and AS+AJ (figure 5*a*; treatment: $F_{2,27} = 11.71$, $p < 0.001$), whereas in CBA mice most gametocytes were produced after day 9 PI, when AJ produced fewer gametocytes than AS and AS+AJ (figure 5*b*; treatment: $F_{2,20} = 5.0$, $p = 0.017$; strain: $F_{1,20} = 6.04$, $p = 0.023$; treatment × strain: $F_{2,20} = 6.1$, $p = 0.009$). In C57 mice, gametocyte peaks in mixed infections mostly resembled those in AJ infections, the numerically dominant clone (figures 5*a* and 1*a*). In CBA mice, mixed infections produced gametocyte dynamics that did not resemble gametocyte production in either AS or AJ single infections, but which peaked during a period when both clones were present in high numbers (figures 5*b* and 1*b*). Summarized over the whole infection, AJ produced most gametocytes in C57 mice, but fewest in CBA mice, in which AS produced most (strain: $F_{1,20} = 8.6$, $p = 0.008$; treatment × strain: $F_{2,20} = 8.3$, $p = 0.002$).

4. DISCUSSION

Our results show that host genotype affects the outcome of within-host competition between pathogen strains. In C57 mice, the avirulent clone disappeared below detectable levels from the peripheral blood after 10 days of the infection and produced far fewer parasites than it would have done on its own. This competitive suppression almost certainly reduced the clone's transmission potential: the period of greatest gametocyte production in single infections occurred after day 10 PI (figure 5*a*). As the avirulent clone never reappeared during the course of our study, it seems that it was competitively excluded, something we have not observed before (Taylor *et al.* 1997*b*; Taylor & Read 1998; Read *et al.* 2002; A. F. Read and M. A. Anwar, unpublished data). By contrast, the virulent clone did not suffer at all from competition in mixed infections.

In CBA mice, the avirulent clone was also competitively suppressed during the acute phase, but it persisted and went on to produce more parasites during the chronic phase than it would have done on its own. Thus, in CBA

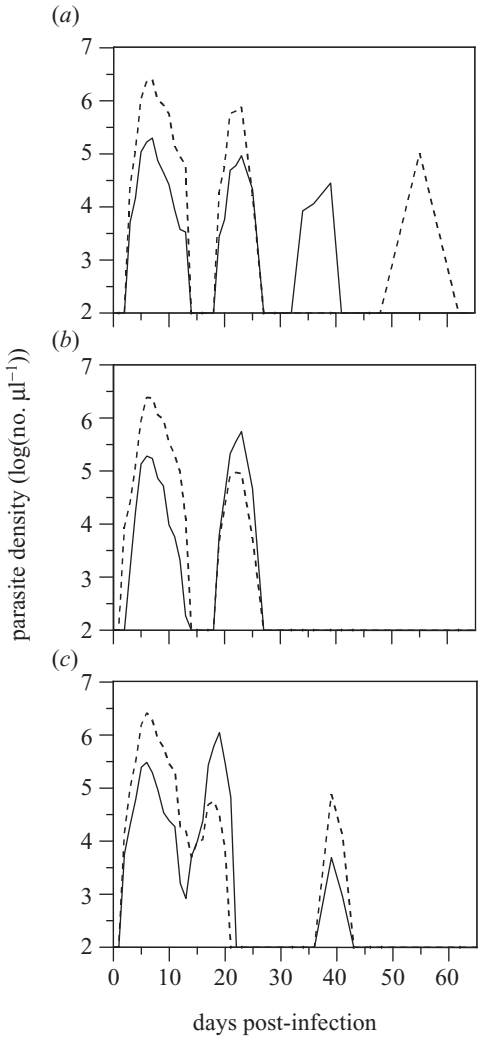

Figure 2. Log AS (solid lines) and AJ (dashed lines) parasite densities over time in mixed AS+AJ infections for the three CBA mice that survived the acute phase. As the limit of detection was 100 parasites µl^{-1} blood, *y*-axes start at 2.

mice, competitive suppression gave way to facilitation. This may have resulted in enhanced transmission: the density of the avirulent clone peaked at around day 20 PI and coincided with a large gametocyte peak (figures 1*d* and 5*b*). Whether the presence of the more virulent clone actually enhanced the overall fitness of the avirulent clone would depend on the host mortality rate induced by the virulent clone and on how many of the gametocytes produced around this time were of the virulent clone.

Our experiment, with just two parasite clones and two mouse strains, generated the whole spectrum of outcomes currently captured in a range of different mixed-strain models of virulence evolution. In extreme coinfection models, clone dynamics are unaffected by the presence of other clones (e.g. May & Nowak 1995; Leung & Forbes 1998); in our experiments, this was so for clone AJ in both mouse strains. Coexistence with competitive suppression of at least one clone, as envisaged in other models (e.g. Sasaki & Iwasa 1991; Frank 1992, 1996; Herre 1995; Van Baalen & Sabelis 1995), occurred within CBA mice. Finally, the competitive exclusion of AS from C57 mice before much transmission-stage production occurred bears substantial resemblance to superinfection models (which are perhaps better called superseding infection models; Van Baalen & Sabelis 1995). These models allow no coexistence and postulate that competitive suppression instantly reduces transmission to zero (e.g. Levin & Pimentel 1981; Bremermann & Pickering 1983; Bremermann & Thieme 1989; Knolle 1989; Nowak & May 1994; Leung & Forbes 1998; Gandon *et al.* 2002). Thus, it may prove difficult to capture the real-world complexities of a disease such as malaria (and others; Hood 2003) in models that assume that the outcome of competition is independent of environmental conditions, such as host genotype.

A general conclusion of the above models is that mixed-clone infections will generate selection for increased virulence. However, the precise details of competition will affect the transient evolutionary dynamics and the magnitude of standing virulence in a population, which is what is relevant to animal and human health. Competitive exclusion of avirulent clones, for instance, will affect potential evolutionary trajectories, rates of evolution and average levels of virulence in a population.

Several recent studies have shown that parasite virulence depends on host genotype (e.g. Ebert & Hamilton 1996; Ebert *et al.* 1998; Imhoof & Schmid-Hempel 1998; Carius *et al.* 2001; Mackinnon *et al.* 2002), and theory has shown that such host-genotype dependence of virulence can explain why polymorphisms in growth rate and virulence are maintained (Regoes *et al.* 2000). If the outcome of competition is as dependent on host genotype as is the expression of virulence itself, this could also contribute to the maintenance of growth-rate and virulence polymorphisms in the parasite population. Wille *et al.* (2002) also suggested this when they found that some strains of the endophyte *E. bromicola* competitively excluded other strains on one genotype of its host *B. erectus*, but not on another.

Clone AS was competitively suppressed by AJ, except during the chronic phases of infections in CBA mice, where AS did better than it did on its own. This could be the result of a combination of strain-specific immunity and antigenic variation. In two of the surviving CBA mice we saw AS overgrow AJ after it had been suppressed (figure 2*b*,*c*). This might suggest that the immune system had been focusing on the clone that had produced the highest number of parasites up until then (AJ), giving AS some advantage. Strain-specific immunity is certainly well known in *P. chabaudi* (e.g. Jarra & Brown 1985; Buckling & Read 2001; R. Carter, unpublished results), and facilitation of one pathogen as a result of the immune response focusing on another has also been suggested for fungal species infecting leaf-cutter ants (Hughes & Boomsma 2004). *Plasmodium chabaudi* is also known to produce antigenic variants at high rates (McLean *et al.* 1982, 1990; Brannan *et al.* 1994; Phillips *et al.* 1997). Novel antigenic variants escape variant-specific host responses, and when there are more clones in an infection, each generating variants, it seems likely that the immune response may be less efficient at controlling the infection. A combination of strain-specific immunity and antigenic variation has also been suggested to play a role in mixed-species and mixed-strain infections of plasmodia in humans (Bruce *et al.* 2000).

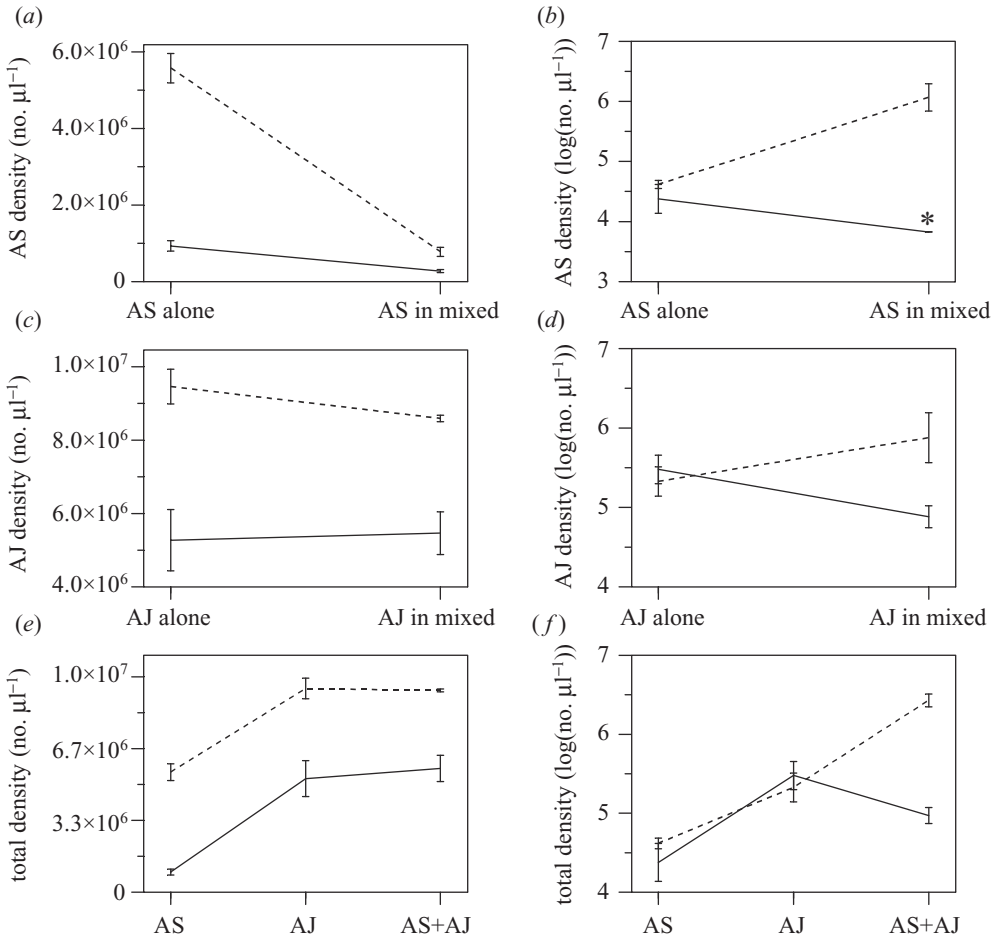

Figure 3. Numbers of parasites produced during (*a*,*c*,*e*) the acute phase and (*b*,*d*,*f*) the chronic phase of the infection for C57 (solid lines) and CBA (dashed lines) mice (mean ± 1 s.e.). Plotted points are: (*a*) and (*b*) AS parasite densities in single AS and mixed AS+AJ infections; (*c*) and (*d*) AJ parasite densities in single AJ and mixed AS+AJ infections; and (*e*) and (*f*) overall parasite numbers in single AS, single AJ and mixed AS+AJ infections. All data points are based on five replicate mice, except for single AJ infections in C57 mice (four mice), mixed AS+AJ infections in C57 mice (four mice) and mixed AS+AJ infections in CBA mice (three mice). The asterisk denotes a parasite density below detection, as AS disappeared from mixed AS+AJ infections in C57 mice.

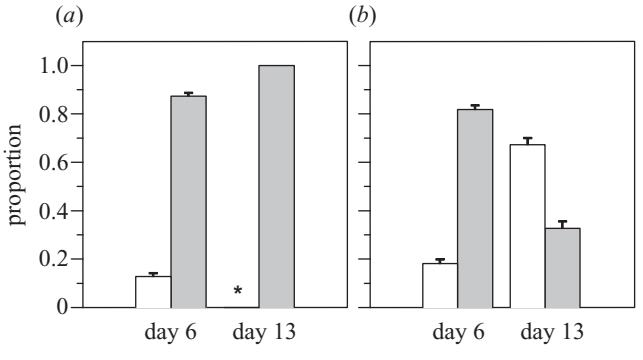

Figure 4. Proportions of AS (open bars) and AJ (filled bars) (mean ± 1 s.e.) in mixed AS+AJ infections in (*a*) C57 and (*b*) CBA mice on days 6 and 13 PI. Data are based on five replicate mice in a follow-up experiment. The asterisk denotes a proportion of 0, as AS disappeared below detection levels from these mixed AS+AJ infections in C57 mice.

Why did infections in C57 and CBA mice show such different dynamics and outcomes of competition? One important possibility is the strain difference in the efficacy of immune control. Mouse strains differ considerably in their resistance to *P. chabaudi* infections (Stevenson *et al.* 1982). Resistance is associated with reduced parasite densities, and appears to be a complex genetic trait, involving many genes affecting immunity and the production and characteristics of red blood cells (Stevenson *et al.* 1982; Yap *et al.* 1994; Fortin *et al.* 2002). Parasite densities were higher in CBA mice (figure 3*e*), and such higher densities could reduce the chance of stochastic loss and increase the chance of producing antigenic escape variants.

If differences in overall immune control do explain the strain differences in the outcome of competition, we would expect to find coexistence of the two parasite clones in C57 mice that were made more susceptible, for example by administration of anti-interleukin 12 monoclonal antibodies (Yap *et al.* 1994). Conversely, we would expect clone AS to disappear in CBA mice that were made more resistant, for example by artificially increasing their interleukin-12 levels (Yap *et al.* 1994).

If overall resistance does affect the outcome of within-host competition, then we would expect to find differences not only between host genotypes, but also between individuals that differ in their sex, nutritional or health status, vaccination status, or any other factor that influences resistance. Semi-immune mice are much more resistant to

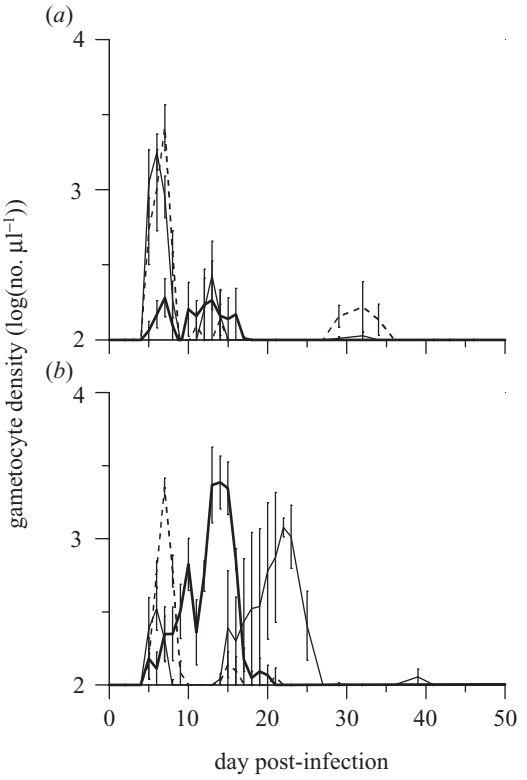

Figure 5. Log gametocyte densities over time (mean ± 1 s.e.) for (*a*) C57 and (*b*) CBA mice infected with single AS (thick solid lines), single AJ (dashed lines) or mixed AS+AJ (thin solid lines) infections. Shown are overall (AS+AJ) numbers of gametocytes only, as our real-time quantitative PCR cannot be used to distinguish between AS and AJ gametocytes. All data points are based on five replicate mice, except for single AJ infections in C57 mice (four mice after day 12), mixed AS+AJ infections in C57 mice (four mice after day 7) and mixed AS+AJ infections in CBA mice (four mice on day 11 and three mice from day 12 onwards). As the limit of detection was 100 gametocytes μl^{-1} blood, *y*-axes start at 2.

P. chabaudi infection than are naive mice (Buckling & Read 2001; Mackinnon & Read 2003). Extrapolating from our results, we would expect that only the virulent clone would survive in such vaccinated hosts. It is important to test this, because this implies that vaccination could increase the frequency of virulent clones in a population, thus leading to unforeseen consequences of vaccination (Gandon *et al.* 2001, 2003; Read *et al.* 2004).

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.