Mixed-genotype infections of malaria parasites: within-host dynamics and transmission success of competing clones

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SUMMARY

Mixed-genotype infections of microparasites are common, but almost nothing is known about how competitive interactions within hosts affect the subsequent transmission success of individual genotypes. We investigated changes in the composition of mixed-genotype infections of the rodent malaria *Plasmodium chabaudi* clones CR and ER by monoclonal antibody analysis of the asexual infection in mice, and by PCR amplification of clone-specific alleles in oocysts sampled from mosquitoes which had fed on these mice. Mixed-clone infections were initiated with a 9:1 ratio of the two clones, with ER as the minority in the first experiment and CR as the minority in the second experiment.

When beginning as the majority, clones achieved parasite densities in mice comparable to those achieved in control (single-clone) infections. When they began as the minority, clones were suppressed to less than 10% of control parasitaemias during the early part of the infections. However, in mosquitoes, the frequency of the initially rare clone was substantially greater than it was in mice at the start of the infection or four days prior to the feed. In both experiments, the minority clone in the inocula produced as many, or more, oocysts than it did as a single-clone infection. These experiments show that asexual dominance during most of the infection is poorly correlated to transmission probability, and therefore that the assumption that within-host population size correlates to transmission probability may not be warranted. They also raise the fundamental question of why transmission rates of individual genotypes are often higher from mixed than single-clone infections.

1. INTRODUCTION

In many microparasitic diseases, genetic diversity in parasite populations within single hosts is common. This can arise because of co-infection, super-infection, or somatic mutation during the course of the infection. A considerable body of theory suggests that where different parasite genotypes are interacting with each other and with the immune system of the host, the outcome of the infection, the epidemiology of the disease and the evolution of the parasite species can all be altered. Length of infection and density of parasites (and presumably therefore the severity of symptoms) can both be increased in mixed-genotype infections (Hellriegel 1992; Antia *et al*. 1996). Parasite genotypes that transmit to other hosts are thought to be those that succeed in competitive situations (Bremermann & Thieme 1989), which will ultimately affect the evolution of characteristics such as growth rate and virulence (Levin & Pimentel 1981; Nowak & May 1994; reviewed by Bull 1994; Ebert & Herre 1996; Frank 1996). However, as several authors have pointed out, our theoretical understanding of interactions within mixed infections has far outstripped our understanding of the facts, so that most of the assumptions of these models remain untested (May & Anderson 1990; Bull 1994; Gupta & Day 1996).

Remarkably few experiments have tested whether competitive interactions do occur between different strains or clones of the same parasite species (Seed 1978; Snounou *et al*. 1992; Read & Anwar, in preparation). These have shown that the infection course of one strain is altered by the presence of another strain sharing the same host, suggesting that infection dynamics are not regulated entirely by strain-specific immune responses. As far as we are aware, only one study has addressed how transmission from mixed infections might be altered (Nakamura *et al*. 1992). This showed that the relative transmission of two lines of *Eimeria tenella* from mixed infections was very different from that from single-line infections. None of these experimental studies examined directly whether transmission rates were correlated with within-host replication rates, an assumption common to many models (e.g. Bremermann & Pickering 1983; Gupta & Day 1996; and reviews by Bull 1994; Frank 1996). Unless we understand which model assumptions are most realistic, there is little hope of deciding how best to control parasitic diseases, or of predicting how selection imposed by control measures will act on

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within-host life history strategies or virulence (Read 1994). In particular, almost nothing is known of the critical link between within-host interactions and the subsequent transmission success of individual genotypes. Here, using immunological and genetic analyses of mixed infections of a rodent malaria, *Plasmodium chabaudi*, in laboratory mice, we begin to rectify this.

In *P. chabaudi*, haploid asexual parasites circulate in the blood stream of the mouse, dividing every 24 hours. A typical infection in the mouse strain used here has a peak asexual parasitaemia around day 8 post infection (p.i.), with up to 40% of red blood cells being infected. Host immune responses (eg. Langhorne *et al*. 1990) and probably also resource limitation (Richie 1988) such as red blood cell shortages (Yap & Stevenson 1994; Hetzel & Anderson 1996), reduce the infection dramatically by day 14 when only a few per cent of red blood cells are infected. A parasite which has recently invaded a new red blood cell can develop into another asexually replicating stage, or into a sexual gametocyte. Gametocytes are the only blood stage parasites able to infect the mosquito vector. The bulk of gametocyte production occurs after the peak of asexual parasitaemia (Wery 1968; Buckling *et al*. 1997). In experiments initiated with the number of parasites used here, very few gametocytes are produced before day 12 of the infection, maximum densities occur around day 14, and they decrease dramatically by day 18. When taken up by the vector, gametocytes rapidly mature into gametes, which fuse to form diploid zygotes. These zygotes exist only briefly, before meiosis and replication resulting in many haploid sporozoites occurs. All the haploid progeny of a zygote are held together in an oocyst during this multiplication phase. By dissecting mosquitoes eight to nine days after they were fed on an infective mouse, oocyst numbers can be counted to assess transmission intensity.

In the experiments presented here, we analyse the success of two clones of *P. chabaudi*, denoted CR and ER, introduced into mice as a mixed infection. Previous work had shown that, where one clone is dominant in the inoculum, very marked alterations of the asexual population dynamics of the minority clone can occur (Read & Anwar, in preparation). We therefore initiated mixed infections with 1:9 and 9:1 ratios of the two clones. Transmission from the resulting infections was compared to that from control single-clone infections which received the same number of CR or ER parasites used in the mixed infections. As we report elsewhere (Taylor *et al*. 1997), these mixed infections were more infectious to mosquitoes than were single-clone infections because gametocyte densities were higher.

Here we examine the performance of the two clones individually within the mixed infections. *P. chabaudi* clones are morphologically indistinguishable, but can be discriminated using immunological and genetic techniques (McLean *et al*. 1991; Ranford-Cartwright *et al*. 1991). Asexual parasites of the two clones possess different alleles of the merozoite surface protein 1 (MSP-1) gene. These can be differentiated in the blood stages by allele-specific monoclonal antibodies linked to fluorescent markers, and in the oocysts by the production of different sized fragments after PCR amplification. Using these markers, we followed the course of infection of each clone in mixed infections in mice, and in the oocyst populations formed after mosquitoes had fed on them. We could then compare the infection dynamics of each parasite clone in a mixed infection with their dynamics when the same number of parasites were inoculated into a host on their own. These data allowed us to assess whether competitive interactions occurred between the clones in the mixed infections and to assess whether these interactions affected the transmission from the infections.

2. MATERIALS AND METHODS

(*a*) *Parasites, mice and mosquitoes*

Two cloned lines of *P. chabaudi* denoted CR and ER (Beale *et al*. 1978), from the World Health Organisation's Registry of Standard Malaria Parasites, Edinburgh University, were used. The mouse hosts were male C57BL/6J/Ola mice (Harlan, England). *P. chabaudi* is usually non-lethal to this strain (Stevenson *et al*. 1982). Mice were fed on SDS rat and mouse maintenance diet. Drinking water was supplemented with 0.05% para-amino benzoic acid (PABA) to enhance parasite growth. Artificial illumination was provided from 07:00 to 19:00 hours. The mosquito host was *Anopheles stephensi*, maintained at 25–28 °C and 70–80% humidity, and fed on 10% glucose in water supplemented with 0.05% PABA.

(*b*) *Inoculation of mice with standard numbers of parasites*

Parasite densities of C57/Bl/6J/Ola mice infected with CR or ER parasites were determined from Giemsa-stained thin blood smears and red blood cell counts made using flow cytometry (Coulter Electronics). Blood from these infected mice was diluted in calf serum–Ringer solution (50% heat-inactivated calf serum, 50% ringer solution (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl), 20 units heparin per ml mouse blood) to give initial dilutions of the two parasite clones. These were then further diluted with calf serum–Ringer solution, or mixed together to give the required number of parasites in a 0.1 ml inoculum (see experimental design below and figure 1).

(*c*) *Parasite counts and mosquito feeds*

The density of asexual parasites (number of asexual parasites per ml of blood) was calculated from thin blood smears and red blood cell counts taken between 17:45 and 18:15 hours on selected days. To sample transmission, five-day old *Anopheles stephensi*, starved for the previous 48 hours, were then allowed to feed on the mice for 20–30 min. Unfed mosquitoes were discarded, and the remainder were dissected 8–10 days later to obtain counts of the number of oocysts on each mosquito midgut. Single oocysts were dissected from the midguts of infected mosquitoes using finely pointed disposable microcapillary tubes (Ranford-Cartwright *et al*. 1991). This was done at random, with oocysts being dissected from a representative set of both heavily infected and more lightly infected mosquitoes. The oocysts were put into tubes containing 50 µl of oocyst lysis buffer (100 mM NaCl, 25 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.8), 0.5% Sarkosyl, 1 mg ml^{-1} Proteinase K), placed at 55 °C for one hour and then stored at −20 °C.

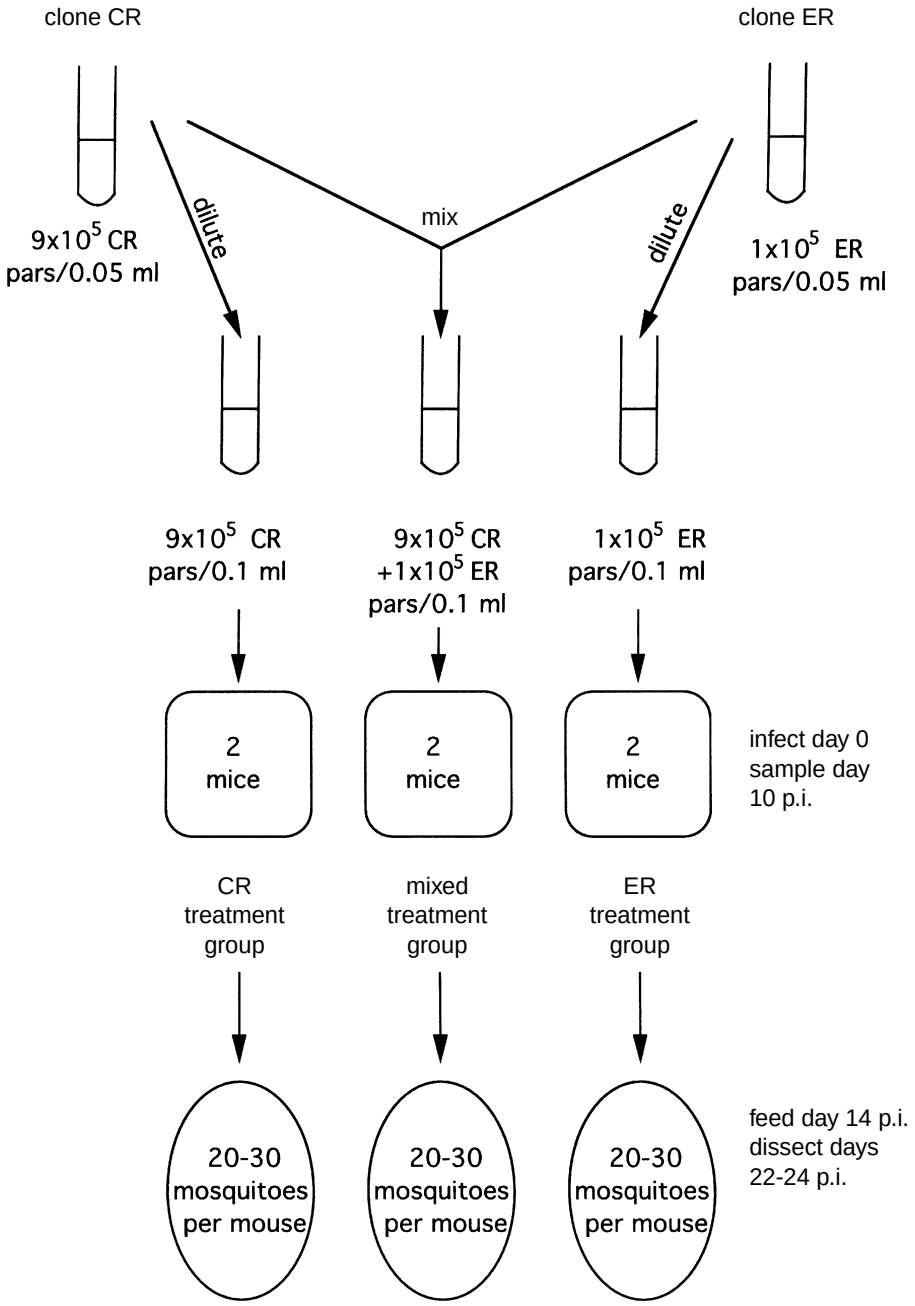

Figure 1. Schematic representation of an experimental block in experiment 1. This protocol was repeated four times (four blocks), giving a total of eight mice per treatment group (where treatment groups were CR, ER or mixed infections). A similar protocol was used for experiment 2, except that the proportion of the two clones in the inocula of mixed infections was reversed, and additional mice were included for a day 18 p.i. mosquito feed. Pars represents parasites.

(*d*) *Immuno-fluorescence testing of MSP-1 alleles*

The monoclonal antibodies (Mabs) used were B18 (IgG3, Boyle *et al*. 1982) and H2 (IgG2b, Hamers-Casterman, unpublished data) which react specifically to clones CR and ER, respectively (McLean *et al*. 1991). Before use, each Mab was diluted 1:1000 in phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.1% sodium azide. The protocol is based on those used by Bruce *et al*. (1990) and McLean *et al*. (1991), and is briefly as follows. 10 µl of infected mouse blood were diluted in 100 µl PBS (pH 7.4), mixed thoroughly and kept on ice. Blood cells were rinsed five times by centrifugation and PBS washes, and then pipetted onto multispot slides (Henley-Essex). These were dried and stored desiccated at −20 °C. A mixed antibody preparation (1:1 mixture of diluted B18 and H2) and a mixed conjugate preparation (1:1 mixture of fluorescein (FITC)-labelled goat anti-mouse IgG3 and rhodamine (TRITC)-labelled goat anti-mouse IgG2b, both from Southern Biotechnology Associates Inc., and diluted 1:100 in PBS) were made.

Slides were fixed with acetone for 5 min. 25 µl of antibody preparation were added to each well and incubated at room temperature for 30 min. Wells were rinsed three times with PBS, and 15 µl of conjugate were added and incubated for 30 min, before being rinsed with PBS three times and PBS–glycerol–DABCO solution (50% PBS, 50% glycerol, 25 mg ml^{-1} 1,5-diazabicyclo[2,2,2]octane) used to mount a coverslip. Under fluorescence microscopy, the two clones gave off different coloured signals, allowing the ratio of the

two in mixed infections to be calculated. An average of 433 asexual parasites (minimum 278) were counted for each sample to obtain a ratio. Bias in these ratios caused by the unequal affinities of the two monoclonal antibodies or unequal production of the target molecule by the two clones was controlled for as detailed in Appendix 1.

(*e*) *PCR typing of MSP-1 in oocysts*

Individual oocyst samples were thawed and DNA prepared from them using the phenol–chloroform technique of Ranford-Cartwright *et al.* (1991). The variable region I of the MSP-1 gene (Deleersnijder *et al.* 1991; McKean *et al.* 1993) was amplified using the nested PCR technique. The primers were prepared by Oswel DNA service, Edinburgh, with the sequences 5′-TTAAAGAAGAACGAAGCC-3′ and 5′-CTAATTCAACTGGATCGAC-3′ (outer), and 5′-GTTACAACAAATCGAAGCT-3′ and 5′-TTGAGCATAAAGTTCAGC-3′ (inner). 1 µl of DNA from single oocysts was amplified by PCR in 20 µl reactions containing 1 × buffer (Gibco BRL), 1.5 mM $MgCl_2$, 100 nM of each primer (50 nM for the outer PCR), 75 µM of each of dATP, dTTP and dCTP, 65 µM dGTP, 10 µM 7-deaza-2′-deoxyguanosine (c^7dGTP) and 1 unit of Taq DNA polymerase (Gibco BRL). Outer PCRs were layered with mineral oil and subjected to 25 cycles at 94 °C for 30 s, 58 °C for 30 s and 70 °C for 30 s, and then 70 °C for 10 min. 1.2 µl of this product were added to a 20 µl inner reaction, covered with mineral oil and subjected to 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 70 °C for 30 s, and then 70 °C for 10 min. Products were visualized on 2 % agarose with TBE gels stained with ethidium bromide. ER alleles produced a band of 359 bp and CR alleles a band of approximately 340 bp.

An average (±s.e.) of 17.7 (±6.94) oocysts (equivalent to 36 alleles) were dissected at random from the mosquitoes which had been allowed to feed on each mixed infection mouse, and analysed by PCR.

(*f*) *Experimental design*

Two experiments were conducted. In experiment 1, the mixed-clone infection group had an initial inoculum of 9×10^5 CR + 1×10^5 ER, while the CR and ER control infection groups were given 9×10^5 CR or 1×10^5 ER *P. chabaudi* parasites respectively (figure 1). Two mice per treatment group (three groups, as above) were infected in each of four replicate experimental blocks, to give a total of 24 mice in experiment 1. Asexual parasite density was measured between 17:45 and 18:15 hours on day 10 p.i., and slides for monoclonal antibody analysis prepared. Mosquitoes were allowed to feed on all mice on day 14 p.i. Between eight and nine days after the feed, mosquitoes were dissected to assess transmission intensity and samples stored for PCR analysis.

Experiment 2 was similar to experiment 1, except that the relative abundances of the two clones were reversed and additional mice were infected to allow a second mosquito feed. The treatment groups had initial inocula of 1×10^5 CR + 9×10^5 ER, 1×10^5 CR or 9×10^5 ER parasites. Again, four replicate experimental blocks were carried out, this time with four mice per treatment group, so that there were a total of 48 mice in experiment 2. Slides (to determine asexual parasite density and for monoclonal antibody analysis) were prepared from half of the mice in each treatment group on day 10 p.i., and mosquitoes were allowed to feed on these mice on day 14 p.i. The remaining mice were sampled for monoclonal antibody analysis on day 14 p.i. and mosquitoes were allowed to feed on them on day 18 p.i. Samples of oocysts for PCR analysis were stored as in experiment 1.

(*g*) *Data analysis*

To analyse the ratios of the two clones in the asexual and oocyst populations, generalized linear models with binomial error structures were fitted to counts of the CR alleles and total alleles sampled from each mouse (Crawley 1993). For asexual parasites, one allele represented one haploid asexual parasite counted. For the transmission stages one allele represented one of the two alleles identified by PCR in a single oocyst (i.e. the equivalent of one successful gamete). The mean and 95 % confidence intervals were calculated for the eight mice in each group of mice with mixed infections, one from experiment 1, and one for each of the two feeds in experiment 2.

The relative success of the clones in mixed and single-clone infections was analysed as follows. The asexual density for each mixed infection mouse was multiplied by the relevant ratio of the two clones as determined from monoclonal analysis of that infection. This determined the asexual densities attributable to the CR and ER clones separately. Similarly, the mean number of oocysts per gut, derived from each of the mixed infection mice, was multiplied by the ratio of the two clones obtained by PCR analysis of the oocysts for that mouse. This gave the contribution to the oocyst populations attributable to each clone. All these values were then $\log_{10}$ transformed (adding 1 to mean oocyst counts to allow zero values to be analysed) to give a normal error distribution for statistical analysis.

For each experiment and sampling point, the asexual or oocyst population densities of CR and ER in the mixed infections were compared to those in the corresponding single-clone control infections using *t*-tests. The four blocks were combined to give eight mice in each of the mixed and single-clone infection groups, and the significance of differences between the two treatments assessed. Ratios of the mean population sizes in the mixed infections to the mean population sizes in the control infections were calculated from the untransformed data to represent these results graphically. Following this, ANOVAs were carried out to assess the significance of differences between blocks within each experiment. However, as the sample sizes per block were small, significant interaction terms must be interpreted with caution.

In order to account for any error involved in estimating the clonal ratios for individual mice the results of these analyses were confirmed as follows. All analyses were repeated using the mean proportions of CR across the eight mixed infection mice fed at that time, and also the upper and lower 95 % confidence intervals of this mean. None of these changes altered the conclusions (analyses not presented).

3. RESULTS

(*a*) *Total parasite numbers*

A full analysis of total asexual parasite and oocyst numbers has already been presented (Taylor *et al.* 1997). Briefly, in both experiments, mixed-clone infections produced higher oocyst burdens than the sum of the two corresponding control groups. Similarly, gametocyte densities in mixed infections on the day of the feed were significantly higher than the corresponding sum of the CR and ER control infections, and probably account for this difference. Oocyst burdens following the day 14 p.i. feed in the two experiments were similar, but in experiment 2, oocyst burdens following the day 18 p.i. feed were only about 10 % of those on day 14 p.i.

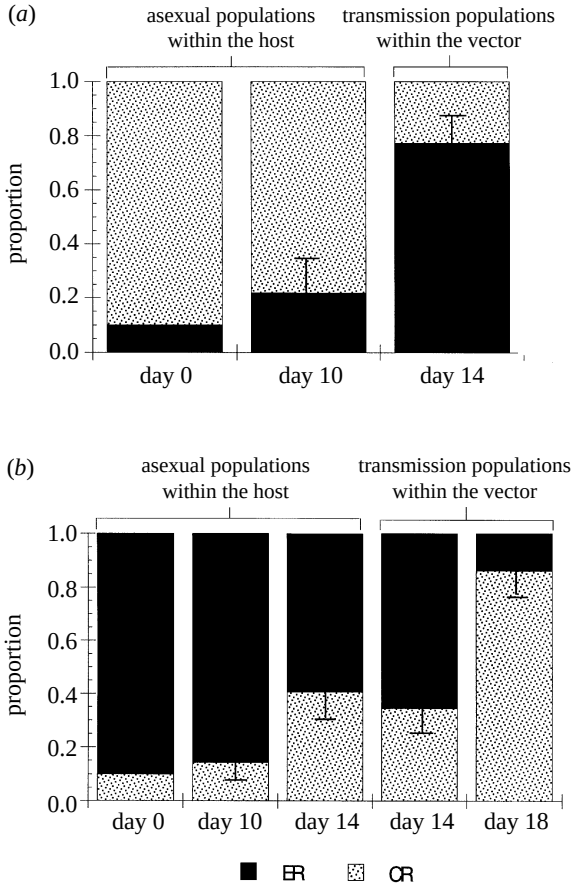

Figure 2. Clonal composition at sampling points throughout the mixed infections in (*a*) experiment 1 and (*b*) experiment 2. The error bars are 95 % confidence intervals. Days refer to the day post infection of the mice in all cases (day 0 values represent the starting inocula).

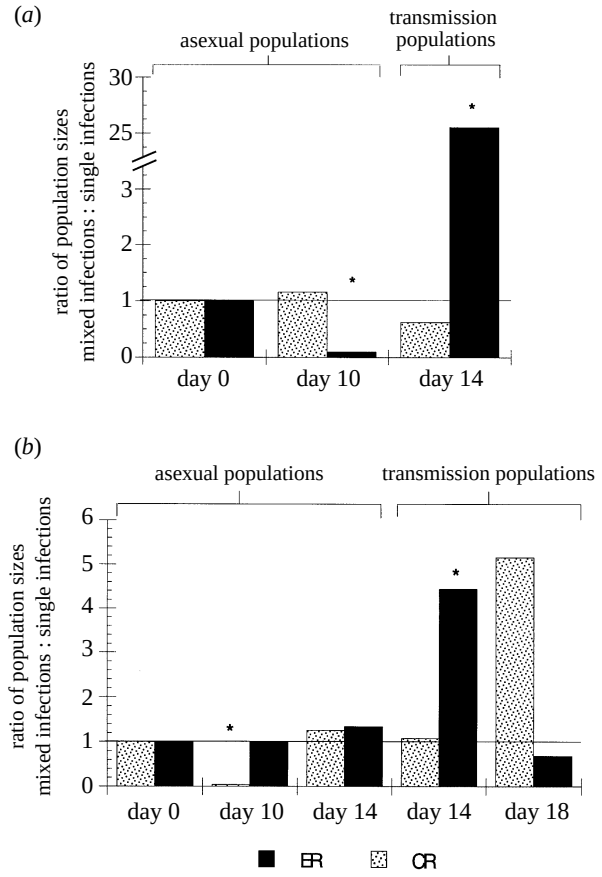

Figure 3. Population sizes of the two clones within the mixed infections relative to the single-clone controls for (*a*) experiment 1 and (*b*) experiment 2. The horizontal line represents the situation where a clone has equal population sizes in mixed and single-clone infections. Days refer to the day post-infection of the mice in all cases (day 0 values represent the starting inocula). The mean oocyst burden produced from the mixed infections was similar for both day 14 feeds (70.9 and 79.0 oocysts per mosquito for experiments 1 and 2, respectively), but much lower for the day 18 feed in experiment 2 (5.9 oocysts per mosquito). An asterisk denotes a significant difference in the population of a clone in the single-clone and mixed-clone infections.

(*b*) *Hardy–Weinberg equilibrium in oocysts derived from mixed infections*

When mosquitoes took a blood meal from mixed infection mice, both self-fertilization and cross-fertilization were expected to occur, resulting in homozygote and heterozygote oocysts respectively. If random mating between the gametes of the two clones occurred, alleles in the oocysts derived from the mixed infections should conform to the Hardy–Weinberg equilibrium. This was the case for each of the three feeds ($\chi^2_2 = 0.36$ (experiment 1), $\chi^2_2 = 0.06$ (experiment 2, day 14), $\chi^2_2 = 0.54$ (experiment 2, day 18); in all cases $p > 0.5$).

(*c*) *Relative frequencies of the two clones in mixed infections*

In experiment 1, ER constituted 10 % of the inocula of the mixed infections. On day 10 p.i., just after peak asexual parasitaemia, this had risen to 22 %. However, in the mosquitoes fed on the infection four days later, 78 % of the alleles of the oocysts were derived from the ER clone (figure 2*a*).

In experiment 2, the CR clone, which comprised 10 % of the initial inoculum, had increased to 14 % of the infection on day 10 p.i. and to 41 % on day 14 p.i. Oocysts which developed in mosquitoes fed on day 14 p.i. contained 34 % CR alleles, and those in mosquitoes fed on day 18 p.i. showed that 86 % of the successful gametes were derived from the CR clone (figure 2*b*).

None of these ratios differed significantly between the blocks of the experiments, except for the proportion of ER in the oocysts of experiment 1 (for all others $p > 0.1$). Mice with mixed infections in the second block of experiment 1 had a higher proportion of CR than the mice in the other three blocks (36.2 % compared to 17.3 %, 17.6 % and 19.3 %, $\chi^2_3 = 7.89$, $p < 0.05$).

(*d*) *Relative success of clones in mixtures and single-clone infections*

(i) *Asexual populations*

In experiment 1, clone ER was suppressed within the mixed infection ($t_{14} = 2.67$ $p < 0.05$) having on average 9.6 % of the asexual density of the ER control infections on day 10 p.i. (figure 3*a*). This effect varied

significantly between blocks ($F_{3,8} = 10.17$, $p < 0.01$). Suppression within the mixed infections occurred in three of the four blocks (where ER asexual densities in the mixed infections ranged from 54% to 1% of the control ER infections), and ER produced the same asexual density in mixed and single-clone infections in the fourth block. At the same time, mean asexual density of the other clone, CR, across all mice was unaltered in the mixed-clone compared to the single-clone control infections ($t_{14} = 0.78$, $p > 0.2$; figure 3*a*). However, the relative success of CR in mixed-clone and single-clone infections varied significantly between blocks ($F_{3,8} = 7.64$, $p < 0.01$), with suppression of CR within the mixed infections occurring in one of the four blocks.

In experiment 2, when the CR asexual densities in the CR control mice were compared with those in mixed infections on day 10 p.i., the initially rare clone was again suppressed in the mixed infection ($t_{14} = 7.63$, $p < 0.0001$). As part of the mixed infection, CR achieved only 3% of the density it could have achieved if ER were not present in the same host. The extent of this suppression varied significantly between blocks ($F_{3,8} = 7.18$, $p < 0.05$), but all four blocks showed high levels of suppression (CR densities in the mixed infections ranged from 14% to less than 1% of the control infections). ER, the majority clone in the inoculum had similar asexual densities in mixed and single-clone infection ($t_{14} = 0.20$, $p > 0.5$), and there was no evidence that this result varied between blocks ($F_{3,8} = 1.20$, $p > 0.2$).

By day 14 p.i. of experiment 2, asexual densities of ER and CR were not significantly different between the mixed and control groups ($t_{14} = 0.65$, $p > 0.5$ and $t_{13} = 0.33$, $p > 0.5$, respectively; figure 3*b*). For the relative successes of CR there was a marginally significant difference between blocks ($F_{3,7} = 4.27$, $p \approx 0.07$), with suppression within the mixed infections occurring in two out of four blocks. There was no evidence of similar processes occurring for the ER populations ($F_{3,8} = 0.30$, $p > 0.5$).

(ii) *Transmission populations*

In experiment 1, the numbers of oocysts derived from the CR clone was similar in single-clone and mixed-clone infections ($t_{13} = 0.06$, $p > 0.5$). However, the ER clone produced more oocysts in mosquitoes fed on the mixed infections than in those fed on the single-clone infections ($t_{13} = 3.55$, $p < 0.01$). On average, mixed infections produced 26 times as many successful ER gametes as those in the ER control mice (figure 3a). There was no evidence of significant differences in the relative success of the clones between blocks (for both clones, $p > 0.5$).

In experiment 2, transmission of clone CR on day 14 p.i. was not altered relative to the control ($t_{14} = 0.94$, $p > 0.2$), but ER had higher transmission success from mixed infections than from single-clone infections ($t_{14} = 2.56$, $p < 0.05$). ER oocyst burdens were on average 4.4 times higher from the mixed infections than from the control infections (figure 3*b*). There was no evidence that the relative transmission success of either clone differed significantly between blocks (in both cases, $p > 0.2$).

Oocyst burdens following the day 18 p.i. feed were low for all three groups of mice, and differences in the transmission successes of both clones between mixed and control infections were not significant ($t_{13} = 0.39$, $p > 0.5$ for ER and $t_{13} = 1.43$, $p > 0.1$ for CR). However, CR within the mixed infection produced on average 5.2 times as many oocysts as in the single-clone control infections (figure 3*b*). Again, there was no evidence of significant differences in the relative transmission success of the clones between blocks (in both cases, $p > 0.5$).

4. DISCUSSION

Mathematical models of mixed parasitic infections predict very complex dynamics, both over the course of an infection (Hellriegel 1992; Antia *et al.* 1996), and over evolutionary time-scales (Levin & Pimentel 1981; Nowak & May 1994). The results presented here show that the relative frequency of two competing clones can alter dramatically over the course of an infection, and that the relative frequency of transmitted genotypes can be very different from that at the start of an infection.

The presence of a more numerous competitor in the inoculum suppressed the parasitaemia of the initially rare clone in both experiments, whereas initially dominant clones achieved parasitaemias comparable to control infections. The mechanism by which this suppression occurs is unclear, but competition for red blood cells and nutrients can limit total parasite densities (Hellriegel 1992; Yap & Stevenson 1994; Gravenor *et al.* 1995; Hetzel & Anderson 1996), as can non-specific host responses (Richie 1988). During the initial rise in parasite numbers, the ratio of the two clones remains approximately the same as the starting inocula in both experiments. This suggests that direct interference between the two clones was not occurring, but that factors limiting total parasite densities resulted in suppression of the minority clone relative to its control infection (resource competition or immune-mediated apparent competition *sensu* Holt 1977). The rate at which an initially rare clone is able to increase in frequency within mixed infections probably depends on the relative competitive abilities of the clones, their growth rates and the relative strengths of strain-specific immunity induced in the host. Of the two clones used here, ER seems to be able to increase its representation the fastest (figure 2).

Within-host population sizes from the main part of the infection poorly predicted transmission success (figure 2). Relative frequencies of the genotypes in oocysts were not directly related to their starting inocula, and could not be predicted from the asexual composition of the infection four days prior to the feed. All three groups of mice showed that the initially rare clone dramatically increased its representation (percentage of all parasites) in the oocyst population relative to its frequency in the asexual population four days

earlier. In all experiments the clone introduced as the minority of a mixed infection produced as many oocysts or more than it did as a single-clone infection (figure 3). When ER was the initially rare clone, for example, its transmission was 20-fold higher than that which it achieved as a single-clone infection. We assayed transmission at specific time points, rather than throughout the infections. Nevertheless, density on day 14 p.i. is probably a reasonable approximation to total transmission: gametocytes are produced for only a short period during *P. chabaudi* infections in mice, with very low densities prior to day 12 p.i. and from day 18 p.i. onwards (Read & Anwar, in preparation; Buckling *et al.* 1997).

These results do not support the assumption made in many models of virulence (e.g. May & Anderson 1990; Gupta & Day 1996; see reviews by Bull 1994; Frank 1996) that within-host replication rate is directly correlated with transmission from the infection. ER forms the majority of the transmission on day 14 of both experiments, despite starting as the minority of the mixed infection in the first and the majority in the second. Furthermore, in the experiment 1 feed and the experiment 2 day 18 feed, the initially rare clone, which was heavily suppressed in the early stages of the mixed infection, contributed the majority of the transmission population. This 'co-infection' is more complex than that assumed by some current models dealing with 'super-infection', where a superior competitor takes over a host to exclude the other from transmission altogether (Levin & Pimentel 1981; Bremermann & Thieme 1989; Nowak & May 1994). In all three feeds, both clones contributed to the transmission population, with random mating occurring between the gametocytes of the two clones as has previously been observed for *P. falciparum* (Ranford-Cartwright *et al.* 1993) and *P. vivax* (Rosenberg *et al.* 1992).

There are at least two explanations for the dramatic differences in relative frequency of the two clones in the transmission population and in the asexual population four days earlier. First, there may be changes in the gametocyte conversion rate of the parasites within the mixed infections. Previous work using *P. chabaudi* in splenectomized rats indicated that gametocytes of *P. chabaudi* take four days to mature (Cornelissen & Walliker 1985). If this is the case in mice, oocysts sampled from a feed would be derived from gametocytes whose development was initiated four days previously. This assumption was the basis of our sampling regime in these experiments. If it is correct, the initially rare clone (which was heavily suppressed within mixed infections) must have produced a higher number of gametocytes, and hence oocysts, per asexual parasite than the initially dominant clone. Such a result could be predicted from life history theory where an organism being suppressed in one type of development (here, asexual proliferation) should divert resources to another, such as transmission stage production (Stearns 1992; Buckling *et al.* 1997).

Second, there may be marked alterations in parasite density and clonal composition between these two points in time, with gametocyte density of each clone correlated with its asexual density. Very recently it has been suggested that, in mice, gametocytes of *P. chabaudi* could take only 48 hours to develop from merozoite to full maturity (Gautret *et al.* 1997). As gametocyte development appears to be decided before the merozoite invades a new red blood cell (Bruce *et al.* 1990), it is still not clear exactly how long the period between commitment to gametocyte development and functional maturity may be. If this period is around two days, the composition of the gametocyte (and hence oocyst) populations would reflect the composition of the asexual populations fewer than four days prior to the feed. Strain-specific immune clearance might allow the initially rare clone to dominate the asexual population later in the infection when conversion to transmission stages occurs, and hence to increase transmission. Rapid changes in the genotypic composition of asexual populations can occur in *P. falciparum*-infected humans (Daubersies *et al.* 1996) and in *P. chabaudi*-infected mice (Read & Anwar, in preparation). Such changes in asexual dynamics are unlikely to be absolutely synchronous between mice, and could therefore contribute to variation between blocks in the clonal composition of the asexual population.

It is not possible to formally rule out either explanation with our data, but the second experiment gives some insight. On day 14 p.i., the clonal frequencies in the asexual populations are remarkably similar to those found in mosquitoes fed that day (figure 2*b*), perhaps most consistent with the second explanation. It seems likely that ER was a superior competitor whether it started as a minority or as a majority of the inoculum, and was consequently able to dominate the asexuals of the mixed infections on day 14 in both experiments, and that the higher transmission results from this. Clone CR also manages to dominate the transmission population in experiment 2, but not until day 18 p.i. This suggests that dominance of the initially rare clone later on in infections may be a general phenomenon, but with the precise timing determined by differences in competitive ability between the clones.

Our results show that a clone can produce as many or more oocysts when it is the initially rare clone in a mixed infection as when it is infecting a host on its own. If initially rare clones can increase transmission by having a later peak of infection, then why do they not do this in single-clone infections? Part of the answer may relate to the ecology of *P. chabaudi* in its natural host, *Thamnomys rutilans*, where it is described as a persistent, chronic infection (Landau & Chabaud 1994) with many hosts harbouring more than one genotype (Beale *et al.* 1978). Mixed infections of the human malaria, *P. falciparum*, are also common (Creasey *et al.* 1990; Day *et al.* 1992). It may be that malaria parasites have evolved to maximize transmission from mixed-genotype infections genotype infections. The fact that asexual success does not correlate with increased transmission begs the question of why parasites produce such a large population of apparently functionless asexuals in both single and mixed infections (Taylor & Read 1997).

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APPENDIX 1

Controlling for differential affinity of monoclonal antibodies

Two factors could produce a biased estimate of the ratio of the two clones assessed by immuno-fluorescent antibodies. The antibodies may vary in their affinities to their respective protein targets, or the expression of the target antigen may vary between clones. Differences between clones in the timing of schizogony, and hence the expression of the target antigen MSP-1, have been observed in *P. chabaudi* (McLean 1986).

To control for any such biases, mixtures were constructed and analysed as follows. Appropriate volumes of blood from mice infected with known parasitaemias of the CR or ER clones were pooled to give 11 mixtures ranging from 5% to 95% ER. These mixtures were rinsed five times in PBS and pipetted onto multispot slides as with the experimental samples. The protocol detailed in the methods section was repeated exactly on these control mixtures. Control mixture slides were made on three separate occasions. All ratios were counted from examination of these slides under UV light and were plotted against the true (known) ratio of the parasite clones in the mixture. The results show a higher affinity of the H2 monoclonal antibody (binding to ER) relative to B18 (binding to CR), leading to an overestimated proportion of ER parasites (see figure 4). The equation for the least squares best fit line through all datapoints and forced through the points 0,0 and 1,1 was then obtained. The counts of parasites obtained from the experimental mouse blood samples were adjusted for the slight bias in affinity using this fitted equation (actual proportion ER $= x + 0.4465x(x-1)$, where $x =$ observed proportion ER).

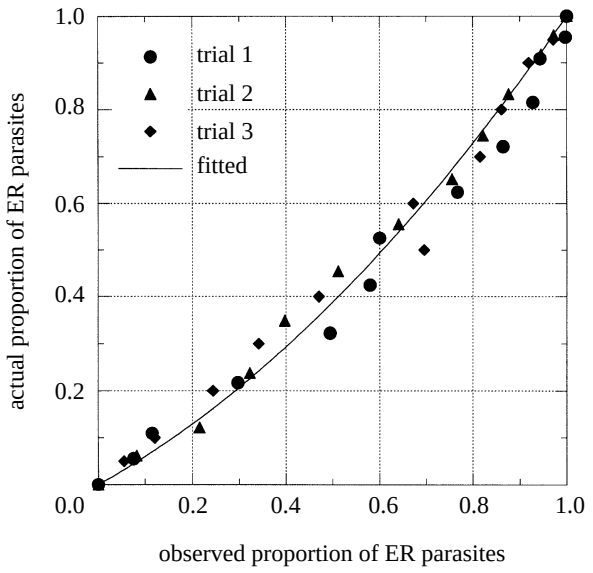

Figure 4. Controlling for differential affinity of two monoclonal antibodies. Observed against true proportions of ER for the three sets of artificial mixtures. The fitted line has the equation $y = x + 0.4465x\,(x-1)$.

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