Competitive release and facilitation of drug-resistant parasites after therapeutic chemotherapy in a rodent malaria model

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Malaria infections frequently consist of mixtures of drug-resistant and drug-sensitive parasites. If crowding occurs, where clonal population densities are suppressed by the presence of coinfecting clones, removal of susceptible clones by drug treatment could allow resistant clones to expand into the newly vacated niche space within a host. Theoretical models show that, if such competitive release occurs, it can be a potent contributor to the strength of selection, greatly accelerating the rate at which resistance spreads in a population. A variety of correlational field data suggest that competitive release could occur in human malaria populations, but direct evidence cannot be ethically obtained from human infections. Here we show competitive release after pyrimethamine curative chemotherapy of acute infections of the rodent malaria *Plasmodium chabaudi* in laboratory mice. The expansion of resistant parasite numbers after treatment resulted in enhanced transmission-stage densities. After the elimination or near-elimination of sensitive parasites, the number of resistant parasites increased beyond that achieved when a competitor had never been present. Thus, a substantial competitive release occurred, markedly elevating the fitness advantages of drug resistance above those arising from survival alone. This finding may explain the rapid spread of drug resistance and the subsequently brief useful lifespans of some antimalarial drugs. In a second experiment, where subcurative chemotherapy was administered, the resistant clone was only partly released from competitive suppression and experienced a restriction in the size of its expansion after treatment. This finding raises the prospect of harnessing in-host ecology to slow the spread of drug resistance.

competition | evolution of drug resistance | *Plasmodium chabaudi* | transmission | in-host ecology

Resistance to antimicrobial drugs is usually detected in pathogen populations within a few years of drug deployment. The subsequent evolution is one of the leading causes of failure to control infectious diseases in humans (1, 2). A key determinant of the time taken for a resistant mutant to spread sufficiently to undermine the clinical usefulness of a drug is the strength of selection for resistance. Even small differences in the relative fitness of wild-type and drug-resistant pathogens can alter the useful therapeutic lifespan of a drug by decades (3). The strength of selection is determined by a number of factors. Best known are those factors affecting parasite exposure to drugs, such as the frequency of drug use (4–9). In most mathematical models of this process, drug use reduces the fitness of drug-sensitive parasites while having no impact on resistant clones. However, the biology of malaria has led several theoreticians to propose that, where sensitive and resistant parasites coinfect the same host individuals, drug use would further increase the relative fitness of drug-resistant clones by removing drug-sensitive competitors (4, 9–13). Similar proposals have been made in the context of antiviral and antibacterial drugs (14–16).

The argument goes as follows. Imagine a person is infected with two clones of malaria parasites, one of which is resistant. If the drug-sensitive clone is removed by chemotherapy, the relative fitness of the resistant clone in the population will increase simply because it survives, whereas the other does not. But if the resistant clone experiences competitive release, whereby it is able to expand to fill the niche space from which it was previously excluded, the increase in relative fitness would be doubled if the clones were equally sharing the niche space and more than doubled if the resistant clone was in the minority. The effect gets even stronger as the number of sensitive clones in an infection increases.

If competitive release translates into increased transmission, it could have extremely large effects on the useful therapeutic lifespan of a drug. Where mixed infections are common, the magnitude of these effects could, in theory, be comparable to or even greater than that arising from the survival advantage of resistance alone (4). Theory also shows that if competitive release occurs, it would be a major determinant of whether drug resistance will spread to fixation or stabilize at intermediate frequencies (12), and of how resistance evolution will proceed when transmission is reduced by malaria-control programs (17).

A key question, then, is whether competitive release occurs. For malaria, there is direct evidence of the cocirculation of multiple *Plasmodium* clones in both acute and persistent human infections, including the coexistence of resistant and sensitive clones (18–23). A body of correlational epidemiological evidence is consistent with crowding effects in human malaria infections (24–28), and some patterns of drug resistance in Africa are more readily explained by invoking competitive release (11, 29). However, unambiguous experimental evidence of competitive release cannot be ethically obtained from human infections. Antimalarials are normally used to relieve suffering, and direct tests for competitive release require that clone performance in treated infections be compared with that in untreated infections.

We have therefore tested for competitive release after chemotherapy by using the rodent malaria model *P. chabaudi* in laboratory mice. Here strong crowding effects occur, whereby parasite and transmission stage densities of individual clones can be severely suppressed by the presence of coinfecting clones (30–36). This competitive suppression substantially reduces the transmission of

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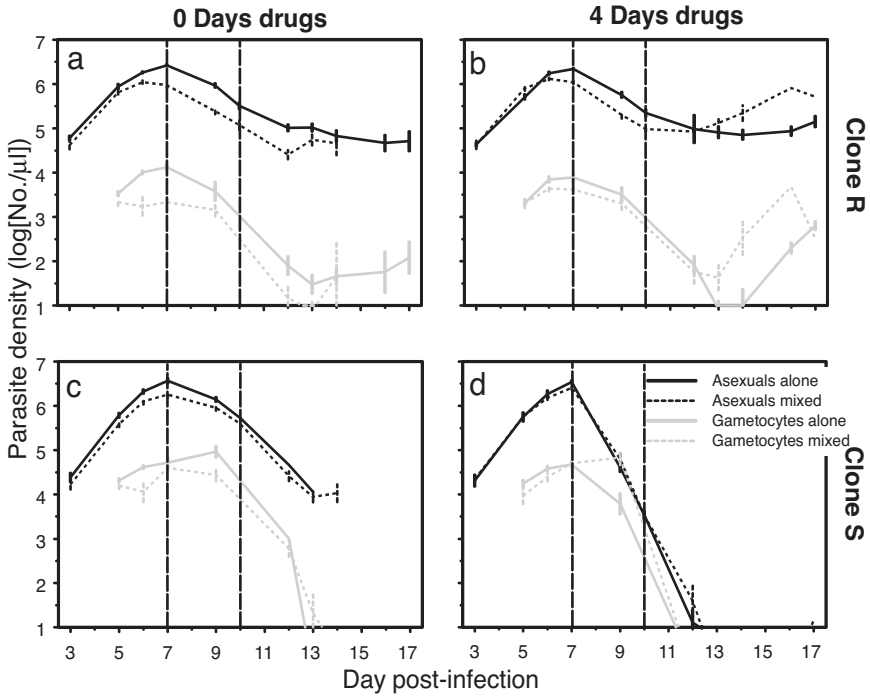

Fig. 1. Parasite densities through time in experiment 1. Asexual density from qPCR (black lines) and gametocyte density from qRT-PCR (gray lines) are given for the resistant (*a* and *b*) and sensitive clone (*c* and *d*) in single (solid lines) and mixed (dotted lines) clone infections. Drug treatment (*b* and *d*) or sham injection (*a* and *c*) was administered on days 7–10 inclusively (marked by hashed vertical lines). Posttreatment sampling began on day 12. Minimum *y* axis value represents the lowest reliable detection threshold of qPCR. Mean densities (± 1 SEM) were calculated from all mice that were alive on the respective sampling day, a maximum of 20 per group at the start of the experiment. Malaria-induced deaths progressively reduced sample sizes, particularly in the untreated groups, and one to three mice were removed per group on days 6, 7, 13, and 14 for other experiments. Numbers of surviving mice during the key posttreatment phase are shown in Fig. 2.

individual clones to mosquitoes (34). Administering chemotherapy immediately after the inoculation of infections of sensitive and resistant clones allows resistant clones to exploit the host in a way they cannot when competitors are present (37). Thus, there is competitive release with experimental protocols that mimic prophylactic chemotherapy.

But the critical issue is whether drug treatment administered during acute infections results in competitive release. Typically, malaria parasites are exposed to antimalarial chemotherapy not at the start of infections, but during treatment of clinical symptoms, which only appear after parasite densities have reached high levels. By the time the parasite becomes established and treatment occurs, the immune system may have become sufficiently primed to control any competitive release of the resistant clone (38). Here we report experiments testing for competitive release during the acute phase of infections. We found that competitive release did occur. Indeed, after curative or near-curative chemotherapy, this release was so great that the resistant clone did better than it did when a competitor had never been present. Moreover, drug regimes that only partially cleared the sensitive clone maintained a degree of competitive suppression, raising the prospect that in-host ecology could be harnessed to slow the spread of drug resistance.

Results

Curative Chemotherapy (Experiment 1). As found previously (33–37), the resistant clone was competitively suppressed by the sensitive clone in the absence of drug pressure so that, over the first 2 weeks of infection, it achieved densities of about half (60.4 ± 16.9%) that achieved when alone (total asexuals days 3–14, $F_{1,8} = 10.7, P = 0.01$; total gametocytes days 3–14, $F_{1,8} = 8.4$, $P = 0.02$) (Figs. 1 *a* and *b* and 2). Four days of pyrimethamine treatment, which was initiated when mice began to lose weight and became anemic, cleared the sensitive clone from all infections (Fig. 1 *c* and *d*). Host mortality also was reduced by chemotherapy in single infections of the sensitive clone (50% untreated, 5% treated; $\chi^2 = 15.5$, $P = 0.002$, df = 3). As expected, the density of the resistant clone was unaffected by drug treatment in single infections (total asexuals days 12–14, $F_{1,10} = 0.5$, $P = 0.5$) (Figs. 1 *a* and *b* and 2).

When the drug-sensitive competitor was removed by chemotherapy from the mixed infections, the resistant clone went on to produce at least twice as many asexual parasites and gametocytes as it produced when the competitor was present or than it did in treated infections when alone (competition–drug treatment interaction: total asexuals days 12–14, $F_{1,15} = 5.9$, $P = 0.03$; total gametocytes days 13–14, $F_{1,15} = 6.0, P = 0.03$) (Figs. 1 and 2). Thus, in the mixed infections, competitive release occurred after the elimination of the sensitive clone by chemotherapy. The resistant clone was able to capitalize on the removal of the competitor to such an extent that it produced significantly more gametocytes after treatment than it was able to do from infections in which a competitor had never been present (total gametocytes days 13–14, $F_{1,7} = 8.54, P = 0.02$) (Figs. 1*b* and 2*b*). We note that chemotherapy had no effect on anemia or mortality of mice harboring single infections of the resistant clone or mixed infections of the resistant and sensitive clones ($P > 0.25$).

Subcurative Chemotherapy (Experiment 2). As in experiment 1, the resistant clone was competitively suppressed by the sensitive clone in untreated infections, producing about half (58.7 ± 9.8%) the number of asexual parasites as it did when the sensitive clone was absent (Figs. 3 and 4). One and 2 days of drug treatment were subcurative: The asexual and gametocyte densities of the sensitive clone were reduced, but the clone was not eliminated (treatment main effect: total asexuals days 12–21, $F_{2,24} = 31$, $P < 0.001$; total

EVOLUTION

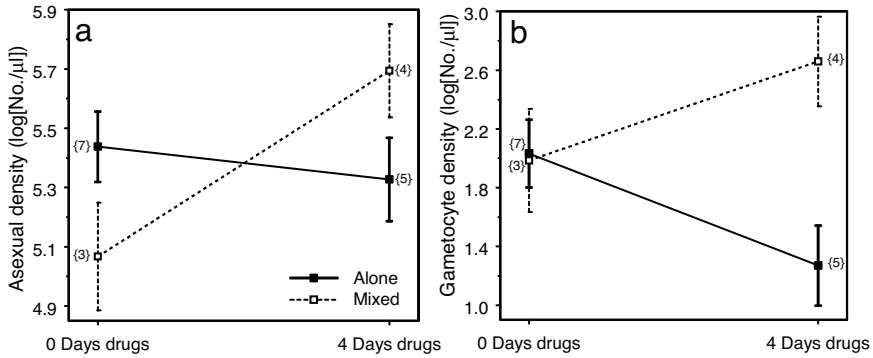

Fig. 2. Mean parasite density of drug-resistant clone in experiment 1. After 4 days of drug treatment, the resistant clone produced significantly more asexual parasites (*a*) and gametocytes (*b*) when the sensitive clone was present (dotted line) than when it was absent (solid line). Thus, elimination of the sensitive clone by chemotherapy resulted in a competitive release so great that the resistant clone performed better than it did in the prior absence of a coinfecting clone (treatment-dependent facilitation). Data points represent least squares mean (±1 SEM) of log-transformed total parasite density over days 12–14 (asexuals) and 13–14 (gametocytes) after infection for the mice surviving until the end of the sampling period. Numbers in brackets beside each point give the number of mice available for this analysis.

gametocytes days 12–21, $F_{2,24} = 47$, $P < 0.001$) (Fig. 3). Drug treatment did not affect the densities of the resistant clone in single infections (total asexuals days 12–21, $F_{2,8} = 1.9$, $P = 0.21$) (Fig. 4).

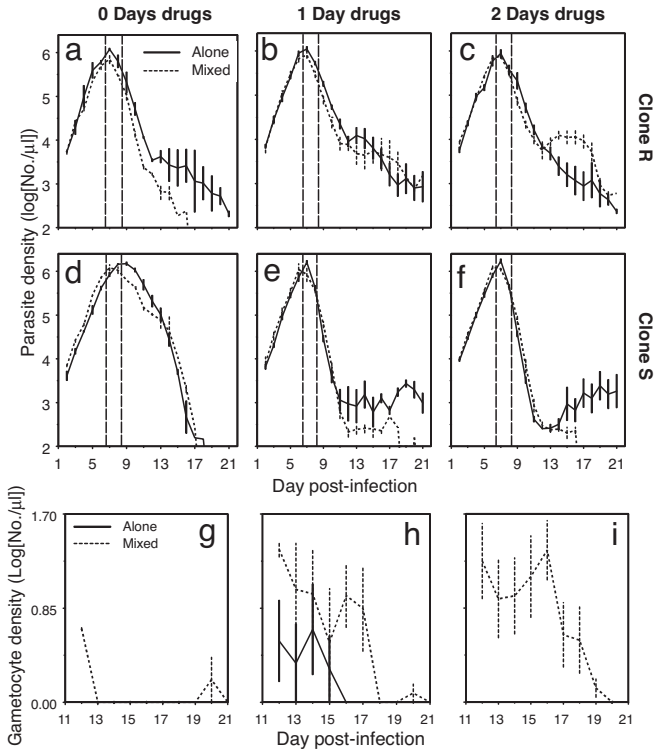

Fig. 3. Asexual parasite (*a–f*) and gametocyte density (*g–i*) over the course of infection for experiment 2 determined by qPCR. The mean asexual densities (±1 SEM) of the resistant (*a–c*) and sensitive (*d–f*) clones in single (solid line) and mixed (dotted line) infections are shown for the 0-, 1-, and 2-day drug treatment groups. Only the gametocytes of the resistant clone are shown. Drug treatment began on day 7 after infection, as indicated by the hashed vertical lines; untreated mice received a sham injection. Posttreatment sampling began on day 11 after infection. The sensitive clone was suppressed by the subcurative chemotherapy, with higher drug dosage resulting in lower parasite densities (*e* and *f*). Two days of drug pressure and high reduction in the density of the sensitive clone resulted in the enhanced growth of the resistant clone in mixed infections (treatment-dependent facilitation) (*c*). One day of drug treatment resulted in competitive release but not enhanced growth of the resistant clone (*b*). Plotted points are a mean of mice surviving until day 21 (see Fig. 4). Absence of lines indicates samples undetected by qPCR.

Suppression of the sensitive clone by drug treatment led to competitive release of the resistant clone, with the magnitude of the release determined by the duration of drug treatment (drug treatment–competition interaction: total asexuals days 12–21, $F_{2,19} = 6.8$, $P = 0.006$) (Figs. 3 and 4). One day of treatment enabled the resistant clone to expand to the densities it was able to achieve when the competitor was absent (total asexuals days 12–21, $F_{1,8} = 0.12$, $P = 0.74$; alone vs. in competition). Two days of treatment allowed the resistant clone to achieve higher densities than it managed when the sensitive clone had never been present (total asexuals days 12–21, $F_{1,9} = 12.3$, $P = 0.007$; alone vs. in competition). Thus, the extent of the competitive release is dose-dependent, with some degree of competitive suppression being maintained at the lower drug dose. We note that anemia decreased significantly after 1 day of drug treatment for both mixed and single infections containing the sensitive clone (mean RBC density days 12–21, $F_{1,12} = 29$, $P < 0.001$). Another day of treatment further alleviated anemia in the single infections of the sensitive clone ($F_{1,8} = 9.3$, $P = 0.016$), but there was no difference in the anemia induced by mixed clone infections after 1 or 2 days of drug treatment (mean RBC density days 12–21, $F_{1,9} = 0.13$, $P = 0.73$).

Gametocyte density of the resistant clone in the second experiment was difficult to assess because of values hovering around the accurate detection threshold of quantitative RT-PCR (Figs. 3 and 4). However, suppression of the sensitive clone by chemotherapy allowed the resistant clone to generate more gametocytes than it produced in the absence of treatment in competition (total gametocytes days 12–21, treatment main effect, mixed infections, $F_{2,13} = 8$, $P = 0.008$).

Discussion

The experiments reported here provide direct evidence of competitive release of a drug-resistant clone after suppression of a competitor by therapeutic chemotherapy. Prophylactic drug treatment has the same effect in this experimental model (37). These data, together with correlational field data consistent with crowding and competitive release (24–29), demonstrate that in-host competition could be an important determinant of the strength of drug selection for resistance in malaria populations. Competitive release of resistance after chemotherapy of mixed infections would greatly accelerate the evolution of resistance and may account for the depressingly short useful lifespans of some antimalarial compounds (4). Similar issues also may affect the evolution of vaccine escape if antimalarial vaccines selectively remove some *Plasmodium* clones and not others.

The mechanism of crowding in malaria infections is unknown, but could in principle arise from competition for limiting resources

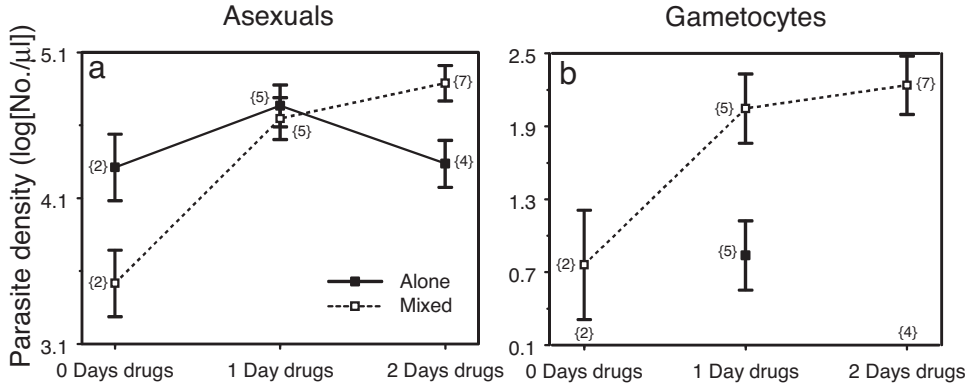

Fig. 4. Mean parasite density of drug-resistant clone in experiment 2. After 2 days of drug treatment, the resistant clone produced significantly more asexual parasites (*a*) and gametocytes (*b*) when in the presence of a competitor (dotted line) compared with the absence of a competitor (solid line), thus experiencing treatment-dependent facilitation. After 1 day of drug treatment, the resistant genotype still underwent competitive release, but it performed only as well as it did on its own. Points represent least squares mean (±1 SEM) of log-transformed total parasite density for days 12–21 after infection. Numbers in bracket show the numbers of mice surviving until the end of the sampling period, which were included in the analysis. Gametocytes were not detected by qRT-PCR in the single-infection 0- and 2-day drug treatment groups.

such as RBCs (39, 40), immune-mediated apparent competition (41), or direct interference competition (42). We are currently trying to determine whether the data we report here on the experimental removal of a competitively superior clone make it possible to distinguish between theoretical models of these possibilities. In the meantime, we note that, as well as enhancing the fitness benefits of resistance in treated hosts, in-host competition also could affect the fitness costs of resistance in untreated hosts. Any endogenous cost of resistance will, if it reduces the competitive ability of resistant clones, result in disproportionate reductions in the frequency of resistant clones in mixed infections. If resistance is generally associated with competitive suppression, the quantitative importance of competitive release we report here would be further magnified. Theoretical models predict that the benefit of competitive release may be particularly high when antimalarial drugs are first deployed in the field and the frequency of resistance in the population is low and then decreases as the frequency of resistance increases (12).

One of the unexpected findings in the current experiments was that competitive release after 2 or 4 days of antimalarial treatment resulted in the enhanced growth of the resistant clone, which was so elevated that it achieved higher densities than it did during the comparable period in infections where a competitor had never been present. What could explain this treatment-dependent facilitation of resistance by the prior presence of the susceptible clone?

One possibility is that the clone-specific component of host immunity focuses on the majority clone. Here, before treatment, that was the sensitive clone. If, after treatment, the host takes some time to respond to the rapid change in the antigenic composition, the resistant clone would experience a period of unchecked growth. Lags in genotype-specific immunity have been suggested by theorists (43) and observed in HIV infections, where minority antigenic types are overlooked because of immune commitment to majority antigens in the population (44). Clone-specific immunity is a feature of malaria infections, including *P. chabaudi*, and immune shielding by a numerically dominant clone would account for other competitive outcomes in *P. chabaudi* (30, 32, 33, 42, 45). If this delayed immune response hypothesis is correct, competitive release of drug-resistant clones would not occur when competing clones are genetically similar, such as when resistance arises *de novo*. The phenomenon we report here would instead be a feature of a resistant clone rising in frequency in a population where mixed clone infections are common, as found in many malaria-endemic regions (19, 46).

To what extent can conclusions derived from an animal model be generalized to human malarias? No models, mathematical or animal, can capture all possible relevant factors, and it is often difficult to assess the relevance of differences between model and reality. *P. chabaudi* infections in laboratory mice share many key features with *P. falciparum*, the most virulent human malaria, but there also are several potentially important differences (47, 48). For instance, *P. chabaudi* infections frequently reach parasitemias an order of magnitude higher than that found in human malaria infections (49–51), and the relative importance of strain-transcending and strain-specific immunity may differ. Unlike people, mice can generate sterilizing immunity against malaria. Likewise, human malaria parasites share a longer evolutionary past with their host, compared with *P. chabaudi* in the laboratory mouse, whose natural host is the thicket rat *Thamnomys rutilans*. It is impossible to determine whether these factors limit the generality of the results we report because they could point to stronger or weaker competitive interactions in mice than in humans. Clearly, the normal caution extrapolating from models needs to apply. But we note again that epidemiological evidence in human malaria infections is consistent with crowding effects in human malaria infections, and some patterns of drug resistance in Africa are more readily explained by invoking competitive release (11, 18–29).

If our experiments are capturing ecological processes in natural malaria populations, they raise an intriguing possibility. After 1 day of chemotherapy, competitive suppression was only partly relieved, resulting in a more restricted expansion by the resistant clone than was seen after 2 or 4 days of treatment. This finding suggests that, with judicious drug regimes, it might be possible to harness the crowding effects to slow the spread of drug resistance. There may be drug regimes that alleviate clinical illness, but do not eliminate sensitive clones. If so, at least some degree of in-host competitive suppression of resistance could be maintained, as seen in our experiments (Fig. 4). We note that, for the mice in our experiments, a second day of treatment did not lead to any further alleviation of anemia beyond that achieved by a single day of treatment, but it did lead to a larger expansion of parasite numbers by the resistant clone. This finding offers the prospect of drug treatment regimes that slow resistance evolution and balance ethical considerations for the well being of infected individuals.

The idea of restricting drug dosage to avoid eliminating parasites contradicts medical orthodoxy that incomplete drug treatment accelerates drug resistance evolution. Overwhelming chemotherapy far beyond what is needed on clinical grounds is frequently said to maximally prolong the useful lifespan of a drug and is a stated

Table 1. Mice in each treatment group from experiments (Exp.) 1 and 2

Clone	No. of mice for each drug treatment duration			
	0 days	1 day	2 days	4 days
Exp. 1 (CBA/Ca mice)				
R	7 [4 + 9 + 0]	—	—	5 [5 + 9 + 1]
S	0 [11 + 7 + 2]	—	—	9 [0 + 9 + 2]
R + S	3 [7 + 9 + 1]	—	—	4 [5 + 8 + 3]
Exp. 2 (C57BL/6J mice)				
R	2 [9 + 0]	5 [5 + 1]	4 [7 + 5]	—
S	3 [8 + 1]	5 [7 + 0]	6 [8 + 4]	—
R + S	2 [10 + 0]	5 [6 + 1]	7 [11 + 0]	—

Values given are the final number of mice used for analysis, with the corresponding numbers in brackets representing mice that were excluded because of death (left value), removed for other experiments in the laboratory (center value, experiment 1 only), or failed inoculation (right value). The total of each cell is the number of mice initially inoculated per treatment. Some treatment groups were not carried out in both experiments (—). Fig. 1 tracks some mice that later died or were removed for other experiments and were excluded from analyses. All mice that died in experiment 2 were excluded from all figures and analyses.

goal in many public health contexts (52–55). Yet basic population genetic theory shows that the strength of selection is proportional to the level of drug pressure. Hence, access to antimicrobial drugs is frequently restricted at a population level. Similar arguments also are relevant to the treatment of individuals. For example, drugs with shorter half lives reduce the number of parasites exposed to drugs, and thus weaken selection for resistance (5, 9, 17). In light of the results reported here, we suggest that there is a strong argument for theoretical and experimental investigations of different chemotherapeutic protocols at an individual host level. It may be that subcurative doses make it easier for resistance involving multiple mutations to arise, but it also may be that crowding effects substantially reduce the rate of spread once resistance has begun to spread. We expect the picture to vary between different epidemiological settings and depend on the frequency of resistance in a population, as well as how chemotherapy impacts on transmission and, hence, clone multiplicity in infections. There need be no simple generality.

Materials and Methods

Parasites, Hosts, and Drug Treatment. A pyrimethamine-resistant *P. chabaudi* clone and a genetically distinct pyrimethamine-sensitive clone were used. Both clones were originally isolated from thicket rats *Thamnomys rutilans* (56). The drug-resistant clone, ASpyr-1B, was derived from the ancestral clone AS by pyrimethamine selection during several rounds of serial passage (57). The drug-sensitive clone was derived from ancestral clone AJ and has never been subject to drug selection. For simplicity, herein we simply refer to each clone as either resistant or sensitive (in the figures, as R and S, respectively). The resistant clone is less virulent than the sensitive clone, achieves lower densities, and is less successful in competition (34, 36, 37, 57).

We performed two experiments: the first to investigate curative chemotherapy and the second to investigate subcurative chemotherapy. In both experiments, mice were inoculated with 10^6 sensitive, 10^6 resistant, or 10^6 sensitive plus 10^6 resistant parasites (2×10^6 total parasites) so that the dynamics of each clone could be compared in the presence or absence of a competitor. Parasites were inoculated into randomized 6- to 8-week-old female mice as described elsewhere (51) and housed by treatment group in cages of three to five mice to create three to five cage replicates of each treatment. The number of mice per treatment group can be found in Table 1. In experiment 1, mice were CBA/Ca; in experiment 2, C57BL/6J mice were used because of the high disease severity and host mortality observed in experiment 1. Mice were maintained as described elsewhere (58).

Antimalarial chemotherapy began on day 7 after infection, when mice first showed significant signs of weight loss and anemia. The antifolate pyrimethamine was dissolved in DMSO, and 100 μl was administered by oral gavage at a concentration of 8 mg per kilogram of mouse body weight, previously shown to clear all sensitive parasites after 4 days of treatment (37). Mice received 0 or 4 days of treatment (experiment 1) or 0, 1, and 2 days of treatment (experiment 2). If not receiving pyrimethamine, mice were gavaged with DMSO alone. All mice were inoculated and gavaged on the same day within an experiment. High levels of mortality occurred in this study across all treatment groups because of a combined effect of highly virulent infection and the stress of drug treatment. For this reason, mice that died were excluded from all analyses. The details of mice inoculated per treatment group and deaths can be found in Table 1.

Monitoring Infection Dynamics. RBC density was measured daily by using flow cytometry (Beckman Coulter), with a baseline taken 1 day before the start of infection. Clone-specific parasite density was estimated by using quantitative PCR (qPCR) on parasite DNA extracted from 5 μl of whole blood taken each day of sampling (35). DNA was extracted with a Prism 6100 machine (Applied Biosystems) according to the manufacturer's instructions. The DNA qPCR was targeted toward the *P. chabaudi ama* gene by using a conserved *Taq*Man probe (5′-6FAM-ATC CTC CTT CTC TTA CTT TC-MGB-3′) and clone-specific primers (resistant clone: forward, 5′-GGA AAA GGT ATA ACT ATT CAA AAT TCT AAG GT-3′, and reverse, 5′-AAT TGT TAT AGG AGA AAT GTT TAC ATC TGT TTG-3′; sensitive clone: forward, 5′-GGA AAA GGT ATA ACT AAT CAA AAA TCT ACT AAA-3′, and reverse, 5′-GTG TTA TAG GAG AAA TGT GTA CAT CTG TTT T-3′). The qPCR was carried out in a final volume of 25 μl containing 2 μl of DNA, each primer at 300 nM, 200 nM probe, 12.5 μl of *Taq*Man Universal PCR Master Mix, and 6.5 μl of H_2O on a Prism 7000 machine (Applied Biosystems).

Clone-specific gametocyte (transmission stage) densities were estimated by using clone-specific qRT-PCR on gametocyte RNA extracted from 20 μl (experiment 1) or 10 μl (experiment 2) of blood (36, 58). RNA was extracted with an Prism 6100 machine (Applied Biosystems), and cDNA was generated by using the high-capacity cDNA archive kit (Applied Biosystems) in a 50-μl reaction. The qRT-PCR was targeted on the *P. chabaudi* gametocyte-specific gene PC108476.00.0 by using clone-differentiating forward primers for the resistant (5′-AAG TTT ACC TGA GAG TAC AAA TAT AAT AGG TGT A-3′) and sensitive (5′-TGA CAG TAC AAA TAT AAT AAG CGC AGT T-3′) clone, with a conserved reverse primer (5′-GCT GCT ATA CGT GTT ATA AAT CCT ATT ACT-3′) and *Taq*Man probe (5′-6FAM -TGT

TAT AAT TGT GTT CAC CCT ATC-MGB-3′). The qRT-PCR was carried out in a final volume of 25 μl by using 7 μl of cDNA, each primer at 900 nM, 250 nM probe, and 12.5 μl of *Taq*Man Universal PCR Master Mix on the Prism 7000 machine.

Typically, <1% of parasites are gametocytes (36), and therefore qPCR counts primarily reflect asexual densities and qRT-PCR counts reflect gametocytes. Validation on this host–parasite system of all sampling and quantification methods used here has been reported elsewhere (34–37, 58). The sampling order of cages was randomly assigned each day. We monitored infection dynamics up to day 21 after infection because drug treatment typically cleared the sensitive clone by day 13 (Figs. 1 and 3). Furthermore, previous studies observed little competition dynamics or transmission after day 21 (35), and drug treatment is unlikely to occur during this phase because infections are frequently asymptomatic.

Trait Definition and Statistical Analysis. Competitive suppression is a reduction of parasite numbers when another clone is present, which we tested for by comparing the performance of a clone in single and mixed infections. The opposite of competitive suppression is facilitation, where clone performance is improved by the presence of a coinfecting clone. Competitive release is improved clonal performance after the removal of a competitor, which we tested for by comparing the performance of the resistant clone in treated and untreated mixed infections. *P. chabaudi* has a 24-h cell cycle, so the total number of parasites present in any defined period can be estimated by summing daily parasite counts.

The effects of competition and drug treatment on the performance of individual clone, and of drug treatment on virulence, were examined by using general linear models (GLM). For GLM analysis, response variables included mean total parasite density, mean total gametocyte density, and mean RBC density, with initial RBC density as a covariate. Using RBC density at the time of drug treatment (day 7) yielded the same conclusions (data not shown). Response variables were summed or averaged for each mouse over the appropriate course of infection to avoid repeated measures on the same mouse in the analysis. Explanatory variables for GLM included drug treatment (0, 1, 2, or 4 days of drugs), clone (resistant or sensitive), and competition (clone alone or in mixed infection). Maximal models (variation in factor = clone + drug treatment + competition + all higher order interactions) were tested in the first instance, and minimal models were obtained by dropping nonsignificant terms successively, beginning with highest order interactions, to obtain the significant minimal model. We present statistical results in the form of $F_{x,y}$, where F is the F ratio, x is the df associated with the stated factor, and y is the error df. A χ^2 test was used to examine percent morality in experiment 1. Data were log (counts) or arcsin (proportions) square-root-transformed to meet the assumption of normality and homogeneity of variance.

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