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Selection for high and low virulence in the malaria parasite *Plasmodium chabaudi*

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What stops parasites becoming ever more virulent? Conventional wisdom and most parasite-centred models of the evolution of virulence suppose that risk of host (and, hence, parasite) death imposes selection against more virulent strains. Here we selected for high and low virulence within each of two clones of the rodent malaria parasite *Plasmodium chabaudi* on the basis of between-host differences in a surrogate measure of virulence—loss of live weight post-infection. Despite imposing strong selection for low virulence which mimicked 50–75% host mortality, the low virulence lines increased in virulence as much as the high virulence lines. Thus, artificial selection on between-host differences in virulence was unable to counteract natural selection for increased virulence caused by within-host selection processes. The parasite's asexual replication rate and number of sexual transmission forms also increased in all lines, consistent with evolutionary models explaining high virulence. An upper bound to virulence, though not the asexual replication rate, was apparent, but this bound was not imposed by host mortality. Thus, we found evidence of the factors assumed to drive evolution of increased virulence, but not those thought to counter this selection.

Keywords: virulence; malaria; evolution; *Plasmodium chabaudi*

1. INTRODUCTION

Most models of the evolution of virulence revolve around parasite adaptation. In these models, it is assumed that the reduction in host fitness following parasitic infection (virulence) is primarily the result of parasite traits favoured by natural selection. Increased rates of host resource use are said to allow parasites more replication or better immune evasion or to induce symptoms that promote transmission. All of these should increase parasite fitness through higher rates of transmission to new hosts but, for horizontally transmitted parasites, are also unavoidably associated with increased rates of host (and, hence, parasite) death. Virulence is thus expected to be some optimal schedule of host exploitation that balances the fitness losses due to possible host death with the fitness gains due to increased between-host transmission (Levin & Pimentel 1981; Anderson & May 1982; Bremermann & Pickering 1983; May & Anderson 1983; reviewed by Bull 1994; Ewald 1994; Read 1994; Frank 1996; Ebert & Herre 1996).

A key prediction of this 'adaptive trade-off' hypothesis is that between-host selection on fitness should genetically alter the virulence of parasite populations. If only less-virulent infections are allowed to contribute propagules to subsequent hosts—a situation which mimics selection against virulence imposed by host death—subsequent infections should become increasingly benign. If only more-virulent infections are allowed to transmit propagules to the next generation, virulence should rise because of the fitness gains through increasing host exploitation. Here we test this prediction by imposing artificial between-host selection for and against virulence using the rodent malaria *Plasmodium chabaudi* in laboratory mice as a model system. To date, empirical work testing the evolution of virulence models has been either correlational (Ewald 1983; Herre 1993; Clayton & Tompkins 1994; Ebert 1994*a*,*b*; Mangin *et al.* 1995; Lipsitch & Moxon 1997) or has involved experimental evolution following manipulation of factors believed to affect selection on virulence (Bull *et al.* 1991; Ebert & Mangin 1997; Turner *et al.* 1998). Importantly, most experimental work on the evolution of virulence has not involved higher vertebrates as hosts, the very situation in which the utilitarian benefits of this branch of evolutionary biology have been most enthusiastically promoted (Williams & Nesse 1991; Ewald 1994; Westoby 1994; Futuyma 1995).

Malaria parasites—one of the most important pathogens of man—feature both rapid replication within their hosts and obligate transmission between hosts via the mosquito vector: they are therefore potentially affected by both between-host and within-host selection. We are interested in the potential evolutionary change in malaria parasite virulence that control measures such as vaccines and drugs might induce. In our earlier studies of the adaptive basis of virulence in malaria parasites, we found from across-clone correlations that *P. chabaudi* has a genetic architecture consistent with the assumptions of the adaptive trade-off hypothesis for between-host selection, i.e. that virulence is positively and genetically related to replication rate, and that replication rate is positively and genetically related to transmission success (Mackinnon & Read 1999). In the present study, to test whether these correlations truly predict the evolution of virulence under selection, we selected for high and low virulence within each of two wild-caught clones of the

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rodent malaria parasite *P. chabaudi* infecting laboratory mice. This paper describes the changes in virulence, replication rate and transmission which resulted from this divergent selection experiment.

2. MATERIAL AND METHODS

(a) *Experimental system*

Plasmodium chabaudi shares strong similarities in life history and pathology with the most virulent human malaria species, *Plasmodium falciparum* (Cox 1988; Taylor-Robinson 1995). The pattern of infection in C57Bl/6 female inbred laboratory mice after inoculation with 10^5 asexual parasites is a phase of rapid replication of asexual parasites to reach a peak parasitaemia between nine and ten days later. This is then followed by a dramatic decrease in parasitaemia, accompanied by *ca.* 5% loss of body weight and an approximately ninefold reduction of red blood cell (RBC) density. If the mouse survives this 'crisis' period, parasitaemia rises slightly further and *ca.* 1% of parasites convert to sexual forms (gametocytes). These are transmissible to mosquitoes two to four days later (Buckling *et al.* 1997; Taylor *et al.* 1997*a*,*b*; Mackinnon & Read 1999).

Infections were initiated by inoculating naive mice with the required number of parasites diluted in a 0.1 ml volume of calf serum buffer. These parasites were obtained from donor mice which had been infected five to ten days previously. Thus, parasite transmission was by serial passage of asexual blood forms: mosquito transmission of sexual forms did not occur.

(b) *Experimental design*

Two parasite clones (denoted BC and CW) were chosen on the basis of virulence from a panel of eight clones which had been previously characterized for virulence and replication rate: over a short series of passages, clone BC was consistently highly virulent and had a high asexual replication rate and clone CW was consistently avirulent with a low replication rate (Mackinnon and Read 1999). These clones were derived by serial dilution of parasite isolates sampled from their natural hosts (*Thamnomys rutilans*) captured from natural forests in the Central African Republic in 1969 (Beale *et al.* 1978). Both clones had undergone a total of nine asexual passages through inbred mice (each passage infection lasting for four to ten days): four of these passages occurred prior to cloning and five after cloning.

Within each clone, divergent artificial selection for high and low virulence was performed for 11 generations (see below) to produce four selection lines: BC-AVIR (avirulent), BC-VIR (virulent), CW-AVIR and CW-VIR. The selection criterion for virulence was the amount of live weight lost by the mouse post-infection (PI), i.e. selection was based on between-mouse differences in virulence caused by populations of infecting parasites. Live weight loss PI was used as a surrogate measure of virulence because in previous experiments, it was positively related to the probability of the mouse dying while under anaesthetic several days after crisis (Mackinnon and Read 1999). In other mouse genotypes *P. chabaudi* infections are often lethal (Stevenson *et al.* 1982).

The procedure for selection of each generation was as follows.

(i) On the day of infection (day 0), two to eight mice per line (mean of 5.5) were each injected with 10^5 parasites: half of these parasites came from each of two different mice in the previous generation. In generation zero the inoculum was 10^6 parasites.

(ii) Live weights were measured on days 0, 6, 10 and 11 for all mice. For each mouse, the difference between the mean weights on days 0 and 6 and the minimum weight reached by day 10 were calculated. Within lines, the two mice with the highest weight loss each donated 5×10^4 parasites to mice in the next generation in the high virulence lines (BC-VIR and CW-VIR) and the two mice with lowest weight loss donated parasites for the low virulence lines (BC-AVIR and CW-AVIR). Passages took place on day 12 PI.

(iii) Parasitaemia (the proportion of RBCs infected with parasites) and RBC density (estimated by flow cytometry, and an inverse measure of anaemia) were also measured each generation on day 10 or 11 PI. These were taken as measures of correlated responses to selection.

After 11 cycles of selection, a separate experiment was conducted to compare directly the virulence, replication rate and transmission rate of parasite populations which had been selected (at generation 12) with their ancestors from generation zero. All parasites were cryopreserved for at least two weeks beforehand. In two independent replicate experiments, five mice per line were inoculated with 10^5 parasites and their live weights, anaemia, parasitaemia and gametocytaemia (the proportion of red blood cells infected with mature sexual forms of the parasite) were measured at regular intervals during the infection.

Parasites from generations zero and 12 were genotyped for the highly polymorphic MSP-1 locus (Taylor *et al.* 1997*a*) to check that there had been no accidental switching of clone identities during the course of the experiment.

(c) *Statistical analysis*

In order to calculate the amount of artificial selection pressure applied during the course of the experiment, realized selection differentials were calculated. This involved calculating the deviations of each mouse from its generation-line mean to produce individual selection differentials. Estimates of the mean selection differential per generation in each line were obtained by fitting a linear model with a factor for selection line within clone to the individual selection differential data from selected mice across all generations. Cumulative selection differentials over generations in each line were calculated by summing the generation-line mean selection differentials over the preceding generations. Correlated selection differentials for parasitaemia were calculated similarly.

To determine the realized genetic trend over generations in weight loss and weight loss as a percentage of starting weight, a linear regression model with fixed effects for clone (BC versus CW), selection line and a covariate for generation within selection line within each clone was fitted to the individual mouse data for all mice alive at the time of measurement. The intercept estimated the clone means at generation zero and the covariates estimated the linear rate of change per generation in each line. Similar analyses were performed for parasitaemia and RBC density after transformation of the data to normalize them and inclusion of day of measurement (day 10 or 11) in the statistical model. Different variances in the two clones made it necessary to analyse the two clones separately for parasitaemia.

For the experiment in which a direct comparison between the selection lines at generation 12 with their generation zero counterparts was made, the traits analysed were the live weight lost between days 0 and 10, RBC density averaged over days 9 and 11, the parasitaemias on days 7, 9 and 13, and the gametocytaemias averaged over days 13 and 15. The data were normalized where necessary and then analysed by fitting a fixed effects model with a factor for replicate and an interaction term for selection line by generation within clone.

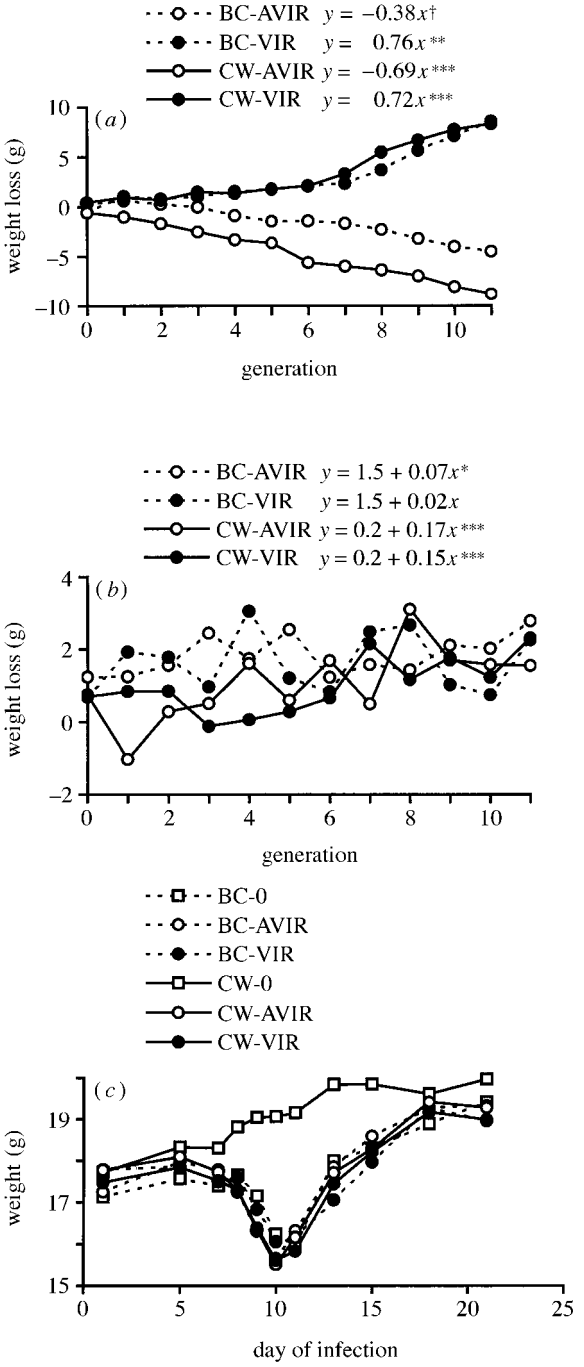

Figure 1. Selection for high (VIR) and low (AVIR) virulence, as defined by weight lost by days 10 and 11 PI in mice inoculated with 10^5 *P. chabaudi* parasites of clone BC or CW. (*a*) Cumulative selection differentials. Each line represents the difference in weight loss between selected mice and unselected mice within the selection line accumulated over generations and, hence, the amount of selection applied. Regression equations show the mean selection differential per generation and significance levels are based on an analysis of individual mouse selection differentials ($n = 22$–24 mice per selection line and s.e. $= 0.23$) and show strong divergent selection within both clones. Significant differences from zero for regression coefficients are indicated as $^{\dagger}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. (*b*) Direct responses to selection for weight loss. Regression analysis revealed that the initially avirulent clone (CW) significantly increased in weight loss in both the AVIR and VIR lines. The initially virulent clone (BC) only slightly increased in weight loss ($n = 63$–65 per selection line and intercept s.e. $= 0.23$ and slope s.e. $= 0.03$).

3. RESULTS

In the current experiment, only 4% of mice (nine out of 248) died apparently due to infection. Deaths occurred evenly across selection lines and generations and, probably due to low numbers of deaths, a relationship between mortality and weight loss was not found ($p > 0.05$).

(a) *Direct responses to selection*

Over the course of 11 cycles of selection ('generations') and despite large between-host selection differentials for increased or decreased weight loss (figure 1*a*), all the lines, both avirulent and virulent, increased in weight loss (figure 1*b*). This increase was smaller ($p < 0.001$) for the clone with initially high virulence (BC) than for the initially avirulent clone (CW). Within clone CW there was no significant difference ($p > 0.05$) between the high- and low-virulence selection lines in the rate of response, although in clone BC the virulent line increased in virulence at a slightly slower rate than the avirulent line (figure 1*b*). Thus, the selection lines did not respond to artificial between-host selection, but instead showed a divergent overall unidirectional increase in virulence.

The separate experiment directly comparing the weight loss caused by parasites from generation zero with parasites from generation 12 from each of the lines confirmed that selection had brought about a permanent increase in virulence in both selection lines of the avirulent clone (CW) ($p < 0.001$) up to the level of the already virulent clone (BC), which itself did not change in virulence over the generations ($p > 0.05$) (figure 1*c*).

(b) *Correlated responses to selection*

The minimum RBC density decreased over generations in both lines of the CW clone ($p < 0.001$) which initially caused much less anaemia than clone BC ($p < 0.001$). The BC lines also slightly decreased in minimum RBC density over generations but only significantly so in the BC-AVIR line ($p < 0.05$). (The estimates of line means with approximate standard errors for RBC density, in units of 10^9 RBC ml^{-1}, were as follows: intercepts, BC 1.14 ± 0.18 and CW 2.23 ± 0.28 and slopes, BC-AVIR 0.04 ± 0.03, BC-VIR 0.00 ± 0.03, CW-AVIR 0.22 ± 0.03 and CW-VIR 0.22 ± 0.03.) The direct comparison between generations zero and 12 confirmed these results. (The estimates ($\times 10^9$ RBC ml^{-1}) were as follows: generation zero, BC 1.10 ± 0.27 and CW 3.51 ± 0.86 and generation 12, BC-AVIR 0.87 ± 0.21, BC-VIR 1.77 ± 0.46, CW-AVIR 0.91 ± 0.24 and CW-VIR 1.12 ± 0.34). Parasitaemia at peak weight loss increased in all lines over the generations despite there being some divergence between lines in correlated selection differentials, particularly in clone BC (figure 2*a*). While there was no significant difference between the clones in rate of this genetic response (figure 2*b*), as for

Figure 1. (*Cont.*) (*c*) Direct comparison of parasites before selection (generation zero, denoted BC-0 and CW-0) and after selection (generation 12) confirmed that CW clones had increased their weight loss to the level of the BC clone which itself did not change ($n = 10$ mice per selection line, five in each of two experimental replicates; line mean weight losses were: BC-0, 1.25 g; BC-AVIR, 1.62 g; BC-VIR, 1.77 g; CW-0, 1.09 g; CW-AVIR, 2.02 g and CW-VIR, 1.83 g and line mean s.e. $= 0.38$ g).

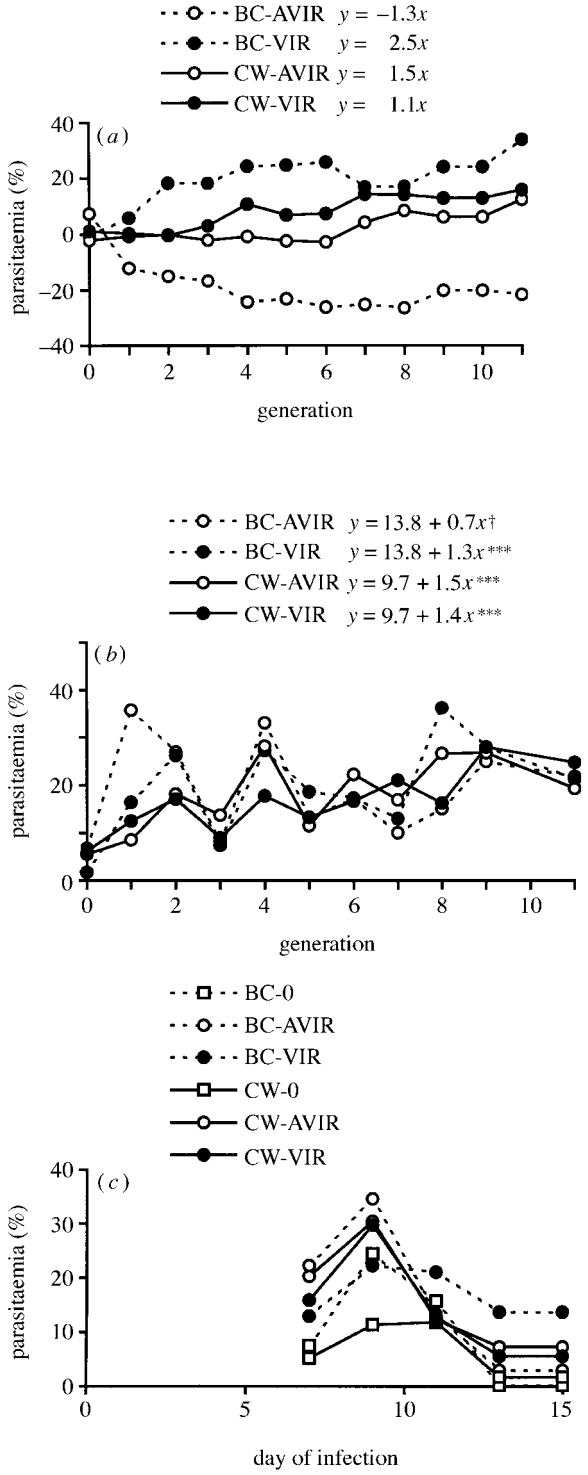

Figure 2. Correlated responses in parasitaemia to selection for high (VIR) and low (AVIR) virulence in BC and CW clones of *P. chabaudi* parasites. (*a*) Correlated cumulative selection differentials illustrate that divergent selection for virulence caused a weak associated divergent selection for parasitaemia on days 10 or 11 which was stronger in clone BC than CW. Regression estimates indicate the mean selection differential per generation (s.e. = 1.9). Significant differences from zero of regression coefficients are indicated by $^{\dagger}p<0.10$, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$. (*b*) Correlated increases in parasitaemia on days 10 and 11 occurred in all lines, particularly in the CW clone. For clone BC the intercept s.e. = 2.3 and slope s.e. = 0.4, and for clone CW the intercept s.e. = 1.4 and slope s.e. = 0.2. (*c*) Direct comparison of parasites before (generation zero) and after (generation 12) weight loss and anaemia, peak parasitaemia in clone BC started at a higher level in generation zero than in clone CW and changed slightly more slowly thus causing the clones to converge (figure 2*b*). This was confirmed in the direct comparison experiment (figure 2*c*). Parasitaemias on day 7 (pre-peak) and day 13 (post-peak) showed the same patterns as peak parasitaemia on day 9. Thus, the correlated responses in anaemia and parasitaemia roughly mirrored the direct responses for weight loss.

Notably, the comparison of generation zero and generation 12 parasites showed that the proportion of cells infected with gametocytes (sexual parasite forms transmissible to mosquitoes) on days 13–15 PI increased between generations zero and 12, more so in the CW lines (from 0.22 ± 0.09 per 1000 RBCs in generation zero to 1.14 ± 0.09 per 1000 RBCs in generation 12; $p<0.001$) than in the BC lines (from 0.06 ± 0.09 per 1000 RBC in generation zero to 0.25 ± 0.09 per 1000 RBCs in generation 12; $p>0.05$). Thus, transmission potential increased alongside parasitaemia and virulence.

4. DISCUSSION

So far as we are aware, this is the first time there has been an attempt to both increase and decrease microparasite virulence using controlled artificial selection. We were unable to reduce virulence using between-host selection. Moreover, the virulence of both lines derived from CW, the initially avirulent clone, converged with that of the virulent clone, BC. This relentless increase in virulence, similar to that reported as a frequent outcome of successive passage of various malaria species in laboratory or clinical situations (James *et al.* 1936; Contacos *et al.* 1962; Chin *et al.* 1968; Alger *et al.* 1971; Yoeli *et al.* 1975; Dearsly *et al.* 1990), occurred here despite a controlled selection regime against it. By restricting the population of parasites to that from only two out of the four to eight mice in each generation, the selection regime mimicked a case fatality rate of 50–75%, far higher than the case fatality rate of malaria infections in human populations (Greenwood *et al.* 1991) and, indeed, most widespread parasites. The steady increase in virulence in the face of selection against it was not due to inaccuracy of selection as selection differentials were high and the response was unidirectional. Instead, it seems that positive within-host selection for access to the parasite population passaged on day 12 PI overwhelmed direct selection at the level of the host for lower virulence. This suggests that selection imposed by competition within a host for access to the transmission population can be highly potent and more than compensate for the reduced fitness through the levels of 'host death' we imposed.

A recent review of serial passage experiments in a wide range of parasites demonstrates that within-host selection for replication rate and, consequently, virulence is usually very potent once the constraints of between-host selection

Figure 2. (*Cont.*) selection confirmed that both clones had increased their peak parasitaemia on day 9, particularly the CW clone. The line mean peak parasitaemias on day 9 were BC-0 24.4%, BC-AVIR 34.6%, BC-VIR 22.3%, CW-0 11.4%, CW-AVIR 30.4% and CW-VIR 29.7% and line mean s.e. = 3.3%.

and/or specific host adaptations are removed, and that such selection is probably driven through within-host competition (Ebert 1998). Our results are in accord with this apparently widespread (though artificial) phenomenon, but moreover show that even when between-host selection is operating strongly it can be virtually ineffective relative to within-host selection. It is possible that the apparent lack of response to between-host selection was because genetic differences between parasite populations in different hosts remained small. This would occur if the mutation rate, population size and strength of selection were large enough such that the full range of possible mutations were generated within each host and were selected in the same direction in each host. While this seems unlikely—and there are no data to support or refute such a proposition—the idea that rapid within-host evolution of rapidly replicating parasites could more than counter between-host selection for reduced virulence challenges most current theoretical models of parasite evolution.

It is usually assumed that reductions in parasite fitness due to host death eventually halt the evolution of increased virulence. Our data do not support this and instead suggest that something else constrains virulence. We do not know what mechanism(s) were responsible for the upper limit onto which all four lines apparently converged. The immunopathology caused by the parasite (White & Ho 1992; Taylor-Robinson 1998) may increase in a nonlinear way as parasite density increases, perhaps because the host exercises a self-preserving upper limit. Alternatively or additionally, perhaps parasite growth becomes limited in some density-dependent manner by factors such as the supply of RBC (Anderson *et al.* 1989; Hellriegel 1992; Yap & Stevenson 1994; Gravenor *et al.* 1995; Hetzel & Anderson 1996) or fever-related, density-dependent release of cytokines and toxins (Kwiatkowski & Nowak 1991; Gravenor & Kwiatkowski 1997; Kwiatkowski *et al.* 1997). Whatever the mechanism, the upper boundary was not set by host death. Reduction in host fitness can clearly select against virulent variants in some circumstances—in the myxoma virus of rabbits, for example, case mortality was initially in excess of 99.9% (Fenner & Ratcliffe 1965; Anderson & May 1982)—and, at the limit, undoubtedly it must. However, our data show that other factors can halt the evolution of virulence at levels below this limit.

If within-host selection was so strong in this experiment, what was its source? The base populations for our experiment were from cloned parasite lines established from a single parasite: mutation and selection must have therefore generated significant genetic change during the five passages prior to and 11 passages during the experiment. The potential for rapid evolution within a single infection would appear to be vast. Exponential growth of the parasite population to 10–40% parasitaemia in this species is followed by a population crash to 1–5% parasitaemia. This crash is probably mediated by a combination of limited host cell supply (Anderson *et al.* 1989; Hellriegel 1992; Yap & Stevenson 1994; Gravenor *et al.* 1995; Hetzel & Anderson 1996) and strong immune attack (White & Ho 1992; Taylor-Robinson 1995), so there is potential for very strong selection on individual parasites for rapid growth before or after the crash, or for immune evasion. Thus, mutations for higher growth rate (e.g. through higher numbers of merozoites per schizont or more efficient invasion) or for immune evasion, e.g. due to mutations in surface antigens (David *et al.* 1985; Klotz *et al.* 1987; Hudson *et al.* 1988), an increased rate of phenotypic switching of variable surface antigens (Brown & Brown 1965; McLean *et al.* 1982; Hommel *et al.* 1983; Roberts *et al.* 1992; Brannan *et al.* 1993, 1994; Baruch *et al.* 1995; Smith *et al.* 1995; Su *et al.* 1995) or higher sequestration of parasites (Gilks *et al.* 1990; Gravenor *et al.* 1998) would be strongly selected within an infection. To illustrate the potential for such genetic change in our experiment, a simple model to predict the frequency change of a new mutant with 20% advantage in growth was constructed (see Appendix A). The model was run under two strengths of immunity—one which resulted in $<0.2\%$ of parasites being killed (weak immunity) and one in which 18% of parasites were killed at peak parasitaemia (strong immunity). Assuming that there was one copy of the mutant at the beginning of the infection in generation zero, after 11 generations, each of 12 days in naive mice, a mutant under weak immune selection with 20% advantage in either its intrinsic reproductive rate (ρ) or its invasion efficiency (β) was expected to have increased in frequency from 10^{-5} to 0.999, giving a large corresponding increase in peak parasitaemia. Under strong immunity, selection of a mutant with 20% growth advantage would bring about a very small increase in peak parasitaemia ($<1\%$). This is because immunity limits peak parasitaemia. Moreover, under strong immunity, the mutant would not become fixed because of the density-dependent immune response incorporated into the model: in this particular case it is expected to oscillate in frequency between 0.60 and 0.90. Thus a combination of immunity and mutation for growth advantage may explain the faster response in the avirulent clone (CW) than in the virulent clone (BC). Alternatively, if a mutation arose which was advantageous because it completely evaded immune killing ($\kappa_2 = 0$), under strong immunity, it is expected to have reached a frequency of 94% by the end of generation 11 with a corresponding large increase in peak parasitaemia. We offer these two examples to illustrate how mutation and selection might explain the observed increase in virulence: no doubt there are many other possible combinations and complexities not taken into account which would alter the outcome in a quantitative way. Nevertheless, the empirical results and theoretical model demonstrate that the potential for within-clone, within-host evolution is high in malaria infections.

An intriguing alternative explanation for the consistent increase in virulence in the lines over the generations is the accumulation of variation *per se*, rather than increased frequency of favourable mutants. Theoretical studies show that microparasite success can be enhanced through diversity either by immune antagonism or immune distraction or overload (Antia & Nowak 1996; Burroughs & Rand 1998; Haydon & Woolhouse 1998). Malaria parasites have the ability to create phenotypic variation within a clone through rapid switching of antigens expressed on the surface of the infected RBC (Brown & Brown 1965; McLean *et al.* 1982; Hommel *et al.* 1983; Roberts *et al.* 1992; Brannan *et al.* 1994; Baruch *et al.* 1995; Smith *et al.* 1995; Su *et al.* 1995). This mechanism provides huge potential for creating diversity within a clone in

addition to any generated by new mutations. It is currently unknown whether any 'immune smokescreen' created by these novel antigens is in fact advantageous to parasite survival or whether antigenic diversity translates to faster growth rates in naive mice. While there is some empirical evidence to suggest that parasite diversity can help in overcoming immune defences in malaria (Gilbert *et al*. 1998; Taylor *et al*. 1998), these studies involved mixtures of distinct clones of parasites. Moreover, where parasite growth rate was measured, it was found not to increase with genetic diversity, even though virulence did (Taylor *et al*. 1998). The role of diversity in promoting parasite growth is an important hypothesis which we are currently investigating empirically.

Although our results question the assumption that host death is the selective pressure acting against increases in virulence, they are consistent with the assumption that benefits to parasites of greater host exploitation accrue through increased transmission stage production, with disease an unavoidable side-effect (Levin & Pimentel 1981; Anderson & May 1982; Bremermann & Pickering 1983; May & Anderson 1983; reviewed by Bull 1994; Ewald 1994; Read 1994; Frank 1996). An alternative reason given for higher virulence is that the symptoms themselves induce higher transmission (Ewald 1983, 1994) for which there is some evidence in malaria in terms of the anti-mosquito behaviour of infected mice (Day & Edman 1983). Our data do not support this alternative, as anti-vector defences were not a target of either our artificial or natural selection and, even if vulnerability to vectors is correlated with weight loss, virulence did not respond accordingly.

The observed increases in gametocyte density as virulence and parasitaemia changed over the course of the experiment are consistent with the positive genetic correlations across clones between replication rate, virulence and transmission in *P. chabaudi* (Mackinnon & Read 1999). This implies that more technically demanding selection regimes involving mosquito passage would have generated a similar picture since, in general and particularly in *P. chabaudi*, gametocyte densities are positively correlated with transmission rates (Carter & Graves 1988; Buckling *et al*. 1997; Taylor & Read 1997; Taylor *et al*. 1997*a*,*b*; Mackinnon & Read 1999). On the other hand, bottlenecks of the parasite population during mosquito passage, as occurs in nature, would probably have occurred, thereby reducing the chance of a mutation being maintained in the population over the course of the experiment. Syringe passage is but one 'non-natural' aspect of our experiment. Another is that the mouse is not the natural host of *P. chabaudi*. How this might matter is not clear. Most models of the evolution of virulence are explicitly not about coevolution and assume that host evolution is largely irrelevant. If that assumption is not true, it causes as much difficulty for theoretical models as for experimental tests of them. Moreover, it may be that by examining parasite evolution in novel environments, more genetic variation is exposed with which to work. It is possible that the increases in replication rate were reflecting adaptation by the parasite to this novel host (Ebert & Hamilton 1996; Ebert 1998), with the BC clone being already more 'pre-adapted' than CW to laboratory mice, e.g. through more compatible parasite ligands to host endothelial receptors (Roberts *et al*. 1985; Berendt *et al*. 1989; Ockenhouse *et al*. 1989*a*,*b*, 1991). If so, the increase in virulence associated with improved 'adaptation' was not halted by high rates of host mortality.

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APPENDIX A

A discrete-time dynamic model to describe the course of *P. chabaudi* infection was constructed based on previous models (Anderson *et al*. 1989; Hellriegel 1992; Hetzel & Anderson 1996; McKenzie & Bossert 1997). Denoting $X(t)$, $Y_i(t)$ and $I_i(t)$ as the numbers of uninfected RBCs, infected RBCs and killer immune cells, respectively, on day t of the infection (equivalent to the tth 24-hour cycle), with subscripts indicating distinct parasite genotypes, our data can be described by the following recurrence equations:

$$\begin{aligned}
X(t+1) &= X(t) + \Lambda - \mu X(t) - X(t)\Sigma_i \beta_i(t)\rho_i Y_i(t) \\
&\quad - \Sigma_i \kappa_i Y_i(t) I_i(t) \\
Y_i(t+1) &= X(t)\beta_i(t)\rho_i Y_i(t) - \kappa_i Y_i(t) I_i(t) \\
I_i(t+1) &= \phi I_i(t) + \gamma Y_i(t) - \varepsilon I_i(t) \\
\beta_i(t+1) &= \beta_i(t) X(t)/X(0),
\end{aligned}$$

where ρ_i denotes the number of merozoites per schizont (reproductive rate) of parasite genotype i, $\beta_i(t)$ is the rate of successful contact of merozoite and RBCs which results in successful invasion for genotype i at time t and is modelled as a variable which declines as the density of uninfected cells decreases, Λ is the rate of production of RBCs, μ is the death rate of uninfected RBCs, ϕ is the normal rate of production of immune cells capable of effecting parasite death, γ is the rate of production of immune cells stimulated by contact with an infected RBC, ε is the rate of clearance of immune cells and κ_i is the rate of parasite killing when infected cells containing parasite genotype i make contact with immune cells specific for parasite genotype i. In addition, there is clearance of uninfected cells at a rate proportional to the total number of immune cells. Here we use this model to examine the rate of change in frequency of a new mutant when initiating the infection with 10^5 parasites of one genotype ($i=1$) and one mutant parasite ($i=2$) with an advantage in reproductive rate (i.e. $\rho_2 > \rho_1$ or $\beta_2 > \beta_1$) or in immune evasion ($\kappa_2 < \kappa_1$). Recurrent mutation was not incorporated into the calculations. After every 12 cycles (one generation), when infections were started again in naive mice, the values of $X(t)$, $Y_i(t)$ and $I_i(t)$ were reset to their initial values which were $X(0) = 1.2 \times 10^{10}$ cells ml^{-1}, $\Sigma_i Y_i(0) = 10^5$ cells ml^{-1}, $\beta_1(0) = 2 \times 10^{-11}$ or 3×10^{-11} cells ml^{-1} for a model with weak immunity or strong immunity respectively (see below) and $\phi I_1(0) = 10^{-3} X(0)$ cells ml^{-1}. The parameter values used were $\Lambda = 1.2 \times 10^9$ cells ml^{-1} day^{-1}, $\mu = 0.1$ cells ml^{-1} day^{-1}, $\rho_1 = 10$, $\gamma = 0.01$ for weak immunity and $\gamma = 0.08$ for strong immunity, $\varepsilon = \gamma$, $\phi = \varepsilon \Sigma_i I_i(0)$ and $\kappa_1 = \gamma$. (Note that in the uninfected state ($t=0$), $\Lambda = \mu X(0)$ and $\phi = \varepsilon \Sigma_i I_i(0)$.) Under conditions of strong immunity and weak immunity the increase in frequency over the 11 generations of the

experiment (132 cycles) was predicted for a mutant with a 20% growth advantage ($\rho_2 = 1.2\rho_1$ or $\beta_i(0) = 1.2\beta_i(0)$) or the advantage of complete immune evasion ($\kappa_2 = 0$). Values of parasitaemia on days 9, 10 and 11 PI resulting from these increases were calculated.

A useful alternative prediction of the rate of increase in frequency of a mutant with an advantage in reproductive rate is from the recurrence equation $p(t+1) = p(t)(1+s)$, where $p(t)$ is the frequency at time t and s is the selective advantage of the mutant compared with the population mean calculated as $s = w_i/\overline{w}$. The fitness of the mutant is w_i and $\overline{w}$ is the mean reproductive rate fitness of the population which can be expressed as $\overline{w} = \rho_2 p(t) + \rho_1(1 - p(t))$ (Kimura & Crow 1978). This equation yields the same prediction as that from the above model when there is no immunity ($\gamma = 0$) and slightly underestimates the frequency change with weak immunity ($\gamma = 0.01$). However, when immunity is strong, prediction of the frequency of a mutant with an advantage in immune evasion is not so straightforward because, under this model, genotype-specific immune selection is frequency dependent, i.e. immune cells are activated and proliferate at a rate which is proportional to the density of the specific parasite genotype.

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