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VIRULENCE OF MIXED-CLONE AND SINGLE-CLONE INFECTIONS OF THE RODENT MALARIA *PLASMODIUM CHABAUDI*

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Abstract.—Most evolutionary models treat virulence as an unavoidable consequence of microparasite replication and have predicted that in mixed-genotype infections, natural selection should favor higher levels of virulence than is optimal in genetically uniform infections. Increased virulence may evolve as a genetically fixed strategy, appropriate for the frequency of mixed infections in the population, or may occur as a conditional response to mixed infection, that is, a facultative strategy. Here we test whether facultative alterations in replication rates in the presence of competing genotypes occur and generate greater virulence. An important alternative, not currently incorporated in models of the evolution of virulence, is that host responses mounted against genetically diverse parasites may be more costly or less effective than those against genetically uniform parasites. If so, mixed clone infections will be more virulent for a given parasite replication rate.

Two groups of mice were infected with one of two clones of *Plasmodium chabaudi* parasites, and three groups of mice were infected with 1:9, 5:5, or 9:1 mixtures of the same two clones. Virulence was assessed by monitoring mouse body weight and red blood cell density. Transmission stage densities were significantly higher in mixed- than in single-clone infections. Within treatment groups, transmission stage production increased with the virulence of the infection, a phenotypic correlation consistent with the genetic correlation assumed by much of the theoretical work on the evolution of virulence. Consistent with theoretical predictions of facultative alterations in virulence, we found that mice infected with both parasite clones lost more weight and had on average lower blood counts than those infected with single-clone infections. However, there was no consistent evidence of the mechanism invoked by evolutionary models that predict this effect. Replication rates and parasite densities were not always higher in mixed-clone infections, and for a given replication rate or parasite density, mixed-clone infections were still more virulent. Instead, prolonged anemia and increased transmission may have occured because genetically diverse infections are less rapidly cleared by hosts. Differences in maximum weight loss occured even when there were comparable parasite densities in mixed- and single-clone infections. We suggest that mounting an immune response against more that one parasite genotype is more costly for hosts, which therefore suffer higher virulence.

Key words.—Malaria, mixed infection, parasite, *Plasmodium chabaudi*, virulence.

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Virulence is the reduction in host fitness caused by parasitic infection. The evolution of virulence has important implications for the control of infectious disease and has stimulated a considerable body of theory (reviewed by Bull 1994; Read 1994; Ewald 1995; Frank 1996; Ebert, in press). However, these ideas have been subjected to remarkably little experimental testing, particularly in the context of vertebrate infectious disease where the utilitarian benefits of the theory have been most stridently advocated (Williams and Nesse 1991; Ewald 1994; Westoby 1994; Futuyma 1995).

Most evolution of virulence models regard virulence as an unavoidable consequence of parasite adaptation. Virulence itself is viewed as detrimental to parasite fitness but genetically correlated to fitness-enhancing traits (reviewed by Bull 1994; Read 1994; Frank 1996; Ebert, in press). Increased virulence (usually modeled as a direct consequence of microparasite replication rate) is assumed to correlate with risk of host death, but also increased transmission stage production. Observed levels of virulence are then said to represent schedules of host exploitation that balance those two factors so as to maximize some measure of parasite fitness. In the simplest models, which consider just one parasite genotype per host, the level of virulence that evolves is that which maximizes the total number of new infections resulting from an infection (e.g., Levin and Pimentel 1981; Anderson and May 1982; Bremermann and Pickering 1983). Optimal virulence may be high if transmission probability is enhanced by host morbidity, or low as prudent parasites attempt to prolong host survival to maximize long-term transmission.

However, many authors have pointed out that where mixed-genotype infections are common, levels of virulence greater than those optimal for single-genotype infections will be favored by natural selection (e.g., Hamilton 1972; Eshel 1977; Axelrod and Hamilton 1981; May and Anderson 1983; Knolle 1989; Frank 1992; Hellriegel 1992; Herre 1993, 1995; Bonhoeffer and Nowak 1994a,b; Nowak and May 1994; van Baalen and Sabelis 1995a,b; Frank 1996; Ebert and Mangin 1997). In the competitive situation of a mixed-genotype infection, parasites that slowly exploit host resources are expected to be outcompeted by those exploiting hosts more rapidly. Thus, optimal levels of virulence are higher in mixed infections, even if this leads to fewer secondary infections than might be otherwise achieved. In the extreme, short-term selection arising from competition within a host can lead to greatly increased levels of virulence, with greatly decreased transmission rates, so-called short-sighted evolution (Levin and Bull 1994).

Where mixed-genotype infections occur, parasites could evolve a genetically fixed strategy appropriate for the frequency of mixed infections in the population. Alternatively, a conditional strategy might evolve where parasites facultatively alter their growth rates, and hence virulence, ac-

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cording to the type of infection they find themselves in (Sasaki and Iwasa 1991; Frank 1992; van Baalen and Sabelis 1995a). A conditional response is adaptive if the level of competition experienced is variable and cannot be predicted in advance. Such responses require that parasites are able to detect other parasite genotypes within a host and respond accordingly. One possible mechanism for this is the detection of a host immune response mounted to an antigenically similar, but not identical, parasite. Whatever the underlying mechanism, both genetically fixed and facultative strategies should result in higher parasite virulence where mixed genotype infections are common.

Whether parasites facultatively alter their within-host growth schedules in the presence of competing genotypes, thus inducing greater virulence in mixed-clone infections, remains to be tested. However, a complexity in such tests is the role of host factors in determining virulence. Although generally ignored in most formal models, virulence must be a consequence not only of what parasites do to hosts but also on the costs and effectiveness of parasite control by hosts. Responses against a more heterogenous parasite population may utilize more host resources. It may also be less effective. It is well known that parasites that create antigenic diversity within infections can evade host immunity and probably prolong the infection (e.g., malaria and trypansome infections; Wakelin 1996). It therefore seems plausible that genetically heterogeneous infections will be less rapidly cleared by the host. Thus, even in the absence of facultative alterations in parasite growth schedules, differences in host responses could result in mixed clone infections being more virulent. The crucial test for the existence of conditional virulence strategies is to compare both virulence and within-host growth schedules in mixed- and single-clone infections. We did this using the rodent malaria parasite *Plasmodium chabaudi.*

In vertebrate hosts, asexually replicating clones of malaria parasites continually infect red blood cells until the host dies or the infection is controlled by host immunity. For a variety of experimental and ethical reasons, it is not practical to directly assay mortality rates in the laboratory. Instead, we assayed weight loss and anemia as measures of virulence. In the strain of mouse used in our experiments, *P. chabaudi* is usually nonlethal (Stevenson et al. 1982), but we assume that in less-resistant host genotypes these parameters are correlated with probability of death. Host responses to anemia have been linked with resistance to infection with *P. chabaudi* (Stevenson et al. 1982; Yap and Stevenson 1994) and both weight loss and anemia induced by single-clone infections of *P. chabaudi* differ repeatably between parasite lines (Mackinnon, unpubl. data; Read and Anwar, unpubl. data).

We infected groups of mice with one of two clones of *P. chabaudi* or mixtures of the two, with all mice receiving the same total number of parasites. Both parasite growth rates and host reponses in mixed infections may be affected by the relative abundance of the parasite clones. Three types of mixed-clone infections were therefore initiated, with inocula composed of 9:1, 5:5, or 1:9 ratios of the two clones. Relative virulence of these three groups is hard to predict a priori: to do so would require detailed information about optimal growth rates and host responses in the three situations, and by how much both could be altered.

These experiments also allow us to test whether virulence is phenotypically correlated with parasite replication rate and transmission. Phenotypic correlations can be indicative of underlying genetic correlations. *Plasmodium chabaudi* parasites replicate synchronously every 24 h in the host blood stream. As all infections were initiated with the same number of parasites, we monitored parasite density early in the infection (before significant parasite mortality due to limiting nutrient or host immunity occurs) as a correlate of replication rate. As a measure of transmission rates, peak gametocyte density was assayed. Gametocytes are nonreplicating parasites that develop from the replicating blood stage (asexual) parasites, and the only stages capable of infecting mosquitoes. Previous experiments with *P. chabaudi* have shown that gametocytes are present for only a brief period, during which gametocyte density is correlated with both the proportion of mosquitoes infected and average parasite burdens per mosquito (Taylor et al. 1997a).

Methods

Parasites and Hosts

Two cloned lines of *P. chabaudi chabaudi* denoted CR and ER (Beale et al. 1978), obtained from the WHO Registry of Standard Malaria Parasites, Edinburgh University, were used. Clones are derived from asexual proliferation of a single parasite obtained from wild isolates by serial dilution. Isolates from infected tree rats (*Thamnomys rutilans*) collected in the Central African Republic in 1965 and 1970 were the sources of the CR and ER clones, respectively (Beale et al. 1978; McLean 1986). Since isolation and cloning, the parasites have been maintained for long periods as stabilates in liquid nitrogen. In total, the CR and ER parasites used for this study had been maintained in asexual passage in rodent hosts for 4.5 and 5 months, respectively. The clones have different alleles of the merozoite surface protein 1, distinguishable by genetic or monoclonal antibody techniques (Taylor et al. 1997b). Hosts were male C57BL/6J/Ola mice (Harlan, England). Mice were fed on SDS rat and mouse maintenance diet, and drinking water was supplemented with 0.05% para-amino benzoic acid (PABA) to enhance parasite growth. Artificial illumination was provided from 0530 to 1730 h.

Inoculation of Mice with Standard Numbers of Parasites

Parasite densities in infected donor mice were determined from Giemsa-stained, thin blood smears and red blood cell counts made using flow cytometry (Coulter Electronics). For inoculations, infected blood 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/ml mouse blood) to give separate dilutions of 10^5 parasitised red blood cells per 0.1 ml volume of the two parasite clones.

Monitoring of Infections and Virulence

Mice were weighed to the nearest 0.01 g on days 0, 2, and 4, then daily until day 22, and on days 24, 28, and 31 post-

infection (PI). Red blood cell counts were taken on day 4, then every second day until day 18, and on day 22 PI between 1700 and 1830 h. Thin blood smears were taken at the same time as the blood count measures on days 6, 8, 10, 12, and 16, and used to calculate the asexual parasite density (parasites per ml) on each of these days and gametocyte density (gametocytes per ml) on day 16 PI. Uninfected mice were subjected to the same sampling procedure.

Experimental Design

Mice were weighed and allocated at random to treatment groups. Six treatment groups, each containing four mice, were used: one control (uninfected), two single-clone (CR or ER parasites), and three mixed-clone infection groups (parasite clones in ratios of 1CR:9ER, 5CR:5ER, or 9CR:1ER). All mice allocated to noncontrol groups received a total of 10^5 *P. chabaudi* parasites in a 0.1 ml inoculum intraperitoneally. Control mice received an equivalent number of red blood cells from an uninfected mouse. Mice were inoculated between 1500 and 1700 h. The whole experiment was repeated 13 days later (with different mice from the same cohort) to give two experimental blocks and a grand total of 48 mice in the whole experiment. (One mouse in the ER treatment group of the second block showed an abnormally high parasite density on day 10 and died on day 12 PI. It was excluded from the analysis.)

Statistical Analyses

Weight, weight loss, red blood cell density, and gametocyte density were analyzed using ANOVA to determine the effects of treatment and experimental block. In most cases, there were significant differences between the two experimental blocks, probably because mice in the second block were 13 days older. Block effects were always included in the models where significant, but are of little interest in their own right; only significant interactions between experimental block and other model terms are reported.

Correlations between virulence parameters and measures of asexual parasite densities were carried out using ANCOVA in GLIM (Crawley 1993). The following seven measures of the asexual infection were used: (1–5) parasite densities on days 6, 8, 10, 12, and 16; (6) the difference between day-8 and day-6 parasite density; and (7) an estimate of the total number of parasites during the infection. This last measure was calculated for each mouse individually from the area under the curve of asexual density through time between days 6 and 16 PI. As *P. chabaudi* replicates once every 24 h, this represents a reasonable approximation to the total number of asexuals produced during this period of infection. In all cases, treatment, experimental block, and all interactions with the asexual measure in question were included as predictor variables in a full model, with the virulence measure as the response variable. No interaction terms were significant when removed from these full models. Significance of the remaining effects was assessed from models containing the asexual measure, treatment, and block effects as predictor variables. *F*-ratios were calculated from the change in deviance per degree of freedom divided by the residual mean square deviance as terms were removed from the minimal model (Crawley 1993). The same approach was used to investigate relationships between virulence and transmission, with gametocyte density as the response variable and the virulence measure as the predictor variable.

Results

Most of the weight loss following infection occurred between days 7 and 18 PI, with the lowest weights typically between day 11 and 13 PI (Fig. 1a). However, within and across treatment groups, mice did not lose weight synchronously, so that comparisons of weight loss at specific points in time were not meaningful. Instead, two composite measures of weight loss were used: mean loss (weight on the day of infection minus average weight from days 7–18 PI) and maximum loss (weight on the day of infection minus the average of the two lowest weights subsequently recorded). Red blood cell density fell from day 6 PI to minima on day 10 or 12 PI (Fig. 1b). Mean blood count over days 4–22 PI, and an average of the two lowest blood counts were calculated. Parasites became detectable in blood films on day 6 PI, densities peaked between days 8 and 10, and had fallen dramatically by day 12 (Fig. 1c). In the later parts of the infection, mixed-clone infections maintained higher asexual densities than single-clone infections, although none were comparable to earlier stages of infection.

The virulence of CR infections was not significantly different from that of ER infections as assessed by mean weight loss, maximum weight loss, mean blood count, or the lowest blood count ($F_{1,11} = 0.27$, $F_{1,11} = 0.26$, $F_{1,11} = 0.53$, $F_{1,11} = 0.28$, respectively; $P > 0.2$ in all cases). Similarly, mixed-clone infections with initial CR:ER parasite ratios of 1:9, 5:5, and 9:1 did not differ significantly in mean or maximum weight loss or mean blood counts ($F_{2,18} = 2.55$, $P > 0.05$; $F_{2,18} = 2.00$, $P > 0.1$; $F_{2,18} = 0.44$, $P > 0.5$, respectively). The effect of the initial ratio on lowest blood count differed significantly between the two experimental blocks (block × treatment interaction, $F_{2,18} = 5.05$, $P < 0.05$), with the rank orders of the three groups different in the two blocks, but there was no main effect of initial ratio ($F_{2,18} = 1.65$, $P > 0.2$). As no consistent differences between the two single-clone or between the three mixed-clone groups could be shown, the remaining analyses compare the virulence of mixed-clone and single-clone infections.

Virulence

Mixed-clone infections were more virulent than single-clone infections. Maximum weight loss was greater following infection with two clones than it was following infection with one ($F_{1,35} = 4.79$, $P < 0.05$). Mice with mixed-clone infections lost about 30% more weight than those with single-clone infections (Fig. 2). Mean weight loss showed a similar pattern ($F_{1,35} = 3.66$, $P \approx 0.07$). By the end of the experiment (day 31 PI), weights of mice given mixed-clone infections and single-clone infections had returned to weights comparable to the uninfected controls ($F_{2,41} = 1.53$, $P > 0.2$).

The reduction in mean blood count was higher for mixed-clone infections compared to single-clone infections ($F_{1,35} = 6.60$, $P < 0.05$, Fig. 3), with mixed-clone infections suffering a further 5% reduction on average. The average of the two

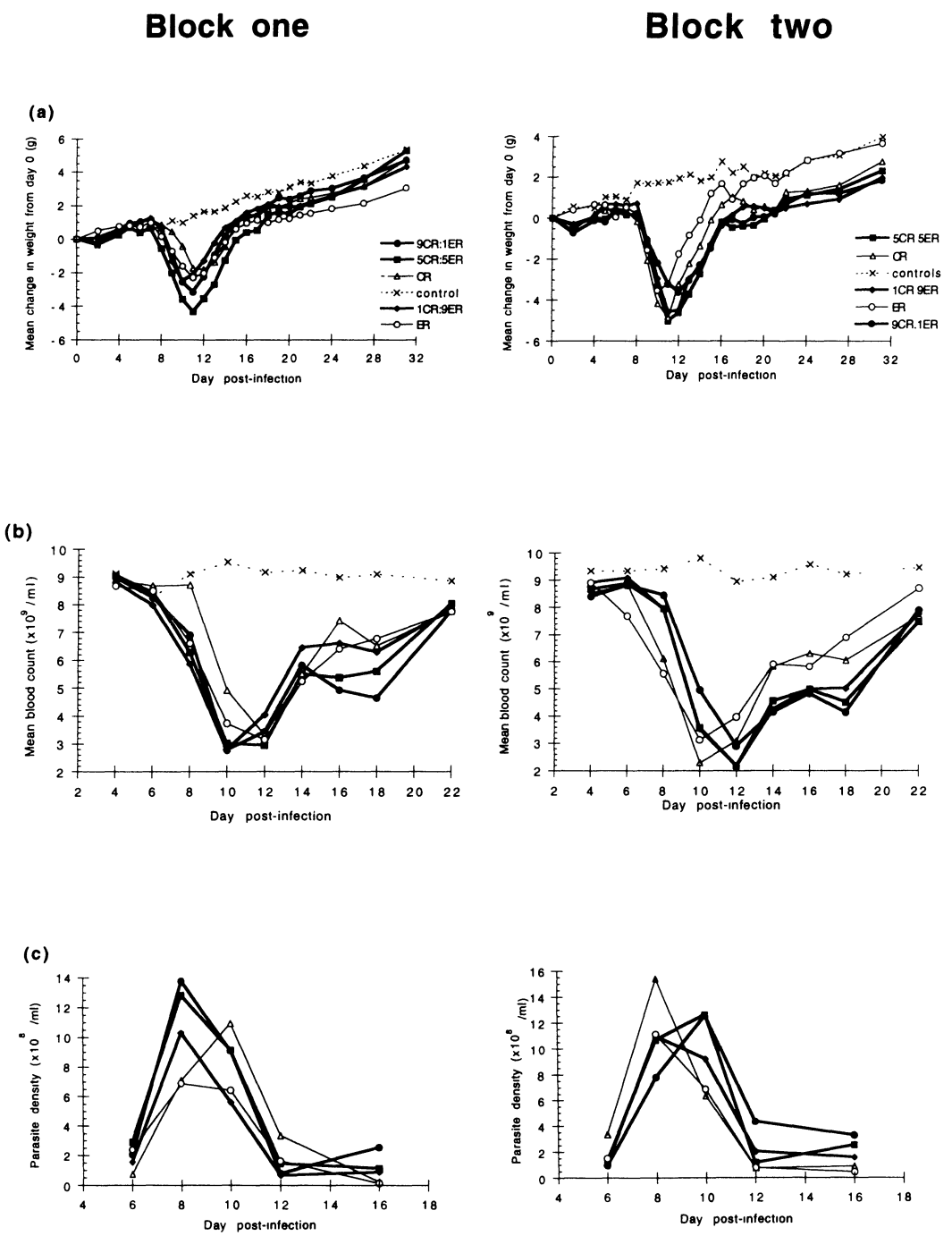

FIG. 1. Mean weight loss (a), mean red blood cell densities (b), and parasite densities (c) of the six groups of mice in experimental blocks one and two during the infections. Day of inoculation = day 0. Each line represents the mean of four mice.

lowest blood counts did not differ significantly between mixed-clone and single-clone infections ($F_{1,35} = 1.86$, $P > 0.1$).

Parasite Densities

The density of parasites achieved by day 6 PI, day 8 PI, and the increase between days 6 and 8 PI. was greater for the mixed-clone infections in block one of the experiment, but greater for the single-clone infections for the second block (treatment × block interactions, $F_{1,36} = 3.39$, $P \approx 0.07$, $F_{1,36} = 9.69$, $P < 0.01$, and $F_{1,36} = 8.04$, $P < 0.01$, respectively). The total number of parasites in mixed-clone and single-clone infections also differed between blocks, being higher for single-clone infections in block one and higher for the mixed-clone infections in block two (treatment × block interaction $F_{1,35} = 5.81$, $P < 0.05$). Thus, there were no consistent differences across blocks in parasite densities or total parasite numbers analogous to those for virulence.

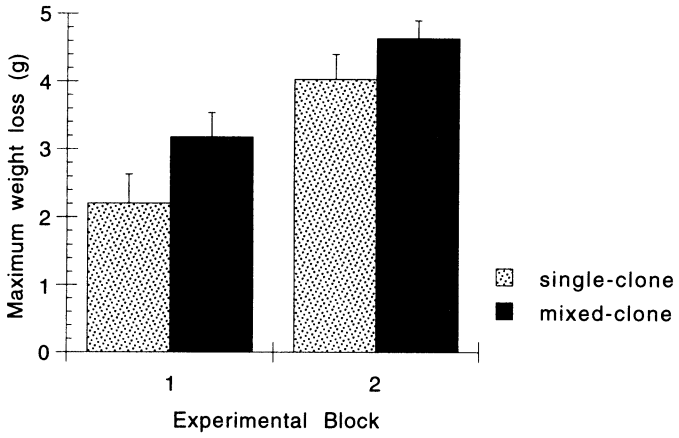

FIG. 2. Maximum weight losses of mixed-clone and single-clone infections. Within each block there were 12 mixed-clone infections and eight single-clone infections. Error bars represent +1 SE.

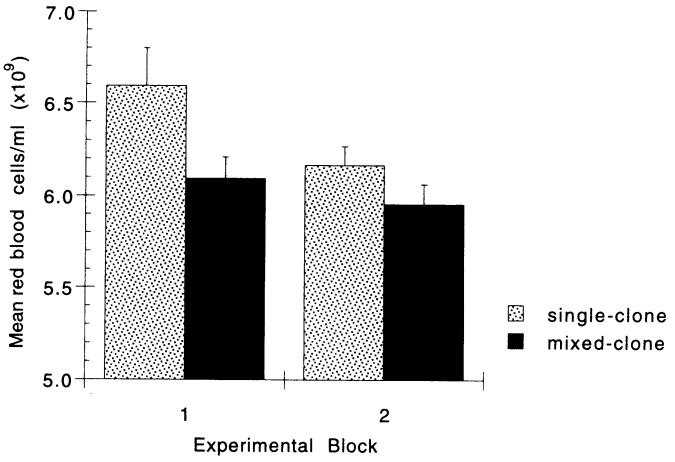

FIG. 3. Mean blood counts of the mixed-clone and single-clone infections over days 4 to 22 of infection. Within each block there were 12 mixed-clone infections and eight single-clone infections. Error bars represent +1 SE.

Virulence and Parasite Numbers

Relationships between parasite densities and virulence were investigated using the virulence measures that showed the clearest treatment effects: maximum weight loss and mean blood count. In none of the following analyses were any interactions between the asexual parameter being tested and either block or treatment significant, demonstrating that any relationships between virulence and the asexual infection dynamics did not differ between single-clone and mixed-clone infections.

Of the seven variables relating to the asexual infection analyzed, only asexual densities on day 6 PI, day 8 PI and the difference between them were positively correlated to maximum weight loss, when controlling for treatment effects (Table 1). Parasite densities later in the infection and the total number of parasites during the infection were unrelated to maximum weight loss. Thus, weight loss was greater when parasite density early in the infection was higher, but was unrelated to the parasite burden later in the infection or to the total number of parasites in an infection.

However, differences in parasite densities did not explain why maximum weight loss was greater for mixed-clone than single-clone infections. On average, mixed-clone infections resulted in an additional weight loss of about 0.8 g compared to single-clone infections with the same parasite density on day 6 PI (Table 1; Fig. 4a). This difference was equivalent to about 3% of the weight of uninfected controls of the same age.

Mean blood count was negatively correlated to day 6 PI, day 8 PI, and day 8 PI minus day 6 PI parasite densities (Table 1), showing that higher parasite densities were associated with lower blood counts. Later in the infection, on days 10 and 12 PI, there were positive associations between parasite density and blood count. But as with weight loss, differences in the mean blood count of mice infected with single-clone or mixed-clone infections were not explained by differences in parasite densities early in the infection (Table 1). Mixed-clone infections showed on average 0.4×10^9 fewer red blood cells per ml than single-clone infections for a particular asexual parasite density at each of the points in time during the infection that were sampled. This difference was equivalent to about 4% of the red blood cell densities of the uninfected control mice (Fig. 4b).

Transmission

In both experimental blocks, gametocyte densities were higher in mixed-clone infections than in single-clone infections ($F_{1,35} = 13.43$, $P < 0.001$, Fig. 5); this difference was

TABLE 1. Relationships between virulence measures and asexual infection dynamics. In ANCOVA models, maximum weight loss or mean blood count was the response variable, with treatment (single- or mixed-clone infection), experimental block, and (when fitted) the asexual measure as predictor variables. Tabulated values are parameter estimates from the full models; the statistical significance of their difference from zero was tested by removing each term from the model (Crawley 1993). Thus, significant terms explain additional variation above that explained by the other terms. In no case did inclusion of higher-order interaction terms significantly improve model fit. † $P \approx 0.07$ (marginal), * $P < 0.05$, ** $P < 0.01$, ns = not significant.

Virulence measure	Asexual measure	Slope of regression	Mixed vs. single intercept
Maximum weight loss	none		0.79*
	day 6 pars/ml	0.029*	0.93*
	day 8 pars/ml	0.011*	0.76*
	day 8 − day 6 pars/ml	0.010*	0.72†
	day 10 pars/ml	−0.0039 ns	0.87*
	day 12 pars/ml	−0.016 ns	0.80*
	day 16 pars/ml	−0.0046 ns	0.86 ns
	Total parasites	0.0023 ns	0.62 ns
Mean blood count	none		−0.36*
	day 6 pars/ml	−0.019**	−0.45*
	day 8 pars/ml	−0.0056**	−0.35**
	day 8 − day 6 pars/ml	−0.0046*	−0.33*
	day 10 pars/ml	0.0065**	−0.49**
	day 12 pars/ml	0.0011**	−0.37**
	day 16 pars/ml	−0.00073 ns	−0.35 ns
	Total parasites	0.00096 ns	−0.43*

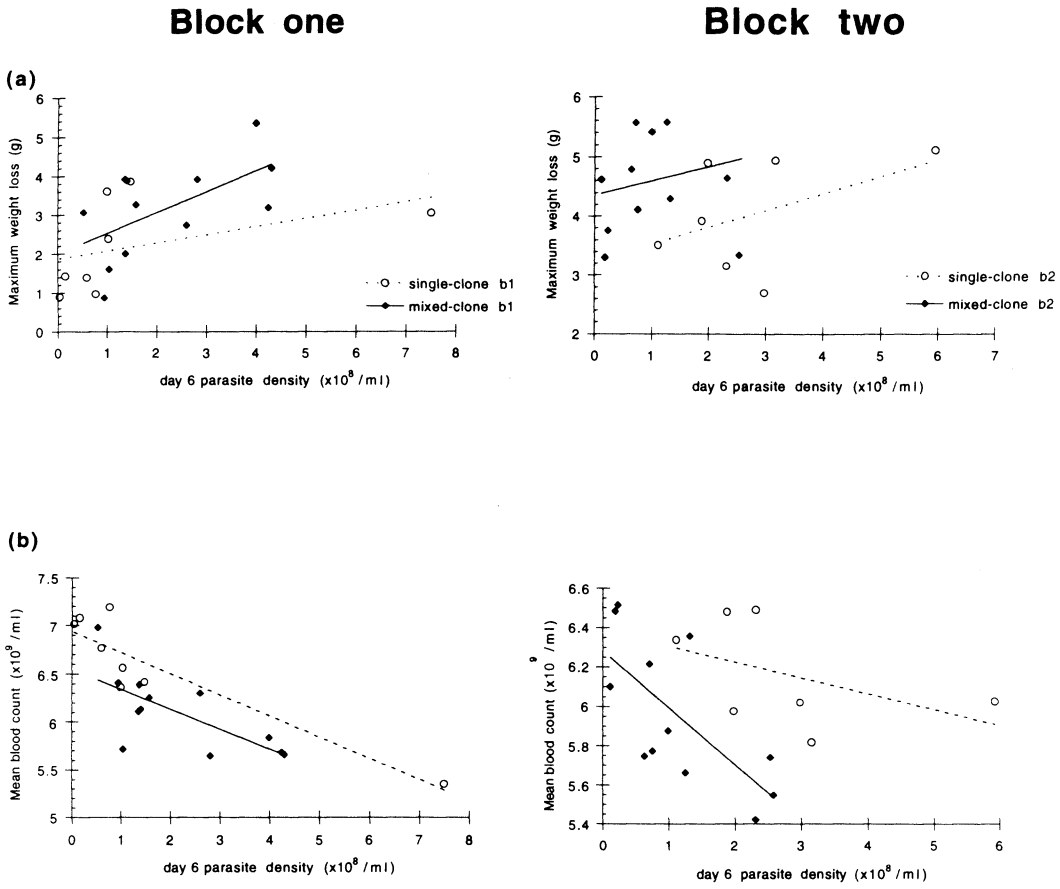

FIG. 4. Relationships between day 6 PI parasite densities and maximum weight loss (a) and mean blood cell count (b) for experimental blocks one and two. OLS regressions were fitted separately for single- and mixed-clone infections. For maximum weight loss, the fitted line for the mixed-clone infections lies above that for the single-clone infections in both blocks; for mean blood count, the mixed-clone line lies below that of the single-clone infections in both blocks.

larger in the second block (treatment × block interaction; $F_{1,35} = 3.60$, $P \approx 0.07$).

Across all mice, both mean and maximum weight loss were positively correlated with gametocyte density ($F_{1,38} = 5.78$ and $F_{1,38} = 6.09$, respectively, $P < 0.05$ in both cases). In neither case did adding a quadratic weight loss term improve the model fit ($F_{1,37} = 0.49$, $P > 0.2$ for mean weight loss; and $F_{1,37} = 0.40$, $P > 0.2$ for maximum weight loss). Thus gametocyte density was linearly correlated with virulence as measured by weight loss. Using gametocyte density, replicate, treatment, and all interactions as predictor variables in the analysis of weight loss and mean blood count, the minimal model contained only the treatment effect in each case ($F_{1,38} = 8.59$, $P < 0.05$). However, weight loss was still positively correlated with gametocyte densities when the difference between mixed- and single-clone infections was controlled for, although the effects were only marginally significant ($F_{1,37} = 3.48$, $P \approx 0.07$ for mean weight loss; $F_{1,37} = 3.48$, $P \approx 0.07$ for maximum weight loss). Thus mixed-clone infections had higher gametocyte densities and caused greater weight loss. However, within single-clone and within mixed-clone infections, there was also evidence that gametocyte density positively correlated with weight loss.

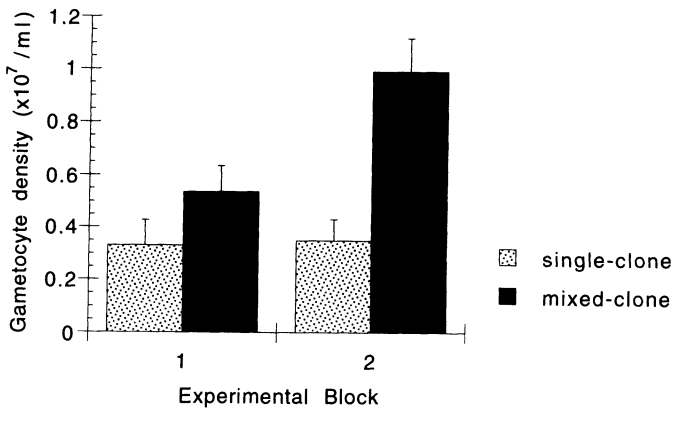

FIG. 5. Gametocyte densities on day 16 PI for the mixed-clone and single-clone infections. Within each block there were 12 mixed-clone infections and eight single-clone infections. Error bars represent +1 SE.

Similar analyses were carried out for the blood count measures. For mean blood count there was no significant correlation with gametocyte density ($F_{1,38} = 3.16$, $P > 0.05$), and no additional effect of the quadratic term ($F_{1,37} = 0.14$, $P > 0.5$). For the lowest blood count measure, the pattern was the same with neither the linear term ($F_{1,38} = 3.64$, P

> 0.05) nor the quadratic term ($F_{1,37} = 0.18$, $P > 0.5$) showing a significant correlation with gametocyte density.

Discussion

Consistent with theory, the virulence of mixed-clone infections was greater than that of single-clone infections, when measured by weight loss and mean blood count. However, there was no consistent evidence that this was due to facultative alterations of within-host growth strategies by parasites in mixed-clone infections. Asexual parasite densities early in the infection of a nonimmune host are correlates of parasite replication rates. Densities later in the infection and the total number of parasites are measures of parasite burden. None of our parasite density measures were sufficient to fully explain the observed patterns of virulence. Within treatment groups, more rapid parasite replication resulted in greater virulence, but for a given parasite replication rate, mixed-clone infections were still more virulent. Thus there is a phenotypic correlation between virulence and parasite replication rate, but there is also an additional effect of genetic diversity, independent of parasite density. If parasite replication rate was the only determinant of virulence, the single-clone and mixed-clone infections should fall along a common fitted line in Figure 4, with the mixed-clone infections concentrated in the upper-right-hand quadrant. Instead, there is considerable overlap in the ranges of parasite replication rates for the two groups, and a significant difference in the intercepts of the fitted lines for single- and mixed-clone infections.

The greater virulence of mixed-clone infections for a given number of parasites may be due to facultative alterations in some aspect of parasite life history other than replication rate. One possibility is that parasites engage in direct interference competition by releasing substances, analogous to allelopathic substances in plants (Rice 1984), to which they themselves are immune but that are toxic to both competing genotypes and the host. Alternately, virulence may be a consequence of the way in which the immune response of the host interacts with the parasites. Blood count and weight loss are directly affected by parasite densities and indirectly affected by the host immune response. Both destroy red blood cells and consume host nutrients. Thus, if there are differences in the immune responses to mixed-clone and single-clone infections, this could explain differences in virulence not attributable to parasite density. For example, diverse parasite populations could stimulate proliferation of a larger number of T- or B-cell clones, and hence a greater immune cascade causing the destruction of more red blood cells, greater consumption of host resources, or higher production of factors such as TNF (tumor necrosis factor), which is associated with many aspects of disease severity (Titus et al. 1991). Under this view, virulence is indeed a side effect of resource extraction from hosts, as assumed by most models of virulence evolution, but with hosts rather than parasites extracting more resources during mixed infections.

Host responses to parasitic infection may play a greater role in determining the virulence of an infection than adaptive parasite evolution (e.g., Ewald 1980; Read 1994; Ebert and Hamilton 1996). The severity of disease incurred by *P. chabaudi* varies greatly with the strain of mouse host (Stevenson et al. 1982), demonstrating that host factors are a major determinant of virulence. Optimality approaches to host responsiveness have yet to be incorporated into theoretical or experimental analyses of the evolution of virulence.

Mixed-clone infections were able to sustain higher parasite densities toward the end of the infection (days 14–16 PI) than single-clone infections (Fig. 1c), as has been observed in previous experiments with *P. chabaudi* (Taylor et al. 1997a). As higher gametocyte densities were also found in mixed infections, this suggests that antigenic diversity in an infection may benefit the parasites in terms of increased transmission from the infections. Genetic analysis of transmission populations in mosquitoes allowed to feed on mixed infections of *P. chabaudi* has shown that both clones were transmitted. Transmission of individual clones was never lower from mixed infections than from single-clone infections, and could be markedly higher (Taylor 1997b). This raises the possibility that, from the parasites' point of view, mixed infections may have beneficial synergisms rather than being strictly competitive. Immune responses against *P. chabaudi* are known to be at least in part strain-specific (Jarra and Brown 1985; Snounou et al. 1989), and it may be that clones that are numerically dominant early in infections shield rarer clones against the onset of strain-specific immunity. Slower clearance of parasites may also account for the greater overall anemia in mixed infections. It cannot, however, explain the greater weight loss: when maximal weight loss occurs, parasite densities were comparable in mixed- and single-clone infections.

Only a few field studies relate malaria morbidity to the number of genotypes in an infection. Those few must be interpreted with caution because of the limitations of the monoclonal (Conway et al. 1991) or PCR (Mercereau-Puijalon 1996) techniques used to assess the number of genotypes, and also the problems of accurately defining morbidity attributable to malaria (Gilles 1988). In The Gambia, groups of patients with mild and severe malaria did not differ in the number of genotypes they were carrying (Conway et al. 1991). In Senegal, two studies carried out in the same village suggest that symptomatic children have fewer genotypes per infection (mean = 1.4, Contamin et al. 1996) than asymptomatics of the same age group subjected to the same transmission intensity (mean = 4, Ntoumi et al. 1995). The interpretation of these and other studies in the same village is that when a child encounters a novel strain, unrestricted parasite growth leads to symptoms (Mercereau-Puijalon 1996). A study in Papua New Guinea suggested that clinical cases more often involve parasites with a particular family of MSP-2 alleles than do asymptomatic controls, suggesting that parasite genotypes differed in their pathogenicity (Engelbrecht et al. 1995). The control group contained a higher percentage of mixed-genotype infections (significance not tested), and a significantly higher proportion of mixed-species infections than were found in the clinical cases. Recent data support these conclusions (Al-Yaman et al. 1997; Beck et al. 1997). Increasingly, data from the field are suggesting that strain-specific immunity is important in developing resistance to malaria, and that multiple infections stimulate protective immunity against a greater range of genotypes. It is therefore likely that in semi-immune hosts, differences in immune sta-

tus are a crucial determinant of virulence, emphasizing the difficulty of applying current ideas of adaptive parasite evolution to biomedical problems. Nevertheless, comparisons of disease severity in people who contract two novel genotypes and in those contracting a single novel strain would be of great interest.

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