The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*

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SUMMARY

Experiments were designed to look at the relationship between infective dose and disease severity using 2 clones of *Plasmodium chabaudi* that differ in virulence. We asked whether there were dose–severity relationships, whether clone differences in virulence were maintained over a range of doses, and whether disease severity could be accounted for by parasite dynamics. Groups of mice were infected with parasite doses differing by an order of magnitude, ranging from 100 to 1×10^8 parasites. Infective dose affected the probability of death, but only with the more virulent clone. Dose also affected morbidity. For both clones, higher doses induced greater anaemia. Larger doses caused greater weight loss, but only for infections with the more virulent clone. Here, for a given dose, mice lost a fixed amount of weight, irrespective of their initial weight. Larger doses induced earlier mortality and morbidity than did lower dose treatments. Finally, dose affected parasite dynamics, with earlier and higher peak parasite densities in larger dose infections. All these effects were small relative to clone differences in disease severity, which were apparent across the range of doses. Dose effects were manifested through the timing and/or magnitude of peak parasite densities, broadly supporting the idea that dose affects disease severity by altering the time the host has to control parasite densities and ameliorate the effects of parasites. We discuss the possible efficacy of intervention strategies aimed at reducing human disease severity by reducing infective parasite dose.

Key words: dose, disease severity, malaria, virulence, *Plasmodium chabaudi*.

INTRODUCTION

The clinical outcome of malaria infection in humans is highly variable. Some people harbour malaria parasites and show no symptoms, while others die rapidly. Many factors may influence this outcome, and one candidate is the inoculating dose of parasites (Greenwood, Marsh & Snow, 1991; Marsh, 1992). Dose might affect disease severity by altering the time available for hosts to mount a defensive response before parasite loads cause clinical disease (Marsh, 1992). Alternatively, lower doses could cause less severe disease by giving the host more time to ameliorate the effects of parasites, rather than just control their densities. For example, the host can, to some extent, offset red blood cell (RBC) destruction by increasing erythropoiesis.

The prevailing view that dose and severity are positively related has been largely encouraged by indirect evidence (e.g. Snow *et al*. 1988; Alonso *et al*. 1991; Greenwood *et al*. 1991; Bermejo & Veeken, 1992; Marsh, 1992), which may have other interpretations (Glynn, Lines & Bradley, 1994). Direct data on the impact of infecting dose on severity of malarial disease in humans are ambiguous. Glynn (1994) has systematically reviewed the medical literature on artificially-induced malaria infections, including malaria therapy administered to neurosyphilis patients. No strong, consistent relationships were found: a few studies suggested a positive relationship between dose and severity (variably measured as duration of illness, experience of chills, type and number of fevers, relapse rates, or whether the patient made a spontaneous recovery), but many found no effect (see also Glynn & Bradley, 1995*a*, *b*; Glynn *et al*. 1995). There are also problems with estimating dose in many of these studies which make it difficult to draw unequivocal conclusions (Glynn, 1994). For instance, in mosquito-induced infections, dose is often equated with bite rates, which is true only if all mosquitoes are equally infected and transmit the same dose of sporozoites.

Experiments have also been performed in birds, monkeys and rodents (see references in Glynn, 1994), but most concentrate on how dose affects the time-course of infection (of pre-patent period or death usually). The only studies of which we are aware that report relevant data do not explicitly test for a dose–severity relationship, or they involve confounding factors (Coggeshall, 1937; Hewitt, 1942; Hewitt, Richardson & Seager, 1942; Ferraroni 1983).

Here, we report the results of a controlled experiment designed to look at the relationship between dose and disease severity using 2 clones of

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Plasmodium chabaudi chabaudi known to differ in virulence. We ask (i) whether there are dose–severity relationships, (ii) whether clone differences in virulence are maintained over a range of doses, and (iii) whether the severity of disease can be accounted for in terms of the time hosts have to develop responses capable of controlling infection.

MATERIALS AND METHODS

Parasitology

Two cloned lines of the rodent malaria parasite, *P. c. chabaudi*, were used to infect 7-week-old female C57 bl6J mice (Harlan, England and B&K Universal, England). The 2 clones, CW and BC, were obtained from different isolates collected in the Central African Republic in 1969 and 1970 from their natural hosts (*Thamnomys rutilans*) and were stored in liquid nitrogen until cloning (Mackinnon & Read, 1999). BC infections peak at higher parasite densities and induce greater weight loss and anaemia than CW infections (Mackinnon & Read, 1999).

Mice were fed on 41B maintenance diet (Harlan, England). Drinking water was supplemented with 0·05 % *para*-amino benzoic acid to enhance parasite growth (Jacobs, 1964). Artificial light was provided from 05·30 h until 17·30 h.

Parasite densities were estimated from the proportion of RBCs infected (calculated from a Giemsa's-stained thin blood smear) and the number of RBCs per ml of blood (counted using flow cytometry: Coulter Electronics™). Blood sampling and measurements of body weight (to the nearest 0·01 g) were carried out daily between 15.00 h and 19.00 h.

Experimental design

Mice were weighed and allocated at random to treatment groups. Within each treatment group, groups of 5 mice were infected with 1×10^2, 1×10^3, 1×10^4, 1×10^6, 1×10^7, or 1×10^8 parasitized RBCs of either clone CW or clone BC. The whole experiment was replicated in a second block that also contained an additional dose treatment of 1×10^5 parasitized RBCs for each clone. Thus, with 1 uninfected control group in each block, there were 13 groups in the first block (a total of 65 animals) and 15 groups in the second block (a total of 75 animals). In the second block, 1 animal from the CW 1×10^6 treatment unexpectedly died on the day after infection, and 2 animals, one from each of the CW 1×10^2 and 1×10^3 treatments, failed to develop patent parasitaemias. Data from these 3 mice were excluded from the analyses.

To administer an exact dose of parasites in a fixed volume of liquid, the appropriate volume of infected blood from the donor mice was diluted in a solution of calf serum-Ringers (50 % heat-inactivated calf serum; 45 % Ringers solution (27 mM KCL, 27 mM $CaCl_2$, and 0·15 M NaCl); 5 % heparin (20 units/ml Ringers)). Each infection was initiated with an intraperitoneal injection of 0·1 ml of this solution. Control mice received the same volume of uninfected calf serum-Ringers.

Disease severity was measured in terms of mortality and morbidity. From surviving mice, acute and chronic morbidity measures were estimated: minimum weight and RBC density, and average weight and RBC density (from day −4 to 35 p.i. for average weight and RBC in block 1 and, in block 2, from day −1 to 35 for average weight, and day 0 to 40 for average RBC).

Statistical analyses

Mortality data were analysed using binary logistic regression. Only the data from surviving mice were included in the morbidity analyses, and these were analysed using general linear models (Crawley, 1993). Maximal models were fitted first and, beginning with higher order interactions, non-significant terms were sequentially removed in a process of backward elimination to generate minimal models.

Fitted terms in the maximal models included dose (specified as a covariate), block and clone (factors), and all linear interactions between factors and covariates. In addition, pre-infection RBC density or body weight was fitted as a covariate to control for initial differences between mice.

Minimal models frequently had weakly significant, biologically uninteresting higher order interactions, usually involving block and/or clone. These interactions involved quantitative but not qualitative differences between blocks. To simplify the analysis, we report the results of separate models for each clone. All *P*-values reported for significant terms are from the appropriate minimal models and *P*-values for non-significant terms are from the step at which they were removed. Our comparison of clone differences in dose–severity relationships is based on the approximate 95 % confidence intervals of the slopes (taken as twice their standard errors).

In most cases there were significant differences between the 2 experimental blocks because both parasite clones were more virulent in the second block (possibly because they had been through 2 additional mice passages). In the current context, block main effects are of little interest in their own right, and so only significant interactions between block and other model terms are reported.

Infective dose was logarithmically transformed to reduce the levering effects of the highest dose treatment, and because this transformation linearized any relationships with virulence. In no cases did $\log_{10}$ dose fitted as a quadratic or cubic term

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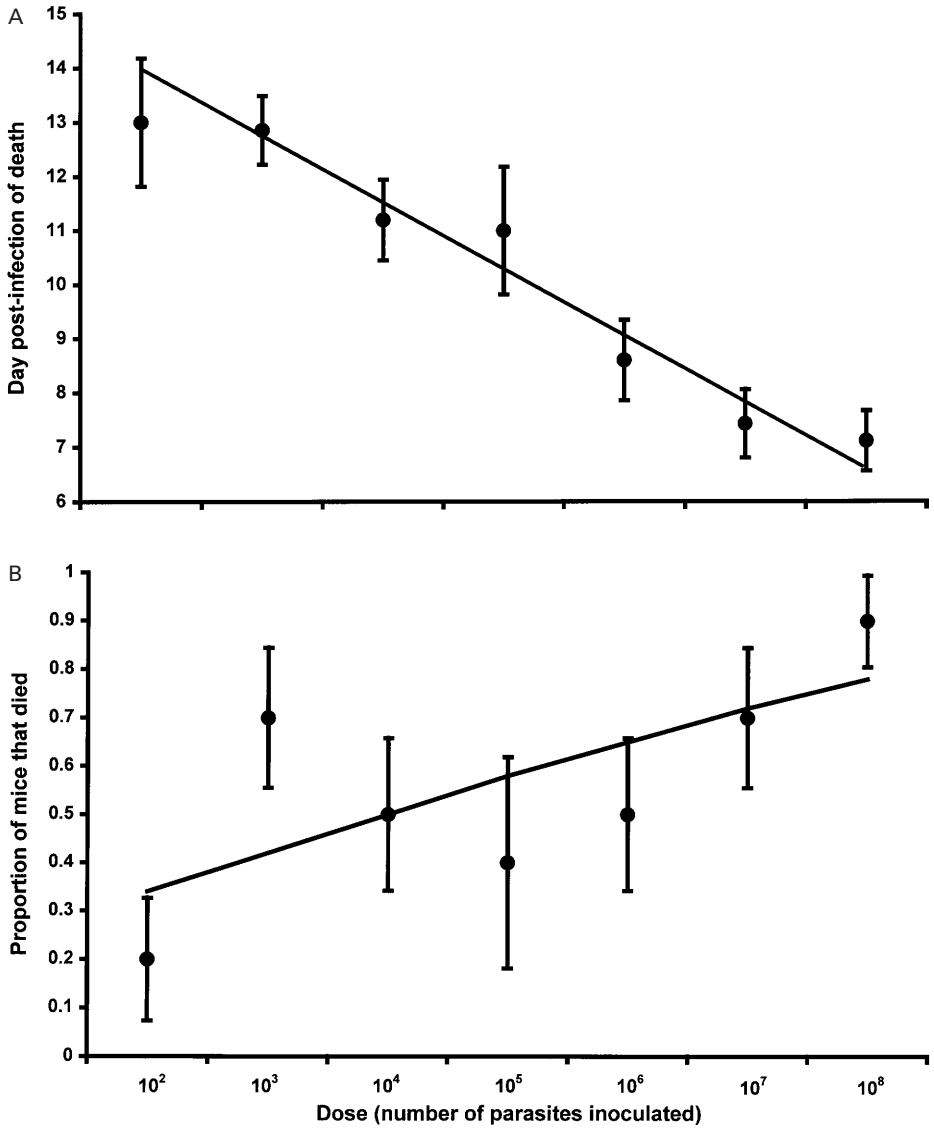

Fig. 1. The effects of dose on (A) the timing of mortality and (B) the mortality rate for BC-infected mice. In (A) points shown are the mean per treatment (±S.E.) and the line is the least-squares regression from the minimal model which contains log dose only. Slope ± S.E. = −1·14 ± 0·13, $n = 37$ mice. In (B) points are the proportion of mice from the 2 blocks that died in each dose treatment (±S.E. calculated using the formula $\sqrt{([p(1-p)]/n)}$, where p = proportion that died), and the line is from logistic regression (see text for further details).

significantly improve model fit. Two of the 3 RBC densities (minimum RBC density and day-1 p.i. RBC density, but not average RBC density) were also log transformed to satisfy the assumptions of the statistical models.

For each infection, the growth rate of the parasite population on day t was calculated by subtracting the log parasite density on day t from the log parasite density on day $t+1$ (McCallum, 1999). Thus, the daily growth rate is the number of new infected RBCs produced by a single parasite after 24 h. The maximum parasite growth rate for all mice (including those that died) was used in the analyses and usually occurred on the first or second day that parasites were detected (parasite growth rates were not different in mice that died or survived). Similarly, data from all mice were used for analyses of maximum parasite density. Mice that died had higher maximum parasite densities (mean parasite density $\times 10^9$/ml ± S.E.: survivors = 1·5 ± 0·06, dead = 2·4 ± 0·09; $F_{1, 121} = 79$, $P < 0{\cdot}001$), but excluding them from the relevant analyses would only serve to strengthen our conclusions. The total number of parasites produced in infections, obtained by summing the daily parasite densities from days 1 to 23 p.i. in the first block and days 1 to 16 p.i. in the second, was calculated for surviving mice only.

RESULTS

Mortality

About half of the mice infected with parasites of clone BC subsequently died (37/70), whereas just

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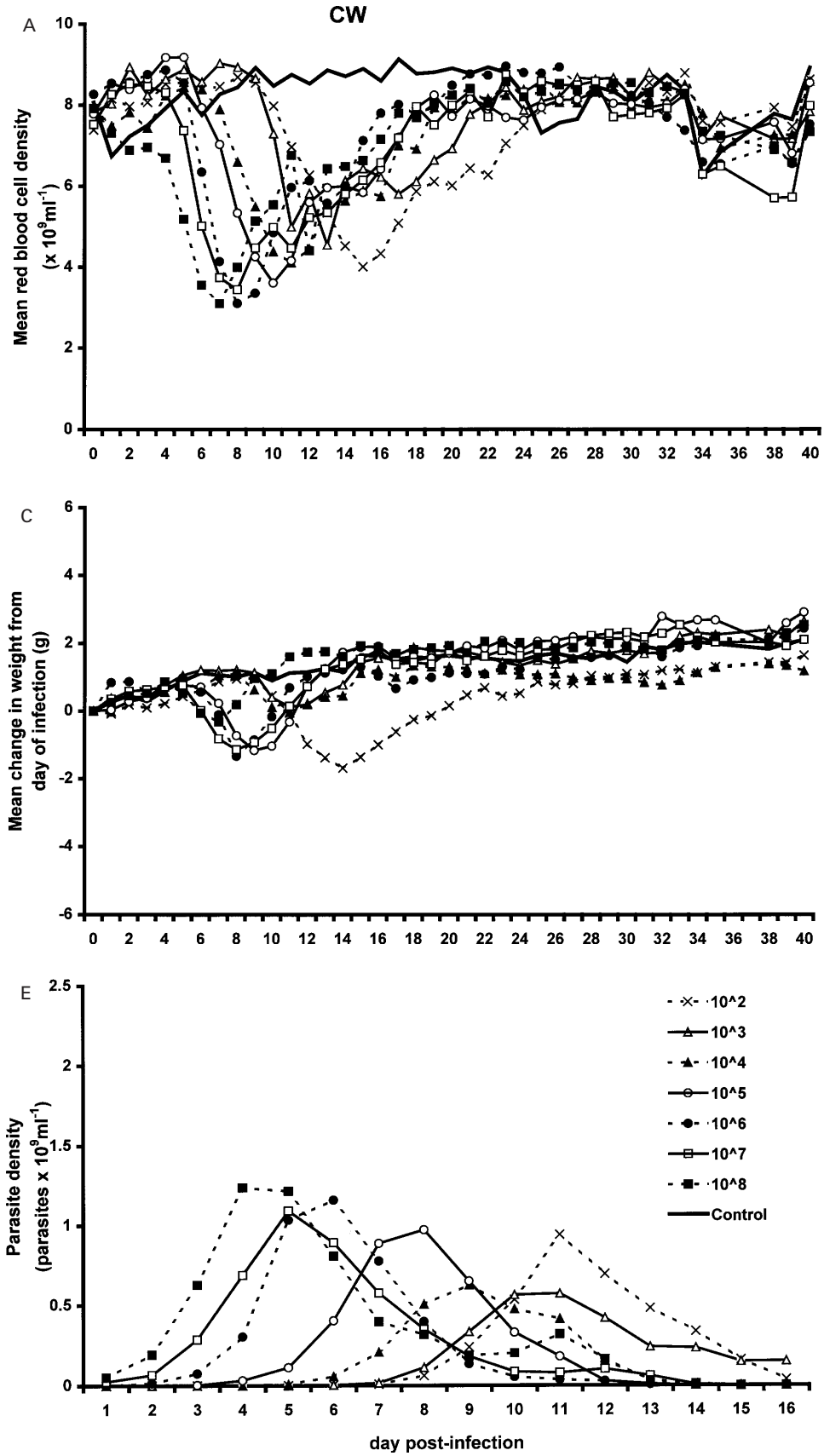

Fig. 2. For legend see facing page.

1 CW-infected mouse died. Among BC-infected mice, death occurred about a day earlier for every 10-fold increase in inoculating dose (Fig. 1A). Mortality rates also increased with inoculating dose (Fig. 1B, $\chi^2 = 4{\cdot}55$, D.F. $= 1$, $P < 0{\cdot}05$). However, dose only explains a small amount of the variation in

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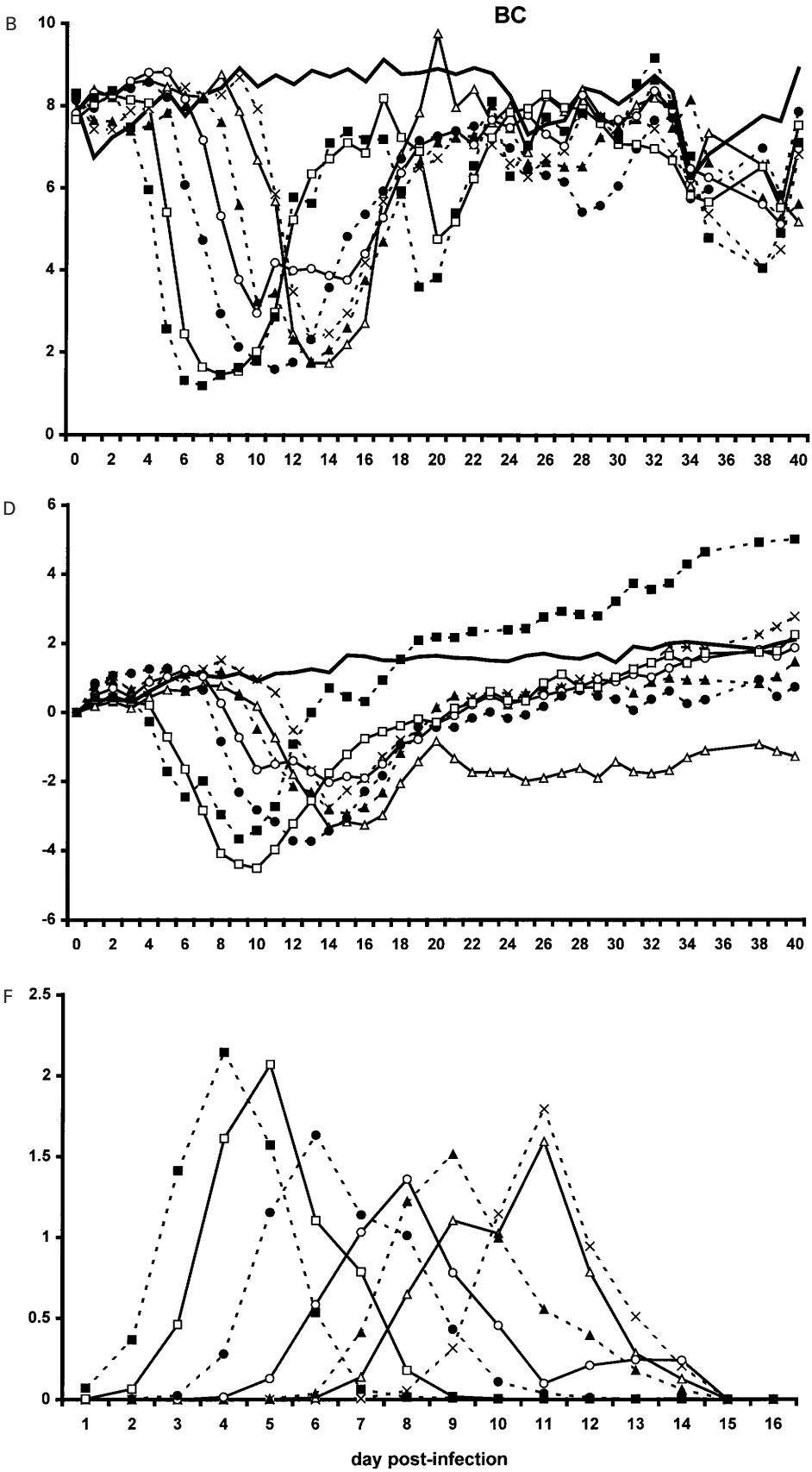

Fig. 2. Mean daily RBC densities (A and B), changes in weight (C and D) and parasite densities (E and F) for mice infected with parasite clone CW (A, C and E) or BC (B, D and F) for each dose treatment in the second experimental block. Each line represents the mean of the mice in the dose treatment. Initially there were 5 mice per group, but subsequently numbers were reduced where mice died. For example, in (D) the 10^8 treatment contains only 1 mouse from day 7 onwards.

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the probability of death (around 7%), and this result is highly sensitive to the 2 extreme dose treatments (10 and 100 million parasites). When either of these were removed, dose no longer explained a significant amount of variation in death rates ($P > 0{\cdot}2$).

Morbidity

The infection kinetics, RBC dynamics and patterns of weight change in one experimental block are illustrated in Fig. 2; the dynamics were qualitatively similar in the other block. BC infections grew faster (mean number of new infected RBCs produced by a single parasite $\pm$S.E.: BC $= 7{\cdot}36 \pm 1{\cdot}05$; CW $= 5{\cdot}07 \pm 1{\cdot}06$, $F_{1,121} = 24{\cdot}2$, $P < 0{\cdot}001$), reached higher parasite densities ($F_{1,81} = 90{\cdot}8$, $P < 0{\cdot}001$), and generated more parasites over the whole infection ($F_{1,81} = 26{\cdot}4$, $P < 0{\cdot}001$) than did infections with clone CW. BC also induced greater maximum weight loss and more anaemia (Fig. 2). The same patterns were also found for average weights and RBC densities p.i. (data not shown).

Dose had a substantial impact on the time-course of infections (Figs 2 and 3). For both clones, maximum parasite densities, anaemias and weight losses occurred later in infections initiated with fewer parasites. Overall, this delay was around 1 day for every 10-fold decrease in dose (Fig. 3).

Dose also had an effect on morbidity (Fig. 4). For both clones, mice inoculated with more parasites were more anaemic (Figs 2A, B and 4A; Table 1). Once initial weight was controlled for, dose affected minimum weight, but only for the more virulent clone (BC). Here, mice lost about a third of a gram more for every 10-fold increase in inoculum size (Figs 2D and 4B; Table 1). Within a dose treatment, mice lost a fixed amount of weight, irrespective of their initial weight ($F_{1,83} = 106$, $P < 0{\cdot}001$, slope $\pm$ S.E. $= 0{\cdot}96 \pm 0{\cdot}09$). The administered dose of CW had no effect on either the minimum weight or the average weight of mice, even allowing for initial mouse weight (Figs 2C and 4B; Table 1).

Dose had an effect on parasite dynamics. The total number of parasites produced in infections was greater in high dose treatments for clone CW (Table 1), and bigger inoculating doses led to greater peak parasite densities for both clones (Figs 2E, F and 4C; Table 1). Nevertheless, it is striking that variation in dose across 6 orders of magnitude generates peak densities that differed by substantially less than 1 order of magnitude. Parasite population growth rates early in the infections were unrelated to inoculating dose (Table 1).

In summary, inoculating dose affected morbidity as measured by maximum anaemia and weight loss. But bigger doses also led to earlier and higher peak parasite densities. In order to investigate whether dose affected morbidity independently of its effects on parasite dynamics, we incorporated peak parasite density and the timing of peak parasite density into models relating dose with anaemia or weight loss.

Once peak parasite density, and the timing of it, had been controlled for, there was no independent effect of dose on morbidity. Similarly, there were no independent effects of parasite dynamics on morbidity when dose was controlled for, with one exception: peak parasite density independently affected minimum RBC density of CW-infected mice ($F_{1,55} = 29{\cdot}6$, $P < 0{\cdot}001$). However, peak parasite density and the timing of it are highly correlated, making it hard to disentangle their effects. Thus, dose affects morbidity only through its effects on the timing and/or magnitude of peak parasite density (or variables highly correlated to these).

DISCUSSION

Fatal infections were common for only 1 of the 2 clones used in these experiments (BC), but there, inoculating dose affected the probability of death (Fig. 1B). Dose also had an impact on morbidity. Anaemia was greater among mice given larger infective doses of either clone (Fig. 4A). Larger doses also led to greater weight loss, but only for infections with the more virulent clone (Fig. 4B). Dose also affected the timing of morbidity, with maximum anaemia and weight loss occurring earlier in infections initiated with larger doses (Figs 3A and B). Death, where it occurred, was also earlier in the higher dose treatments (Fig. 1A). Finally, inoculating dose affected parasite dynamics, with earlier and higher peak parasite densities in larger dose infections (Figs 3C and 4C). In so far as death, anaemia and weight loss are measures of disease severity, we have thus demonstrated positive dose–severity relationships. As far as we are aware, these are the first controlled experimental demonstrations of these relationships in mammalian *Plasmodium*.

The 2 clones used in this study, CW and BC, were chosen because they were known to differ in virulence (Mackinnon & Read, 1999). This difference was also evident in our experiments: BC was more often lethal and induced greater anaemia and weight loss. The clone difference is, at least in part, because BC populations grow more rapidly (see Results section), and achieve higher parasite densities than do CW (Mackinnon & Read, 1999). That earlier work (Mackinnon & Read, 1999) involved infections initiated with 10^4, 10^5, and 10^6 parasites in different experiments, with the onset of symptoms being earlier in the experiments involving larger doses. The experiments we report here confirm that the dose effects on the timing of symptoms reported there were not due to uncontrolled confounding variables across experiments. They are also consistent with the widespread finding

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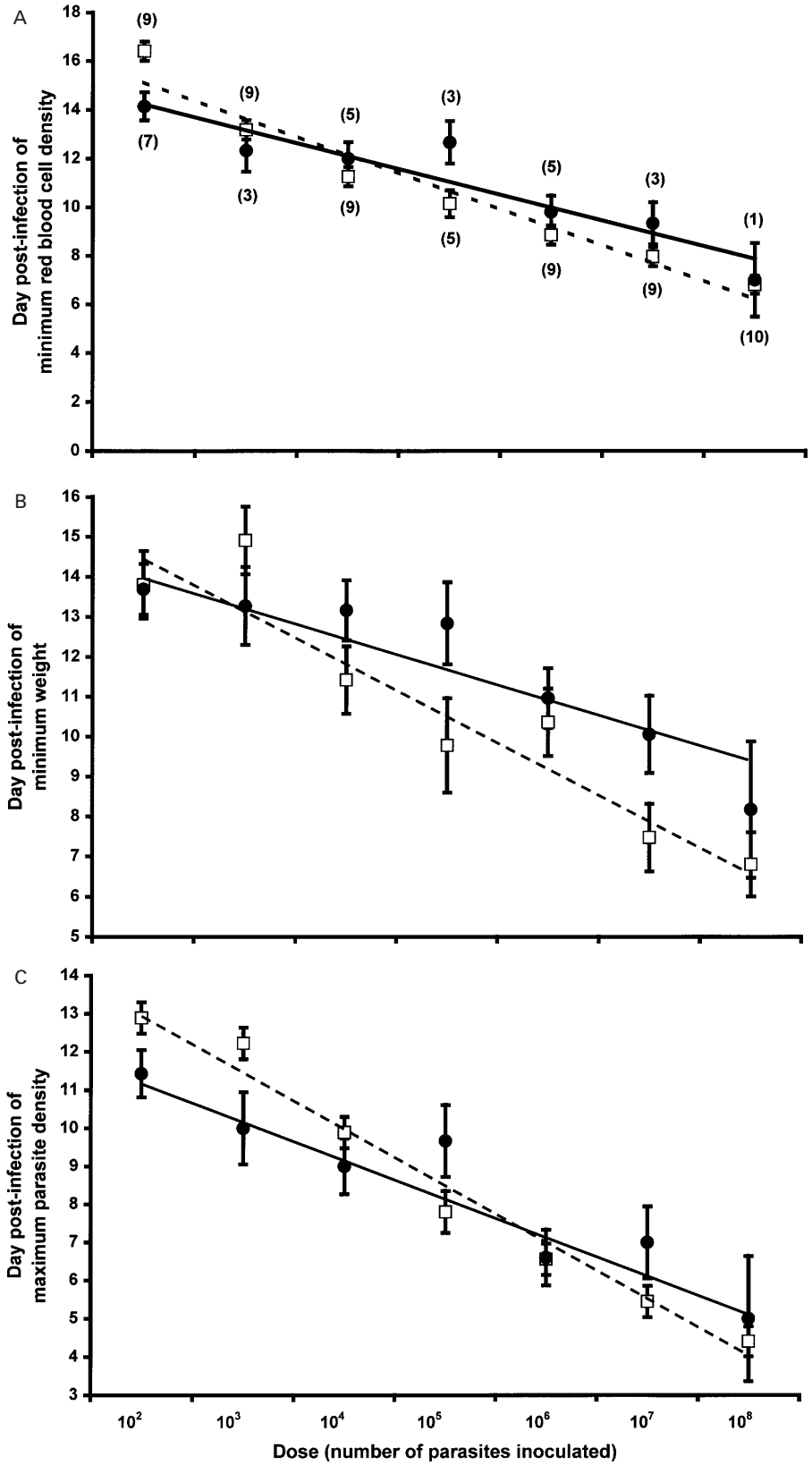

Fig. 3. The effect of dose on the timing of (A) minimum RBC density, (B) minimum weight and (C) maximum parasite density, for BC- (—●—) and CW-infected (--□--) mice. Lines are the least squares regressions from separate minimal models for each clone. Only data from surviving mice are included. In (A) the numbers in parentheses represent the number of mice the means represent, and are the same for Figs 3B, C and 4A, B. The least squares slopes ($\pm$S.E.) and P values for log dose from models controlling for block are: (A) BC: $-0{\cdot}99 \pm 0{\cdot}16$, $P < 0{\cdot}001$; CW: $-1{\cdot}48 \pm 0{\cdot}083$, $P < 0{\cdot}001$; (B) BC: $-0{\cdot}76 \pm 0{\cdot}17$, $P < 0{\cdot}001$; CW: $-1{\cdot}32 \pm 0{\cdot}16$, $P < 0{\cdot}001$; (C) BC: $-0{\cdot}99 \pm 0{\cdot}17$, $P < 0{\cdot}001$; CW: $-1{\cdot}51 \pm 0{\cdot}074$, $P < 0{\cdot}001$.

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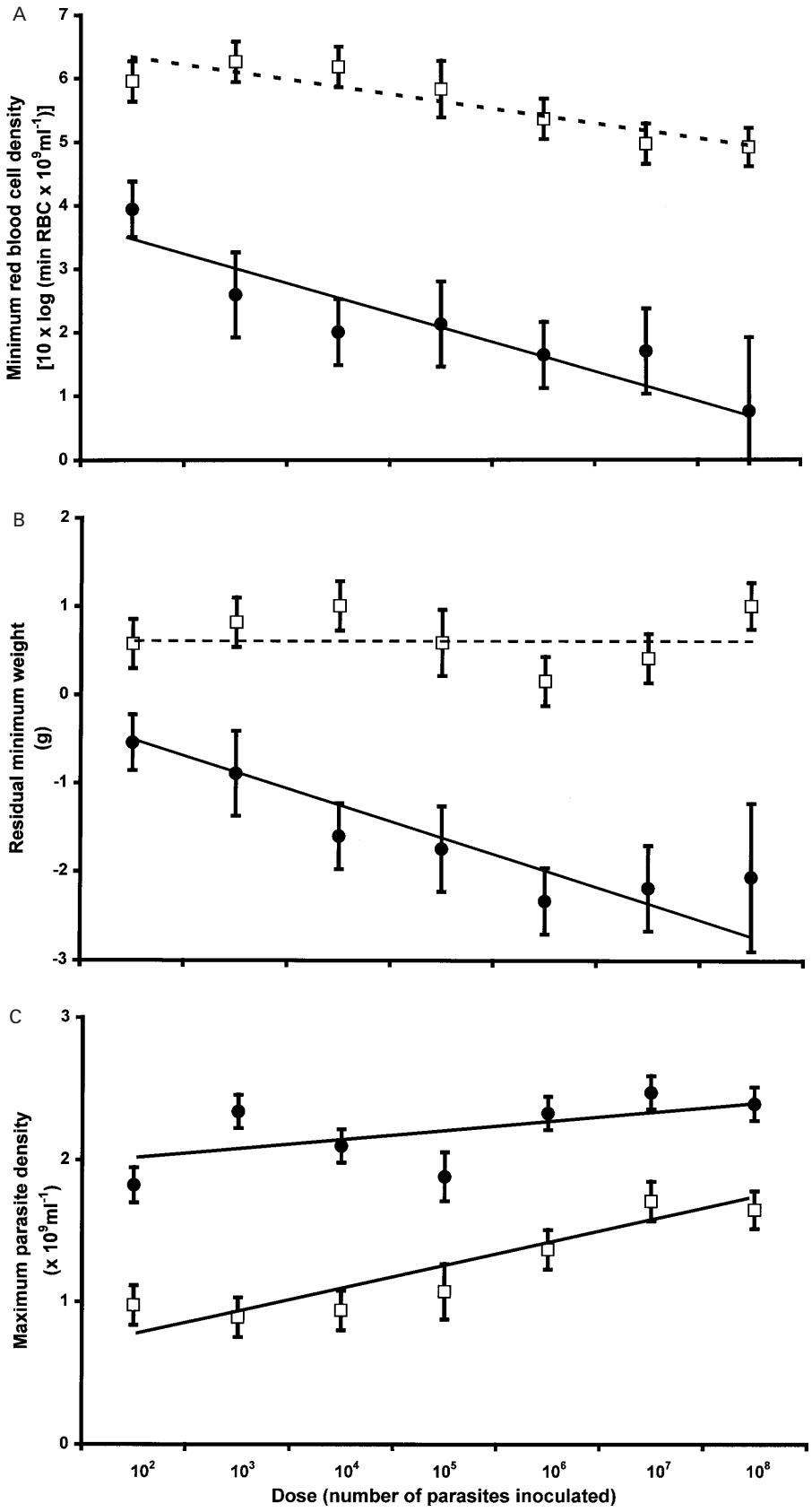

Fig. 4. The effect of dose on (A) minimum RBC density, (B) minimum weight and (C) maximum parasite density, for BC- (—●—) and CW-infected (--□--) mice. Lines are the least squares regressions from separate minimal models for each clone, except for (B). Here, to produce the figure, maximum weight and block were included in the first model for BC and CW simultaneously (to correct for initial body size and block), and the residuals from this model were then run against log dose and clone. Thus, the fitted values come from the same model. Only data from

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Table 1. The effects of inoculating dose on morbidity and parasite densities

(Tabulated values are least squares slopes (±S.E.) from models controlling for block, and incorporating the relevant covariate (initial weight or RBC density) if present in the minimal model (shown as ◆).)

	BC	CW
Minimum RBC density (log (RBC × 10^9/ml))	−0·039±0·01**◆	−0·023±0·006***
Average RBC density (RBC × 10^9/ml)	−0·13±0·04**	−0·090±0·02***
Minimum weight (g)	−0·38±0·09***◆	−0·084±0·07 N.S.◆†
Average weight (g)	−0·11±0·06 N.S.◆	0·032±0·05 N.S.◆†
Maximum parasite density (parasites × 10^9/ml)	0·076±0·02**	0·14±0·03***†
Total number of parasites (parasites × 10^9/ml)	0·18±0·1 N.S.	0·70±0·09***†
Initial parasite population growth rates	−0·026±0·03 N.S.†	0·0026±0·02 N.S.

† Significant block × dose interaction, but the relationship between dose and the relevant variable is in the same direction in each block. * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
N.S., Not significant.

from several other studies that larger doses lead to earlier symptoms (for example, Hewitt *et al*. 1942; Cox, 1966; Glynn, 1994).

For most of our measures of morbidity, timing of morbidity and parasite dynamics, the form of any relationship with dose is quantitatively similar for the 2 clones (comparing the slopes of the relationships given in Fig. 3 and Table 1 for the 2 clones). The relative virulence of the 2 clones is unaltered by dose and the more virulent clone is always more virulent. Thus, an avirulent clone cannot be transformed into a virulent clone by simply giving it a numerical boost early in the infection. Models of the evolution of virulence (for reviews see Ewald, 1983; Bull, 1994; Levin & Bull, 1994; Read, 1994; Frank, 1996) assume that virulence phenotypes are at least partly determined by heritable genetic parasite variation on which selection can act. Substantially more complex models would be required if the relative virulence of strains is qualitatively altered at different doses, and/or if the severity of disease rose with dose and hence the force of infection. The broad picture which emerges from our experiments is that, at least to a first approximation, the implicit assumption that infective dose is irrelevant to evolutionary models of virulence is probably justified. We are currently exploring the effects of dose on transmissibility.

It has been postulated that dose may affect the severity of disease by altering the time the host has to control parasite density before it reaches a clinical disease threshold (Marsh, 1992). In the particular scenario sketched out by Marsh (his Fig. 5), he envisages a set time-interval before an immune response can be activated, and implicitly assumes this response will control parasite growth after a further fixed delay. This hypothesis generates 2 testable predictions. First, that peak parasite density will increase with increasing dose, and second that across dose treatments, all parasite peaks will occur on the same day.

Our results support the first of these, but there is no support for the second: the timing of peak parasite density was not constant, and instead occurred later with decreasing dose. Nevertheless, our data do bolster the idea that dose effects are dependent on parasite dynamics if the assumption of a fixed time-interval from immune activation to parasite control is relaxed.

The timing of peak parasite density across treatments is consistent with the suggestion made by Cox & Millott (1984) that, for *P. vinckei*, parasite-controlling mechanisms are triggered at a fixed threshold level. However, if the speed of this response, once activated, was similar across dose treatments, then one would expect peak parasite densities to be of similar magnitudes. Our data suggest that this is not the case, and something more complex must be involved. We are currently exploring theoretical models that can account for the

surviving mice were included in the analyses for (A) and (B), and each point represents the mean (±S.E.) with sample sizes as shown in Fig. 3(A). For (C) data from all mice (including those that died) were used – see Materials and Methods section for sample sizes.

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dual effects of dose on the timing and magnitude of peak parasite density.

The effect of dose appears to be manifested through the timing and magnitude of peak parasite density: higher dose treatments have earlier and higher parasite densities, which cause greater morbidity. This implies that there is a per parasite effect of virulence, because more parasites cause greater morbidity. It also suggests that the number of parasites that mice are initially exposed to are secondary to how much time they have to mount a defensive immune response which can temper parasite growth. This might be because having more time to mount an immune response allows the host to reallocate resources that aid this response, and help to minimize the damage that parasites can potentially cause. For example, experiencing RBC destruction induces erythropoiesis. If this is stimulated before too many RBCs are destroyed (i.e. when there are few parasites causing it), then the host might be better able to withstand the parasitic assault on RBCs. However, if hosts experience high parasite densities before they can mobilize these processes, then the damage they endure could be more devastating.

The different dose treatments in this experiment cover 6 orders of magnitude. However, the effects of dose on mortality were highly sensitive to the 2 extreme dose treatments (100 and 1×10^8 parasites), and overall, dose accounted for just 7% of the probability of death. And while the effects of dose on morbidity are statistically highly significant, dose explains only 20% of the between-mouse variation in anaemia for both clones, and just 10% of weight loss for the most virulent clone. These effects are small in comparison to those caused by clone differences.

In the field, it is extremely unlikely that naturally infected hosts experience this range of inoculating doses. The average number of *P. falciparum* sporozoites transmitted is typically less than 25 (Rosenberg *et al*. 1990; Beier, 1993), and more than 1000 has never been observed (Beier *et al*. 1991; Lines & Armstrong, 1992). Liver schizonts produce up to 30000 merozoites (Garnham, 1966). Thus, a biologically realistic dose of merozoites released into the blood from a single infective bite is in the region of between 1×10^5 and 1×10^6, and exceptionally 1×10^7. If only these treatments are considered, then dose has no effect on the severity of disease ($P > 0{\cdot}5$ in all cases).

However, the differences in disease severity for the 1×10^5 and 1×10^6 treatments predicted from our regression lines (fitted across our full range of doses) are rather small (for instance, a 10% difference in mortality and minimum RBC density). If these differences did exist, we would almost certainly not detect them, given the within-group variability we observed and our sample sizes: standard power calculations (Armitage & Berry, 1987) show that we had a $< 8\%$ probability of detecting such differences. Indeed, in excess of 100 mice would be needed to afford an 80% chance of detecting such differences as significant. Nonetheless, differences of these magnitudes, which would also not be statistically significant in small clinical trials, could be biologically significant given the world-wide malarial disease burden: interventions capable of achieving a 10% reduction in mortality are highly desirable. To the extent that the results of our experiments across a large range of doses extrapolate to mosquito-induced human infections, our data provide grounds for believing that interventions that reduce infective dose will have an effect on the subsequent severity of disease.

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