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The effect of partial host immunity on the transmission of malaria parasites

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Experiments were carried out to determine the effect of partial host immunity against the rodent malaria parasite *Plasmodium chabaudi* on the transmission success of the parasite. There was a fourfold reduction in both the blood-stage, asexually replicating parasite density and the gametocyte (transmissable stage) density in immunized hosts. Some of the reduction in asexual parasite densities was due to strain-specific immunity, but there was no evidence that strain-specific immunity affected gametocyte densities. However, immunity did affect transmission in a strain-specific manner, with a fivefold reduction in gametocyte infectivity to mosquitoes in homologous challenges compared with heterologous challenges or non-immunized controls. This implies the existence of a mechanism of strain-specific infectivity-reducing immunity that does not affect the density of gametocytes circulating in peripheral blood. The proportion of asexual parasites that produced gametocytes increased during the course of infection in both non-immunized and in immunized hosts, but immunity increased gametocyte production early in the infection.

Keywords: partial immunity; gametocyte; gametocytogenesis; transmission; *Plasmodium chabaudi*; vaccination

1. INTRODUCTION

The effect of partial host immunity on the transmission of malaria parasites is crucial to malaria epidemiology. In humans and laboratory models, partially protective acquired immunity against malaria parasites reduces the density of asexually replicating blood-stage parasites and reduces the duration of infection (Boyd 1942; Jeffery 1966; Sadun *et al*. 1966; Cadigan & Chaicumpa 1969; Graves *et al*. 1988*a*; Jarra & Brown 1985; Jarra *et al*. 1986; Bates *et al*. 1988). Subsequent infectivity to mosquito vectors is likely to be reduced for two reasons. First, there will be fewer asexual parasites to produce the transmissable stages (gametocytes), the density of which generally shows a positive correlation with infectivity to mosquitoes (Carter & Graves 1988; Taylor & Read 1997; Taylor *et al*. 1997). Second, antibodies raised against asexual parasites are likely to have some anti-gametocyte activity (Motard *et al*. 1995; Goodier & Targett 1997; Hayward *et al*. 1999). Partial immunity can further reduce *Plasmodium* transmission success by reducing gametocyte infectivity through inactivation of gametocytes and/or of the resultant gametes in the mosquito midgut ('transmission-blocking' immunity; Mendis *et al*. 1987; Graves *et al*. 1988*b*, 1991; Mulder *et al*. 1994).

However, partial host immunity might result in an increase in the proportion of asexual parasites that produce gametocytes. The rate of gametocyte production (gametocytogenesis) increases when conditions do not favour asexual replication (reviewed in Carter & Graves 1988); in the case of subcurative chemotherapy, this response can result in treated infections being as infectious as untreated infections (Buckling *et al*. 1997, 1999*a*,*b*). Increased gametocyte production *in vivo* has been reported in the rodent malaria, *Plasmodium yoelii*, after immunization with a recombinant malarial heat-shock-like protein that is expressed throughout the life cycle (Motard *et al*. 1995) and has been reported in *Plasmodium falciparum* after the addition of immune serum and lymphocytes (Smalley & Brown 1981) and anti-*P. falciparum* antibody (Ono *et al*. 1986).

Studies using the rodent malarial parasites *P. chabaudi* and *Plasmodium berghei* have shown that partial anti-parasite immunity has a strain (genotype)-specific component. Reductions in peak asexual densities and the duration of the patent infection are greater when animals are challenged with the genotype used for immunization (homologous challenge) than when animals are challenged with a different genotype (heterologous challenge) (McLean *et al*. 1982; Jarra & Brown 1985; Jarra *et al*. 1986; Bates *et al*. 1988). Strain-specific immunity has also been shown experimentally for the human malarial parasites *P. falciparum* and *Plasmodium vivax* (Boyd 1942; Jeffery 1966; Sadun *et al*. 1966; Cadigan & Chaicumpa 1969). The effects of strain-specific immunity on transmission remain to be determined.

We carried out experiments with the rodent malaria *P. chabaudi* to address the following questions. First, does partial immunity reduce transmission? Second, does strain-specific immunity that acts against asexual parasites also act against gametocytes? Third, does immunization change gametocyte infectivity and, if so, does it do so in a strain-specific manner? Finally, does immunization change patterns of gametocytogenesis?

2. MATERIAL AND METHODS

(a) *Host immunization and challenge*

All work was carried out in accordance with the Animals (Scientific Procedures) Act 1986 (UK). Male C57/BL/6J mice (Harlan-Olac, England) aged 8–14 weeks were immunized with 1×10^4 red blood cells (RBCs) infected with a *P. chabaudi* clone (either CR or ER, from the WHO Registry of Standard Malaria Parasites, maintained at the University of Edinburgh, UK) in a

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0.1 ml intra-peritoneal inoculum of 50% Ringer's solution (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl), 45% heat-inactivated calf serum and 5% 200 units ml^{-1} heparin solution. ER and CR probably differ at several loci, and are known to differ at the immunogenic surface antigen MSP1 (McLean *et al.* 1991). Control animals were inoculated with uninfected RBCs in the same volume of inoculum solution. Four days after infection, all mice were treated orally, using a lubricated catheter, with a single dose of 25 mg kg^{-1} mefloquine solution, prepared by dissolving mefloquine sulphate in distilled water that was heated to 60 °C. Treatment was repeated over the next 2 d, to ensure infection clearance, which was confirmed by examination of 500 microscopic fields of Giemsa-stained blood smears (see § 2b) taken weekly until the challenge. Four weeks after the first day of drug treatment, mice were challenged with 1×10^6 parasites of either clone CR or ER. Mice challenged with each clone were thus classed as belonging to one of three treatments: non-immune controls, homologous challenge and heterologous challenge. The experiment was carried out twice, resulting in a total of eight animals per treatment per challenge clone. (As a result of host mortality, sample sizes were reduced to six in CR homologous challenge and seven in both ER homologous and heterologous challenge treatments). Mice, which were age-matched in experiments, were housed in cages of 3–5 animals at a temperature of 25 ± 1 °C with a 07.00–19.00 light cycle, and provided with 41B rat and mouse maintenance diet (Harlan-Teklad, England) and water containing 0.05% p-aminobenzoic acid (PABA), *ad libitum*.

(b) *Parasite counts*

Every second day, between days 5 and 17 post-infection (pi) and during the period 17.00–18.00, thin blood smears from the tail vein were stained with Giemsa and asexual parasites counted per 1.5×10^3 RBCs. At low asexual densities, parasites were counted per 1.25×10^4 RBCs. Mature gametocytes were counted per 1.25×10^4 RBCs. Parasite densities were calculated from the product of RBC densities (measured every second day using flow cytometry; Coulter Electronics, Luton, UK) and parasites per RBC.

(c) *Mosquito feeds and dissection*

On day 7 pi (when preliminary experiments showed the presence of gametocytes in the majority of mice in all treatments) in each experiment, four mice per treatment group per clone (a total of 24) were assayed for infectivity by allowing *Anopheles stephensi* mosquitoes to feed on them. Mice were then anaesthetized by an intramuscular injection of 0.5 ml per 20 g of body weight comprising 3:2:1 of distilled water: Vetalar, Pharmacia & Upjon S.A., Luxembourg: Rompun (Bayer plc, Bury St Edmunds, UK) (Buckling *et al.* 1997), and placed onto pots covered with nylon mesh containing *ca.* 40 × 4–5-day-old female *A. stephensi*, that had been starved for the previous 24 h. Mosquitoes were left to feed in the dark for 30 min, between 18.45 and 19.45. After removal of unfed mosquitoes, the remainder were maintained at 25–30 °C, 70–80% humidity, with a 12 L:12 D cycle, and provided with 5% glucose, 0.05% PABA solution *ad libitum*. After 8–9 d, approximately 25 mosquitoes per mouse were dissected to determine the presence of oocysts on midguts.

(d) *Statistical analyses*

Comparisons of control and immunized hosts were made by analyses of the following summary measures for each infection.

(i) *Total asexual parasites*. This was estimated from parasite densities between days 5 and 17 pi by integrating under the 'parasite density through time' curves for each infection. This is a reasonably accurate measure of the total numbers of asexual parasites (between these days) because the asexual cycle of *P. chabaudi* is known to be 24 h (Cox 1988). Before day 5 pi and after day 17 pi, parasite densities are negligible relative to the total produced in the intervening period.

(ii) *Total gametocytes*. This was estimated for the same time period as for total asexual parasites (days 5–17 pi), during which the bulk of gametocyte production occurs. This measure is a reasonable estimate of total gametocyte numbers because the half-life of a mature gametocyte is estimated to be less than 12 h (Buckling *et al.* 1999a). In addition, the sum of the gametocyte densities on each day is likely to be well correlated with total transmission probability: gametocyte densities at any point in time correlate with both the proportion of mosquitoes infected and densities within mosquitoes (Taylor & Read 1997).

(iii) *The proportion of mosquitoes infected*.

(iv) *Daily rates of gametocytogenesis*. We used a simple exponential growth model to estimate the proportion of asexual parasites that produced gametocytes on individual days (Buckling *et al.* 1999*a*). A summary of the assumptions follows. The maturation period of *P. chabaudi* gametocytes is *ca.* 2 d (Gautret *et al.* 1997), such that a proportion, g, of the asexual parasites at day t will produce gametocytes that will mature on day $t + 2$. *P. falciparum* gametocytes become less vulnerable to immune clearance as they mature, probably because they express less-immunogenic antigens and have reduced metabolic activity (Carter & Graves 1988; Hayward *et al.* 1999). Age-dependent mortality of *P. chabaudi* gametocytes is likely to be similar. It is therefore assumed that survival probability (s) in the first 24 h after asexual densities were measured is the same for gametocytes and asexual parasites. Zero gametocyte mortality is assumed in the second 24 h period.

From these assumptions, the proportion of asexual parasites at time t whose progeny develop into gametocytes, g, can be estimated from observed densities of asexual parasites and gametocytes as

$$g = \frac{\frac{G_{t+2}}{A_t}}{\sqrt{\frac{A_{t+2}}{A_t}} + \frac{G_{t+2}}{A_t}}, \qquad (2.1)$$

(Buckling *et al.* 1999*a*), where G_{t+2} is the density of mature gametocytes on day $t + 2$, and A_t and A_{t+2} are the density of asexual parasites on days t and $t + 2$, respectively. Rates of gametocytogenesis (g) on day t were calculated for days 5, 7, 9, 11 and 13 pi.

Before analysis, estimates of total asexual parasites and total gametocytes were $\log_{10}$ transformed to bring their distributions close to normal. All analyses were carried out using generalized linear models (Crawley 1993). Starting with the highest-order interactions, all factors and their interactions were individually removed in turn from the complete model. Non-significant interaction terms were removed and test statistics obtained from the resulting minimal model.

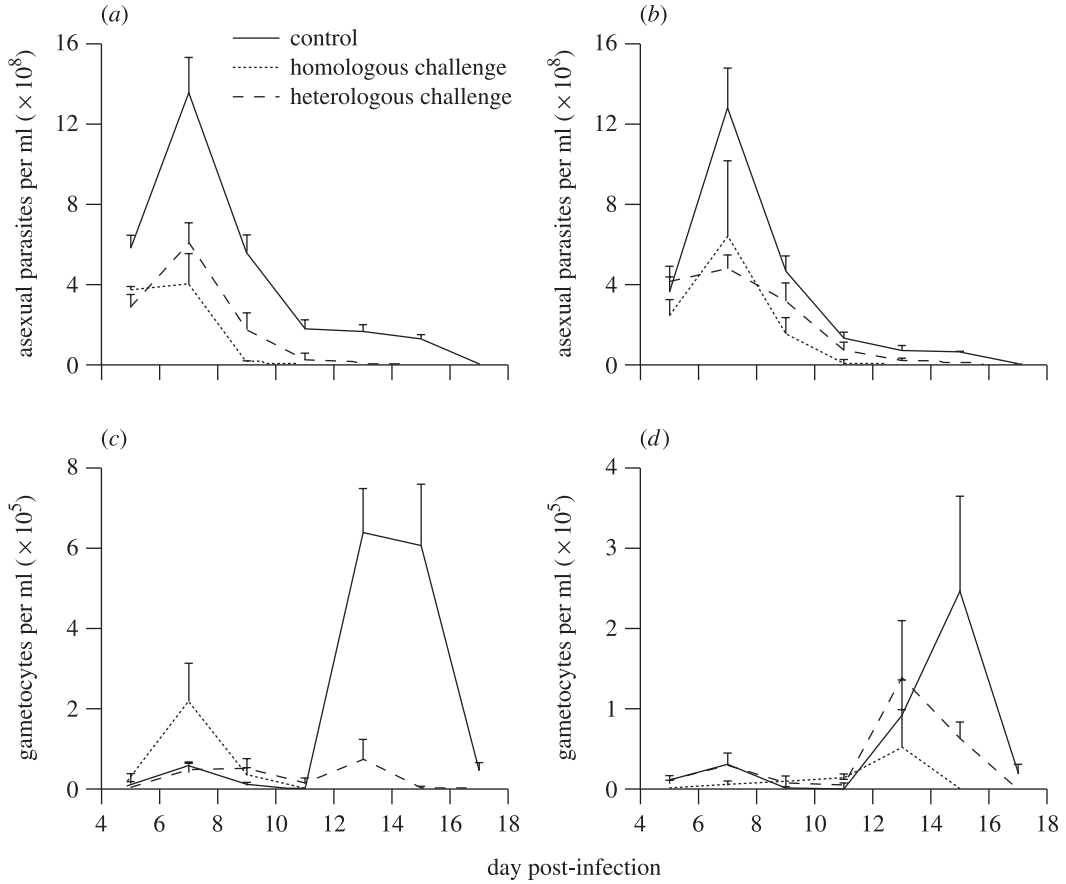

Figure 1. Mean ± 1s.e. asexual parasite densities through time, of (*a*) clone CR, and (*b*) clone ER, and gametocyte densities of (*c*) clone CR and (*d*) clone ER.

The effect of immunization *per se* on (i) total asexual parasites, and (ii) total gametocytes was determined by ANOVA, with treatment (immunized or control), clone (ER or CR) and experiment (1 or 2) fitted as factors. The effect of strain-specific immunity on these measures was determined by carrying out separate analyses using only immunized animals, with immunizing clone (ER or CR), challenge clone (ER or CR) and experiment fitted as factors. Detection of a significant interaction between the immunizing clone and the challenge clone is evidence that strain-specific immunity has occurred (Read & Viney 1996).

A binomial error structure was used for the logistic regression of the probability of a mosquito becoming infected (Crawley 1993). Treatment, clone and experiment were fitted as factors. Square-root arcsine-transformed gametocytes per RBC on the day of the feed (the best correlate of infection probability on an individual day; Buckling & Read 1999) was fitted as a covariate. The data did not require correction for overdispersion.

Daily rates of gametocytogenesis (g, in equation (2.1)) were highly over-dispersed and could not be normalized by transformation—the data consisted of many zeros and very low values, as well as much higher values. Even the use of non-parametric ranking procedures is unlikely to provide an appropriate solution to this problem, given that variation in the very low values will often represent the presence or absence of a single gametocyte in the surveyed microscopic fields. A nominal classification system was therefore used, with each rate of gametocytogenesis classified as high or low, based on whether the value was less or greater than the median of the entire dataset. To control for multiple sampling from the same infection, this value was calculated as the median of the medians for each infection in the entire dataset. Data for each day were analysed using χ^2-tests of independence (with Yates's correction for continuity) to determine whether the proportion of infections that had high rates of gametocytogenesis (median > 0.0006) differed between immunized and naive animals. Given the nominal classification of the data, this analysis was not powerful enough to detect differences in the rates of gametocytogenesis between clones, experiments and the different methods of immunization.

3. RESULTS

Immunization resulted in a threefold reduction in the mean number of total asexual parasites (figures 1*a*,*b* and 2*a*, $F_{1,41} = 58.77$, $p < 0.0001$), and a fourfold reduction in total gametocytes (figures 1*c*,*d* and 2*b*, $F_{1,41} = 7.78$, $p < 0.01$). The reduction in asexual parasite densities had a strain-specific component: parasites densities were lower after homologous challenge than heterologous challenge (figure 2*a*, challenge clone immunizing clone: $F_{1,23} = 10.6$, $p < 0.01$). There was, however, no evidence that the reduction in gametocyte densities in immunized animals had a strain-specific component (figure 2*b*, challenge clone immunizing clone: $F_{1,23} = 1.42$, $p > 0.1$).

Rates of gametocytogenesis in control animals were at a low level until day 11 pi, when they rapidly increased. There was a similar pattern in immunized animals, although rates of gametocytogenesis were generally higher than in control animals before day 11 pi—notably so on day 7 pi (figure 3, $\chi^2_1 = 5.4$, $p = 0.02$). There were no

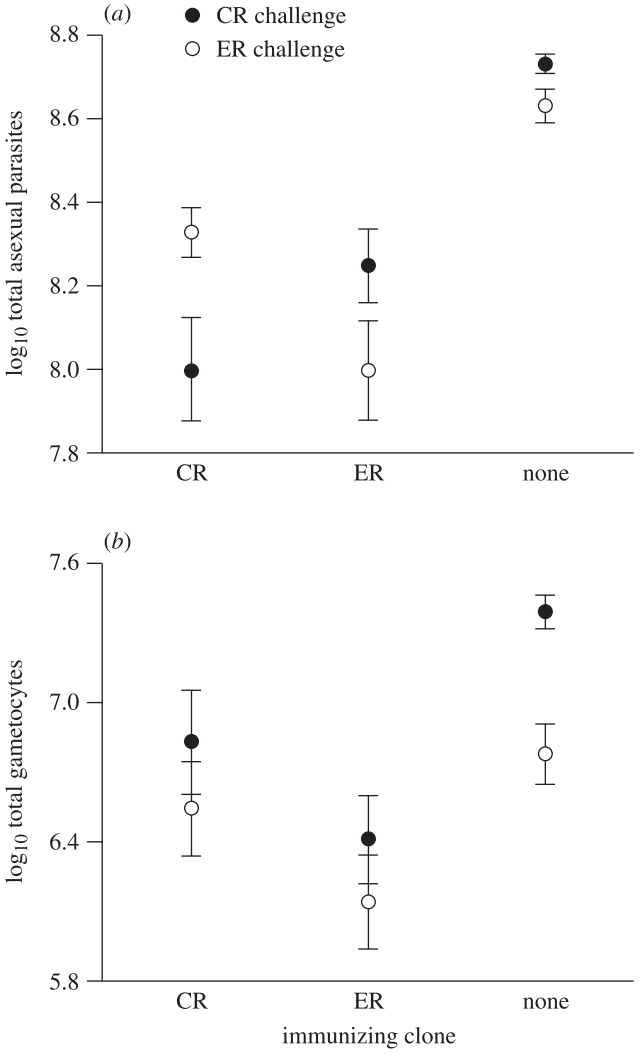

Figure 2. Estimates of (*a*) total asexual parasites and (*b*) total gametocytes, between days 5 and 17 post-infection for all treatments. Plotted points are means ± 1s.e.

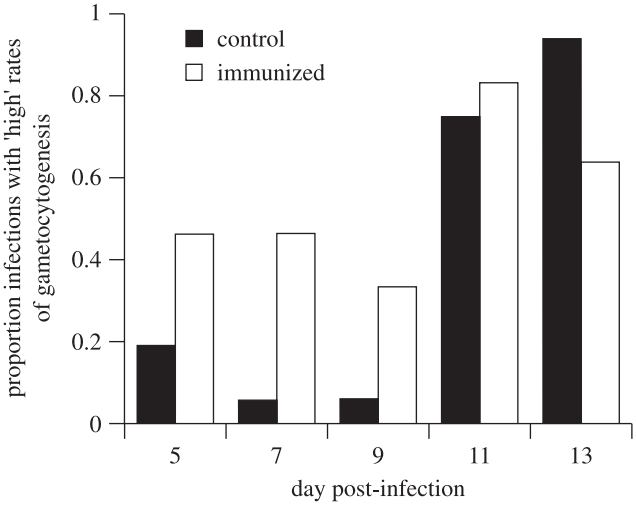

Figure 3. Proportion of all control and immunized infections with 'high' (greater than the median) rates of gametocytogenesis through time. See text for details.

other significant differences in rates of gametocytogenesis between control and immunized animals on the other days (figure 3, $p > 0.1$ in all cases).

The proportion of mosquitoes infected increased with gametocytaemia at the time of the feed ($\chi^2_1 = 37.98$, $p < 0.001$), and at the same rate for clones and all three types of treatment (clone and treatment interactions with gametocytaemia: $p > 0.1$ in all cases). The relationship between the proportion of mosquitoes infected and gametocytaemia was linear: the addition of the quadratic term to the untransformed data did not explain a significant amount of deviance ($p > 0.1$). When gametocytaemia had been controlled for, infectivity per gametocyte was reduced fivefold (at the mean gametocytaemia) in homologous challenges, compared with that in heterologous challenges and controls (figure 4, $\chi^2_1 = 15.31$, $p < 0.001$) which did not differ (figure 4, $\chi^2_1 = 0.73$, $p > 0.1$). Gametocyte infectivity was 2–3 times higher for clone ER than CR (main effect of clone: $\chi^2_1 = 6.8$, $p < 0.01$).

4. DISCUSSION

As in previous studies (McLean *et al*. 1982; Jarra & Brown 1985; Jarra *et al*. 1986; Bates *et al*. 1988), we found evidence of strain-specific immunity directed against asexual parasites (figure 2*a*). Immunization also reduced gametocyte densities, but there was no evidence that this reduction had a strain-specific component (figure 2*b*). Gametocyte densities were reduced fourfold, independent of whether the challenge was homologous or heterologous.

The proportion of mosquitoes infected increased with the gametocytaemia. But although the infectivity of a given number of gametocytes was not reduced by heterologous challenge (relative to controls), it was reduced fivefold in homologous challenge (figure 4). Thus, immunity affecting the infectivity of gametocytes, like immunity against asexual parasites, seems to be strain specific. Given that the total numbers of gametocytes were reduced similarly by both homologous and heterologous immunization, the overall transmission success of a particular parasite strain in a partially immune host is therefore determined by the relative infectivity of gametocytes.

How could differences in gametocyte infectivity between homologous and heterologous challenges arise? It would be surprising if an effective response could have been raised against (polymorphic) gametocyte or gamete antigens, given the short duration of the immunizing infection and the inevitably small numbers of gametocytes that would have been present. It is tempting to speculate that strain-specific anti-asexual immune factors cross-react with gametes in the mosquito midgut, thus blocking infectivity. Inactivation of gametes by humoral immune factors inside the vector is a well-known phenomenon (Sinden 1997), although the relevant gamete/gametocyte antigens are apparently not expressed by asexual parasite stages. However, recent work on *P. falciparum* by Hayward *et al*. (1999) indicates a candidate antigen: the antigenically variable and highly immunogenic PfEMP1, which is encoded by the *var* multigene family. *P. chabaudi* is likely to express similarly variable antigens (Phillips *et al*. 1997). Gametocytes transcribe the same *var* genes as the asexual parasites from which they originated. If PfEMP1 is exposed to humoral immune factors in the mosquito, strain-specific reductions in gamete survival are possible.

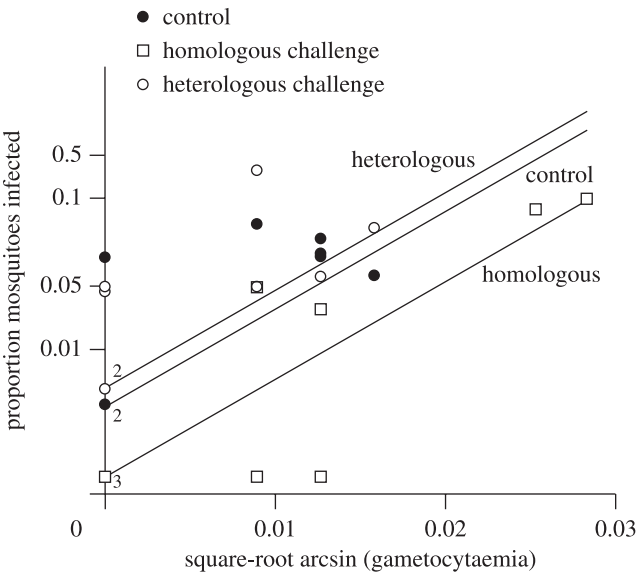

Figure 4. The relationship between the proportion of mosquitoes infected on a logit scale and square-root arcsine-transformed gametocytaemia on day 7 post-infection. Each point represents the infectivity of one mouse. Lines are ordinary least-squares regressions. Numbers in the graph show multiple data points.

In immunized and control animals, rates of gametocytogenesis increased towards the end of the infection (from day 11 pi, figure 3). Before this period, parasites had higher rates of gametocytogenesis in immunized than in control animals. These data support the hypothesis that conditions unfavourable for asexual growth result in increased gametocytogenesis: in all treatments, asexual growth decreased as the infection progressed, and asexual growth for the first 9 d of the infection was less in immunized than in control animals (figure 1*a*,*b*). This pattern was the same for both clones. However, the increase in gametocytogenesis in immunized animals during the early parts of the infection was insufficient to compensate for the effects of immunity: four times more gametocytes were produced in non-immunized animals.

The results from this study on *P. chabaudi* might have implications for understanding the epidemiology of human malaria and the effects of immune intervention. Partial immunity significantly reduced transmission, even though there were compensatory increases in gametocytogenesis, implying that even partially protective vaccines might be of value in reducing transmission. Strain-specific immunity might also have a large impact on transmission, not, however, as a consequence of strain-specific reductions in the densities of asexual parasites, but because of strain-specific reductions in gametocyte infectivity. Effective vaccine development must also take into account possible gametocyte and gamete antigenic polymorphisms.

Strong strain-specific reductions in transmission will also affect the population structure of *Plasmodium*, by reducing the average number of clones transmitted to mosquitoes and hence from mosquitoes back into new hosts. This has several potentially important implications, including further reductions in transmission (Taylor *et al*. 1997), and a decrease in the probability of parasites evolving resistance to multiple anti-malarial drugs (Mackinnon & Hastings 1998).

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