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THE IMPACT OF IMMUNIZATION ON COMPETITION WITHIN *PLASMODIUM* INFECTIONS

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Evolutionary theory argues that ecological interactions between pathogens within an infection can be a potent source of selection shaping traits such as virulence, drug resistance, and infectiousness. In humans, malaria infections are frequently genetically diverse, with mixed genotype infections the norm. A wide variety of evidence shows that crowding occurs within infections, with the population densities of individual genotypes suppressed by the presence of others. Public health interventions are expected to impact on levels of immunity experienced by pathogens, indirectly by reducing the rate of acquisition of natural immunity by reducing the force of infection, and directly in the case of vaccination programs. Here we ask how enhanced host immunity affects competitive interactions between malaria parasites within hosts and thus the strength of in-host selection on traits such as virulence. We used a model malaria system, *Plasmodium chabaudi* in laboratory mice, where it has been previously shown that less virulent parasites are competitively suppressed by more virulent strains, generating within-host selection for increased virulence. We found that immunization with either a recombinant antigen or with live parasites suppressed parasite densities, but that there was no evidence that immunization relieved or exacerbated competitive suppression, or affected the relative frequency of clones within infections. There is thus no reason to think that immunization strengthens or alleviates the potentially very potent selection on parasite traits arising from interactions between pathogen genotypes within infections.

KEY WORDS: **Malaria, multiple infection, *Plasmodium chabaudi*, virulence, within-host competition.**

For organisms living in a group-structured environment, within and between group selection shape the evolution of life-history traits (Frank 1998; West et al. 2006). This is particularly obvious in the case of pathogens, where there can be selection among pathogens within hosts as well as selection among parasites transmitting from different hosts. A substantial body of evolutionary theory has shown that within-host selection can be a highly potent source of selection on pathogen traits, including those of biomedical importance such as virulence, infectiousness, and drug resistance. For instance, it is frequently argued that virulent genotypes will be competitively superior within hosts, so that within-host selection will favor increased virulence (Levin and Pimentel 1981; Ewald 1983; Bonhoeffer and Nowak 1994; Nowak and May 1994; van Baalen and Sabelis 1995; Frank 1996; Mosquera and Adler 1998; Gandon et al. 2001a; Adler and Mosquera Losanda 2002). Similarly, if drug-resistant mutants are competitively inferior, competition with sensitive strains in untreated hosts will greatly strengthen selection against resistance. In contrast, removal of sensitive strains by drug treatment will result in competitive release, substantially enhancing the fitness

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of resistant lines above that due simply to surviving chemotherapy (Hastings and D'Alessandro 2000; de Roode et al. 2004a; Hastings 2006; Wargo et al. 2007a). The rate of spread of resistance, and hence the useful life span of a drug, is therefore greatly shaped by within-host selection.

Thus, key to understanding pathogen evolution is the social context in which parasites exist. How do parasites within hosts interact and if these interactions are competitive, what determines their strength and outcome? In the applied context, an important issue is whether animal and human health programs such as large-scale vaccination and mass chemotherapy affect within-host selection, and if they do, in which direction and at what rate key pathogen traits will consequently evolve. Here we experimentally investigate the effects of immunization on within-host competition in the rodent malaria model *Plasmodium chabaudi* in laboratory mice.

Human malaria infections frequently consist of more than one *Plasmodium* genotype (e.g., Babiker et al. 1991, 1999; Conway et al. 1991; Arnot 1998; Smith et al. 1999; Tanner et al. 1999; Jafari et al. 2004). Mixed infections arise from inoculations of genetically diverse parasites by a single mosquito, or from contemporaneous bites by multiple mosquitoes infected with different parasites. A substantial body of correlational epidemiological evidence is consistent with crowding effects within infections, where population densities of individual genotypes are suppressed when other genotypes are present (Daubersies et al. 1996; Mercereau-Puijalon 1996; Arnot 1998; Smith et al. 1999; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006). However, direct experimental evidence of crowding cannot be ethically obtained from humans infections because deliberate infections and/or comparisons with untreated infections are required.

In the rodent malaria model *P. chabaudi* in laboratory mice, strong crowding effects occur, with replicative and transmission stage densities of individual clones within an infection being severely suppressed when coinfecting strains are present (Jarra and Brown 1989; Snounou et al. 1989; Taylor et al. 1997; de Roode et al. 2003, 2004a,b, 2005a,b, Bell et al. 2006; Raberg et al. 2006). Removal of sensitive strains by chemotherapy leads to competitive release of resistant strains (de Roode et al. 2004a; Wargo et al. 2007a). Importantly, competitive suppression within hosts substantially reduces transmission of individual clones to mosquitoes (de Roode et al. 2005b). In general, avirulent clones suffer more from competition than virulent clones in this system (de Roode et al. 2005b; Bell et al. 2006), so to the extent that these results generalize to the field situation, within-host selection promotes the evolution of virulence.

Thus, correlated field evidence and direct experimentation with animal models point to strong competitive interactions within malaria infections. Changes in disease ecology, which alter the strength or frequency of these interactions could therefore affect pathogen evolution. In theory, widespread vaccination would have very significant effects on between-host selection, with major implications for the evolution of virulence (Gandon et al. 2001b). But immunization of individual hosts could also have major impact on competitive interactions within hosts, and hence on within-host selection (Read et al. 2002; Raberg et al. 2006; Barclay et al. 2008). The impact will depend on the mode of action of the vaccine. For instance, vaccines that target subsets of the malaria population, such as those parasites bearing antigens responsible for pregnancy-associated malaria (Gamain et al. 2007), would reduce competition by selectively removing some strains. However, most anti-malaria vaccines currently under development are intended to elicit strain-transcending immunity to protect hosts against all strains circulating in a population (Mahanty et al. 2003; Matuschewski 2006). Where the vaccines work by eliciting immunity against blood stages of malaria parasites, contrasting effects on in-host competition are likely, depending on the nature of the competition.

In principle, in-host competition can be immune-mediated, where increased densities of one parasite genotype elicit immune responses that negatively affect the densities of another (immune-mediated apparent competition; Read and Taylor 2001; Råberg et al. 2006). Experimental immune manipulations with *P. chabaudi* have revealed mixed evidence for a T-cell-dependent component to parasite competition. Depletion of CD4+ T-cells does not alleviate competition (Barclay et al. 2008), but competitive suppression is enhanced when immunodeficient mice are reconstituted with T-cells (Råberg et al. 2006). Enhancing T-cell-dependent immunity is a major aim of many candidate malaria vaccines (Good et al. 2005) and so these vaccines could strengthen within-host selection, and thus select for enhanced virulence. Consistent with this, serial passage of *P. chabaudi* through immunized hosts increases virulence more rapidly than does serial passage through immunologically naïve mice (Mackinnon and Read 2004).

Even if competition is mediated by nonimmunological mechanisms, alterations in host immune status could affect competitive outcomes. For instance, coinfecting malaria parasites may be competing over limiting resources such as red blood cells or glucose (Hellriegel 1992). If resources are limiting—and blood transfusion during peak parasitaemia has been shown to prolong *P. chabaudi* infection (Yap and Stevenson 1994)—suppression of parasite densities by immunization would likely ease such resource-based competition. Direct chemical warfare could also be occurring between clones, analogous to the allelopathic mechanisms seen in some viral and bacterial infections (Hart and Cloyd 1990; Riley and Wertz 2002). If such interference competition is occurring in malaria parasites (and there is no evidence either way), it is easy to envisage a variety of competitive outcomes that could occur if host immunity interferes with

the recognition, effector or resistance pathways that would be involved in chemical attack.

Rather than further analyze the mechanisms of competition, and then try to indirectly deduce the effects on vaccination on within-host competition, we used the *P. chabaudi* model to directly determine whether immunization exacerbates or alleviates competitive suppression, and hence its effects on the relative frequency of a focal clone in mixed clone infections. Immunity to malaria arises following natural infection with live parasites and, in animal models, following immune stimulation with recombinant parasite antigens. The vast majority of candidate malaria vaccines are based on recombinant parasite antigens, and some of these elicit at least a degree of protective immunity in humans (reviewed by Matuschewski 2006). In our experiments, we included immunization with live parasites to mimic the effects of parasite-induced immunity, and immunization with a recombinant antigen to mimic the effects of vaccine-induced immunity.

Materials and Methods

Table 1 summarizes the design and treatments of each of our three experiments.

PARASITES, HOSTS, AND PARASITOLOGY

Three genetically and antigenically distinct *P. chabaudi* clones were used in our experiments. All clones were originally derived from wild-caught thicket rats (*Thamnomys rutilans*) from the Central African Republic and are, so far as we know, unrelated clones because they came from different isolates (hosts) from different locations (Beale et al. 1978). In experiment 1, we used clones $AS(pyr1A)_{12055}$ and AJ_{4787}. Subscripts denote the precise point in the clonal histories from which they come. Clone AS (pyr1A) was derived through pyrimethamine selection of the original AS clone (Walliker et al. 1975; de Roode et al. 2005b). For simplicity AS (pyr1A) is referred to as AS hereafter. In experiments 2 and 3, we also competed AS against clone CB_{1155}. Clone CB was used in these experiments due to high sequence homology between AS and CB within the AMA-1 region present in the recombinant AMA-1 antigen used for immunization (Grech 2006). We included these two clones in the analogous live parasite immunization experiment for comparability. Previous work showed directly that both CB and AJ suppress AS in mixed infections, as expected because both are more virulent than AS and virulence is positively related to competitive ability (de Roode et al. 2005a,b, Bell et al. 2006).

In all experiments, hosts were *c*. 8-week-old C57BL/6J inbred female mice fed on 41B maintenance diet (Harlan, Blackthorne, UK). Their drinking water was supplemented with 0.05% para-amino benzoic acid to enhance parasite growth and they were kept in a 12L:12D cycle. Thin blood smears and red blood cell counts were used to determine the parasite densities (flow cytometry; Beckman Coulter, High Wycombe, UK). For all

Table 1. Experimental design and sample sizes. In experiments 1 and 2, immunization involved infection with live parasites or uninfected red cells (sham-inoculated controls), with all animals given curative chemotherapy four days later. In experiment 3, immunization involved inoculation of recombinant AMA-1 antigen in adjuvant, with a boost four weeks later. Control groups received only adjuvant at both time points. Most deaths were mice euthanized at predetermined levels of morbidity prescribed by animal care protocols (see text).

	Treatments	Immunization	Parasite challenge	Number of mice	Number of deaths
Experiment 1	1	Sham inoculated	AS	5	0
Live parasite immunization	2	Sham inoculated	AS+AJ	5	0
	3	AS	AS	5	0
	4	AS	AS+AJ	5	0
	5	AJ	AS	5	0
	6	AJ	AS+AJ	5	0
Experiment 2	1	Sham inoculated	AS	4	0
Live parasite immunization	2	Sham inoculated	AS+CB	5	0
	3	AS	AS	4	0
	4	AS	AS+CB	4	0
	5	CB	AS+CB	5	0
Experiment 3	1	DK antigen+adjuvant	AS	5	0
Recombinant antigen immunization	2	DK antigen+adjuvant	CB	6	3
	3	DK antigen+adjuvant	AS+CB	6	1
	4	Adjuvant	AS	5	1
	5	Adjuvant	CB	6	6
	6	Adjuvant	AS+CB	6	6
Total				86	17

experiments, parasite inoculations were prepared from donor mice by diluting blood in calf serum solution (50% heat inactivated calf serum, 50% ringer solution [27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl], 20 units of heparin per mouse). Each mouse was injected intraperitoneally, with a volume of 0.1 mL. Where necessary, mice were euthanized according to predetermined animal care protocols defined in conjunction with University of Edinburgh veterinarians and the UK Home Office.

STIMULATION OF IMMUNE RESPONSE

In experiments 1 and 2, mice were injected a dose of 10^4 parasites of either parasite clone AS, CB, or AJ. Four days after infection, all the mice were dosed orally with 20 mg/kg mefloquine using a lubricated catheter. This procedure was repeated over the next two days. Previous studies have shown that this protocol results in semi-immunity; immunizing infections allowed to run for longer generate near-sterilizing immunity (Buckling and Read 2001; Mackinnon and Read 2003, 2004). Thin blood smears were taken to ensure that the treatment had cleared the infection. Control mice received 10^4 red blood cells from a naïve hosts, followed by mefloquine as above.

In experiment 3, refolded recombinant AMA-1 derived from parasite clone DK was emulsified in the adjuvant Montanide ISA 720 (Seppic, Paris, France). Mice were injected intraperitoneally with 10 μg of protein in a 100 μl emulsion. Boost immunizations were conducted four weeks after the primary immunization. Control mice were injected with a 100 μl emulsion of PBS in Montanide ISA 720. This immunization protocol was adapted from Anders et al. (1998).

PARASITE CHALLENGE AND MONITORING OF INDIVIDUAL CLONE DYNAMICS

For experiments 1 and 2, mice were challenged four weeks after immunization with 10^6 parasites of either AS alone, AJ alone, CB alone, or a mixture of 10^6 AS + 10^6 AJ or of 10^6 AS + 10^6 CB (Table 1). For experiment 3, mice were challenged two weeks after recombinant antigen immunization with either 10^5 parasites of AS alone or CB alone, or a mixture of 10^5 AS + 10^5 CB. Thus in all experiments, mice infected with both clones received twice as many parasites as did those infected with one clone. This was so that we could directly compare the performance of an individual clone in the presence or absence of a coinfecting clone. Previous work has shown that a twofold difference in total parasites numbers has a negligible effect on parasite dynamics and virulence (Timms et al. 2001).

In all experiments, individual clones in mixed infections were monitored by collecting 5 μl samples of tail blood into citrate saline. After 1-min centrifugation at 13,000 rpm, the supernatant was removed and the pelleted blood was stored at −70°C for subsequent DNA extraction using Instagene Matric (BioRad, Hemel Hempstead, UK). Real-time quantitative PCR was used to measure the DNA concentration of both clones in these samples (Cheesman et al. 2003) with primers published elsewhere (Bell et al. 2006). Our real-time protocols cannot distinguish between replicative (asexuals) and transmission (gametocytes) stages. Less than 10% and most frequently less than 1% of total parasites are gametocytes (Taylor and Read 1997; Wargo et al. 2007b) so that the parasite counts we report very largely reflect the density of asexual parasites.

TESTING THE EFFECT OF IMMUNIZATION ON COMPETITIVE SUPPRESSION

We are concerned here with the question of whether immunization alleviates or exacerbates competitive suppression. Hence, the experiments (and analyses) focus on the performance of the competitively inferior clone (AS). Competitive suppression is defined as a reduction of parasite numbers when another strain is present, and we tested for this by comparing the performance of clone AS when another clone was present or absent. Thus, in all experiments, the density of clone AS was the response variable in the statistical analyses. If competition is occurring, AS densities will be lower in a mixed clone infections than in a single infection (tested with the term "multiplicity," a two-level factor describing the number of clones present in an infection). If competition is enhanced or alleviated in immunized hosts, this would appear as a significant interaction between immunization and multiplicity. To investigate whether competition was influenced by the genotype of the clone(s) used in the live parasite immunizations (experiments 1 and 2), we also analyzed immune animals only. Competition mediated by genotype-specific immunity would be seen as significant interactions between immunizing clone and multiplicity.

TESTING THE EFFECT OF IMMUNIZATION ON WITHIN-HOST SELECTION

To determine if the relative density of the two clones in mixed infections was affected by immunization, we compared the frequency of AS in the parasite populations in the mixed clone infections in naive and immunized animals. Alterations in the strength of within-host selection would be seen as significant differences between these treatment groups. To investigate whether the proportion of AS was influenced by the genotype of the immunizing parasites (experiments 1 and 2), we also analyzed immune animals only.

STATISTICAL ANALYSES

To meet normality and homogeneity of variance assumptions, all density data were logarithmically transformed [$\log_{10}$ (density +10)] and all proportion data were transformed using arcsine (sqrt). All analyses were performed as repeated measures using

PROC MIXED in SAS 8.2 (SAS Institute 1999) with the repeated statement (subject = mouse) and the Satterthwaite approximation of the denominator DF (Råberg et al. 2006). The main effects were immunization, day, and their interactions. For all analyses, maximal models including all two- and three-way interactions were fitted. Minimal models were then fitted by removing nonsignificant terms ($P > 0.05$), beginning with the highest-level interaction. In no cases were any of the three-way interactions significant, so we do not report them. The main effect of "day," as well as all two-way interactions with "day" are not reported as the well-known dynamic kinetics of malaria infections are not the focus of this study.

For the density data, all the repeated measures analyses were complemented with analyses of total parasite densities. Due to the 24-h replication cycle of *P. chabaudi*, summation of daily parasite counts allow the quantification of all of the parasites present during a particular period. These data were analyzed using General Linear Models (JMP in 5.1); we report only the multiplicity × immunization interaction terms for these analyses because (1) unlike the main effects, this term directly addresses the hypothesis under test, and (2) conclusions from the main effect terms in these analyses was always the same as those drawn from the corresponding terms in the repeated measures analyses.

As competitive effects are most intense during the acute phase (days 3–11) of the infection (de Roode et al. 2005b; Bell et al. 2006), we focus the statistical analysis on these days. After the acute phase, parasite densities are much lower, and there can be weak competitive suppression, no apparent interaction, and even weak facilitation, depending on the particular clones involved (e.g., Bell et al. 2006).

Results

IMMUNIZATION AND COMPETITION

Experiment 1: Live parasite immunization (clones AS and AJ)

Immunization and the presence of clone AJ both reduced the parasite densities of AS parasites (Fig. 1A,B; repeated measures analysis, immunization: $F_{1,44} = 37.4$, $P < 0.001$; multiplicity: $F_{1,44} = 11.8$, $P < 0.001$). However, the extent of the competitive suppression was similar in immunized and sham-inoculated hosts, with AS densities reduced by comparable amounts (Table 2,

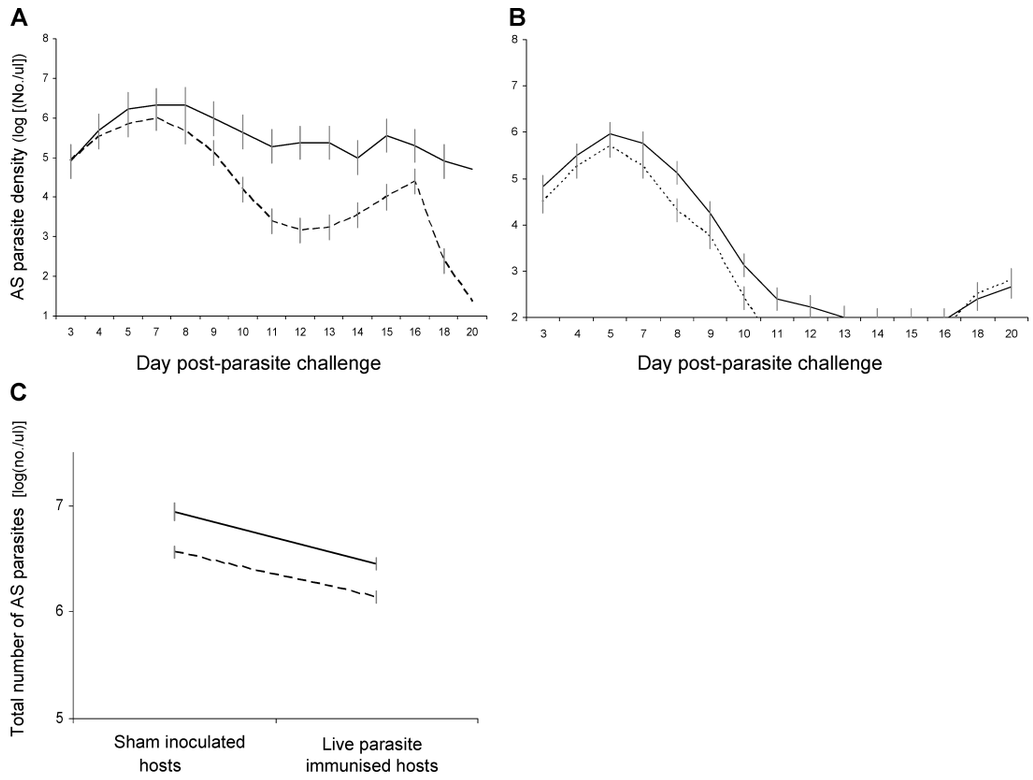

Figure 1. Density of clone AS parasites over time in experiment 1 in sham-inoculated hosts (A) or host immunized with live parasites of either type (B), and the total number of AS parasites present during the acute phase (C). In all panels, solid lines denote densities when the competitor clone (AJ) was absent and dotted lines denote densities when it was present; the magnitude of competition is the area between the solid and dashed lines. Plotted points are the average parasite density (least-squares mean ± SE from analysis) of up to five mice.

Table 2. Tests for an effect of immune status on the extent of competitive suppression of the parasite clone AS. Reported values are for the host immunity × multiplicity interaction terms from repeated measures analysis (middle column) and acute phase total parasite density analyses (right column). Multiplicity is the number of clones per host (1 or 2). Lack of significant immunity × multiplicity interactions indicates that there was no evidence that the level of competition was affected by the immune status of the host.

	Repeated measures	Total parasite density
Experiment 1 (AS+AJ)		
Immunized vs. sham inoculated	$F_{1,44.5}$=0.39, P=0.53	$F_{1,30}$=0.26, P=0.61
Immunized hosts only	$F_{1,37.9}$=1.04, P=0.32	$F_{1,16}$=1.3, P=0.26
Experiment 2 (AS+CB)		
Immunized vs. sham inoculated	$F_{1,36.9}$=0.91, P=0.34	$F_{1,19}$=1.26, P=0.27
Experiment 3 (AS+CB)		
Vaccinated vs. sham inoculated (including 2 low-level infections)	$F_{1,28.9}$=1.1, P=0.31	$F_{1,17}$=0.56, P=0.43
Vaccinated vs. sham inoculated (excluding 2 low-level infections)	$F_{1,21.2}$=1.1, P=0.31	$F_{1,15}$=0.92, P=0.35

Fig. 1C; 58% and 51% respectively). Thus, there was no evidence that competitive suppression was exacerbated or alleviated by immunization.

Considering immunized hosts only (Fig. 2), the parasite density of clone AS was influenced by the presence of the coinfecting clone, but not the genetic identity of the immunizing clone (Fig. 2: multiplicity; $F_{1,39.1} = 5.78$, $P = 0.02$, immunizing clone; $F_{1,39} = 0.07$, $P = 0.78$, respectively). The competitive suppression of AS was slightly less severe in the face of heterologous immunity (i.e., when AJ was the immunizing clone) than when it was

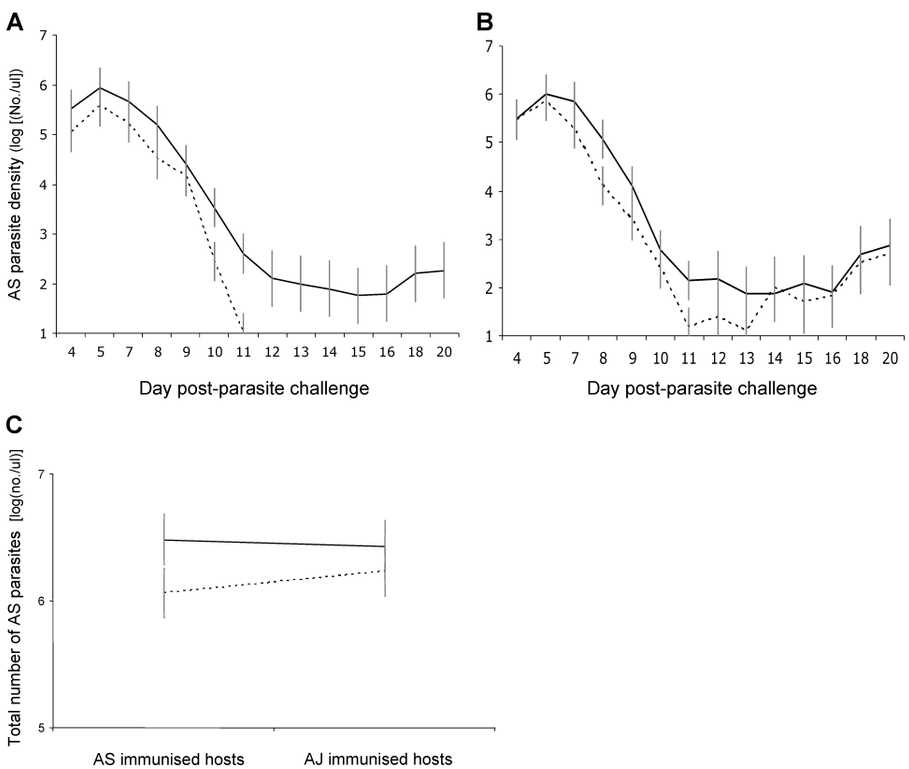

Figure 2. Density of clone AS parasites over time in experiment 1 in hosts immunized with live AS parasites (A) or with live AJ parasites (B), and the total number of AS parasites present during the acute phase of those infections (C). In all panels, solid lines denote densities when the competitor clone (AJ) was absent and dotted lines denote densities when it was present; the magnitude of competition is the area between the solid and dashed lines. Plotted points are the average parasite density (least-squares mean ± SE from analysis) of up to five mice.

homologous (i.e., when AS was the immunizing clone), but this difference (37% vs. 62% respectively) was not statistically significant during the acute phase of infection (Table 2). During the chronic phase, AS was competitively excluded in mice that had been immunized with AS parasites (below detection thresholds at least), whereas in AJ-immunized mice, AS was competitively suppressed but not excluded (Fig. 2).

Experiment 2: Live parasite immunization (clones AS and CB).

As with experiment 1, immunization reduced AS densities, and these densities were further suppressed when the other clone was present (Fig. 3; multiplicity; $F_{1,36.3} = 5.4$, $P = 0.028$, immunity $\times$ day; $F_{7,111} = 10.4$, $P < 0.001$). However, the magnitude of this competitive suppression was similar in both sham-inoculated (90%) and immunized (65%) hosts (Table 2). Thus there was also no evidence from this experiment that immunization enhanced or alleviated competition.

Experiment 3: Recombinant antigen immunization (clones AS and CB)

During the experiment, 17 of 34 mice died or had to be euthanized (Table 1), after their parasite densities had peaked. We can offer no explanation for these mortality rates. They were substantially higher than normal in experiments of this sort (e.g., Mackinnon and Read 2003; de Roode et al. 2005; Bell et al. 2006), and marked different from what we found in experiments 1 and 2 (Table 1).

One of the deaths was a sham-immunized host infected with the normally avirulent clone AS. This mouse also had an uncharacteristically low parasite density, so was removed from all analyses. All other mice that died are included in relevant analyses because their parasite densities had peaked prior to death. In addition, two of the five vaccinated hosts infected with the less virulent parasite clone AS had extremely low level parasite densities (maximum of 2×10^4 parasites/μl), two orders of magnitude lower than we normally observed (e.g., Mackinnon and Read 2003; Bell et al. 2006) and substantially lower than the remaining three mice in this group (maximum of 2×10^6 parasites/μl). To provide a conservative analysis, we therefore report the following results with these two mice both included and excluded.

With those two mice in the analyses, recombinant antigen immunization reduced the densities of AS parasites (Fig. 4A,B,D: repeated-measure analysis, recombinant antigen immunization: $F_{1,31.1} = 7.3$, $P = 0.01$), but the presence CB parasites did

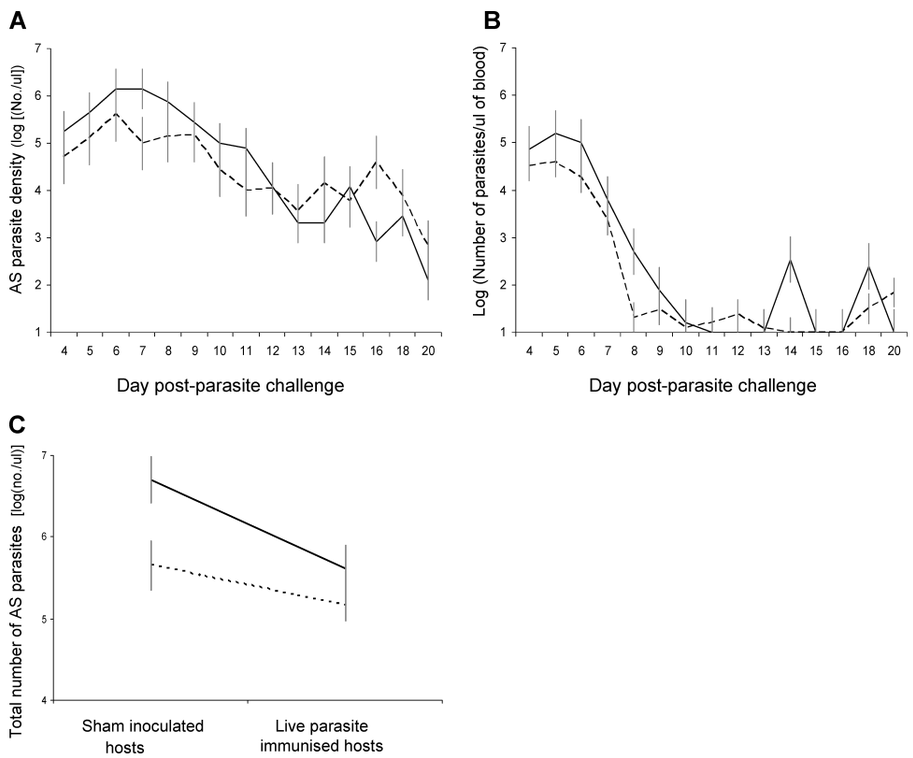

Figure 3. Density of clone AS parasites over time in experiment 2 in sham-inoculated hosts (A) or host immunized with live parasites (B), and the total number of AS parasites present during the acute phase (C). In all panels, solid lines denote densities when the competitor clone (CB) was absent and dotted lines denote densities when it was present; the magnitude of competition is the area between the solid and dashed lines. Plotted points are the average parasite density (least-squares mean ± SE from analysis) of up to five mice.

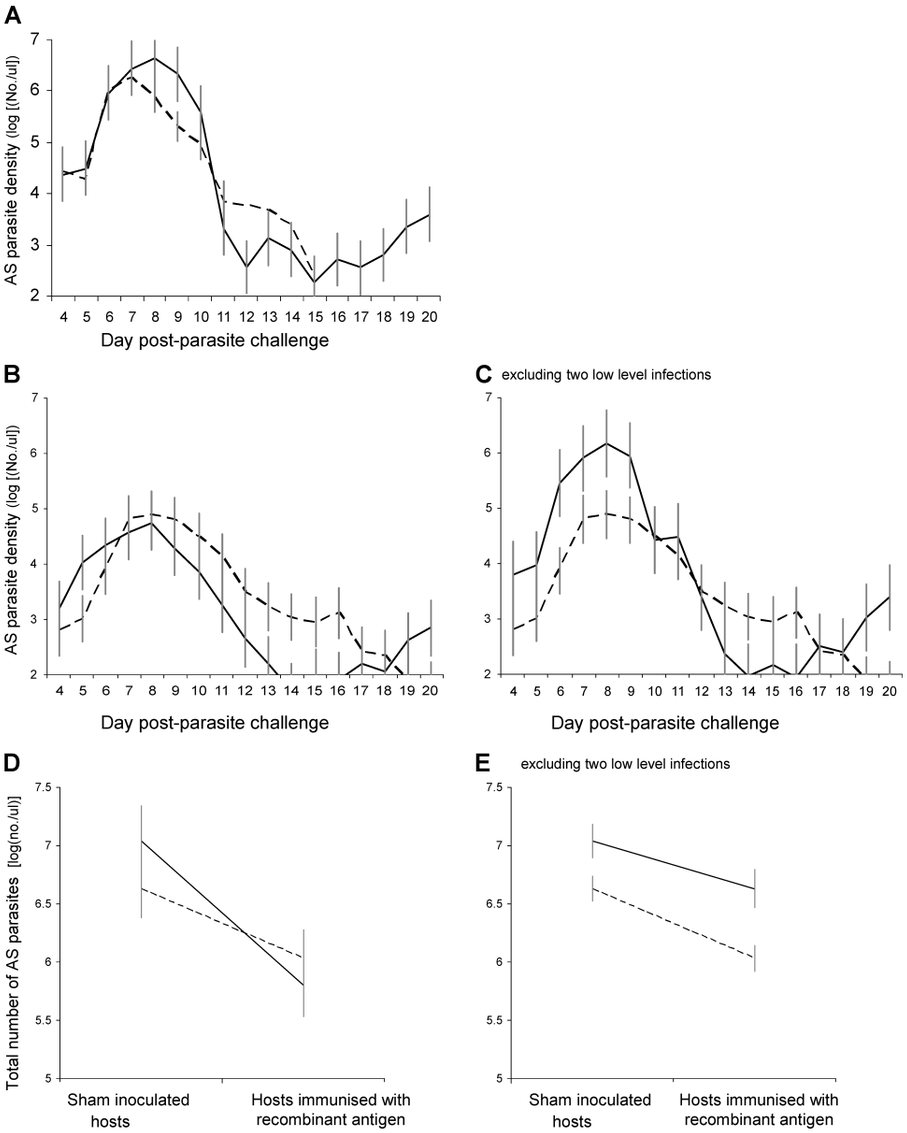

Figure 4. Density of clone AS parasites over time in experiment 3 in sham-inoculated hosts (A) or host immunized with recombinant antigen (B, C), and the total number of AS parasites present during the acute phase (D, E). In panels C and E, the abnormally low-density single-clone infections are excluded (see text for further details). In all panels, solid lines denote densities when the competitor clone (CB) was absent and dotted lines denote densities when it was present; the magnitude of competition is the area between the solid and dashed lines. Plotted points are the average parasite density (least-squares mean ± SE from analysis) of up to six mice.

not (multiplicity: $F_{1,29.6} = 0.22$, $P = 0.64$; multiplicity × immunization interaction n.s.; Table 2). However, there was evidence of competitive suppression when the two mice with perversely low density parasitemias were excluded from the analysis: AS densities were reduced by immunization and by the presence of a competing clone (Fig. 4C,E : vaccine × day; $F_{7,87.1} = 6.1$, $P < 0.0001$, multiplicity; $F_{1,23} = 4.5$, $P = 0.045$). There was no evidence that the extent of competitive suppression was affected by immunization (Table 2): competition reduced parasite densities in sham-inoculated hosts by 60% and host immunized with the recombinant antigen by 68%. Thus, as with the other two experiments, there was no evidence that competitive suppression was exacerbated or alleviated by immunization.

Of the three experiments, only this one was designed to test for the effect of competitive suppression and immunization on the competitively dominant clone. Recombinant antigen immunization reduced the density clone CB, but the presence of AS did not (recombinant antigen immunization: $F_{7,26.9} = 7.9$, $P = 0.009$, multiplicity; $F_{1,26.4} = 2.3$, $P = 0.14$, recombinant antigen immunization × multiplicity; $F_{1,25.4} = 1.5$, $P = 0.22$). Thus, there

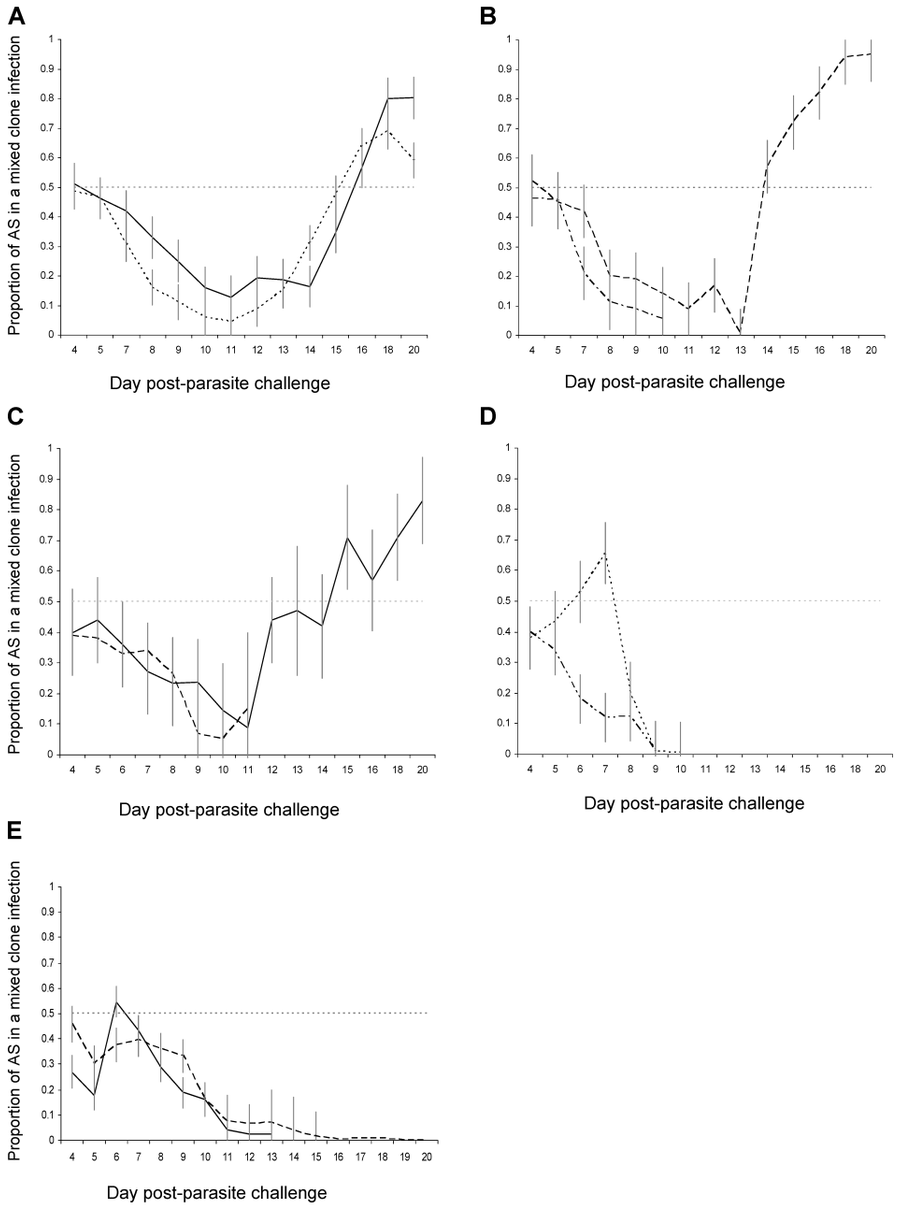

Figure 5. **Proportion of AS parasites within mixed clone infections: (A) sham-inoculated (solid line) and live parasite immunized mice (dotted line) in Experiment 1; (B) AS-immunized (dotted and dashed line) and AJ-immunized hosts (dashed line) in Experiment 1; (C) sham-inoculated (solid line) and live parasite immunized hosts (dotted line) in Experiment 2; (D) AS-immunized hosts (dotted and dashed line) and CB-immunized (dotted line) in Experiment 2; and (E) sham inoculated (solid line) and recombinant antigen immunized hosts (dashed) in Experiment 3. All hosts immunized with parasite clone AS were parasite negative for parasite clone AS after day 10. The horizontal gray dashed line shows the 50% frequency at inoculation. Plotted points are the average proportion of AS in a mixed infection (mean ± SEM).**

was no evidence that immunization with recombinant antigen led to competitive suppression of the dominant clone.

IMMUNIZATION AND WITHIN-HOST SELECTION

In the mixed infections, the frequency of AS in the parasite populations did not differ between immunized and naive hosts in any of the experiments (Fig. 5, Table 3). Among the immunized hosts in experiments 1 and 2, there was some suggestion of frequency differences associated with the identity of the immunizing clone, with AS being somewhat rarer in AS-immunized hosts. However, given that these effects were weak ($P = 0.06$, $P = 0.08$, respectively; Table 3), and that there was no evidence that immunization

Table 3. The effect of host immunity on the frequency of clone AS in mixed-clone infections.

Experiment 1 (AS+AJ)	
Immunized vs. sham inoculation	$F_{1,17.7}$=2.0, P=0.17
Immunized hosts only	$F_{1,6.9}$=4.88, P=0.06
Experiment 2 (AS+CB)	
Immunized versus sham inoculation	$F_{1,8.4}$=0.02, P=0.89
Immunized hosts only	$F_{1,6.4}$=4.2, P=0.08
Experiment 3 (AS+CB)	
Recombinant antigen immunization vs. sham inoculation	$F_{1,9}$=0.64, P=0.44

per se had an effect ($P > 0.15$ in all cases, Table 3), we conclude that there was no evidence that immunization affected the strength of within-host selection imposed by coinfection.

Discussion

In all three of our experiments, the population densities of the focal clone (AS) during acute phase infections were suppressed by immunization, and by the presence of a coinfecting parasite clone. But in none of the experiments was there evidence that immunization alleviated or exacerbated the competitive suppression (Figs. 1–4), or that it altered the clonal composition of mixed infections (Fig. 5). Given that the frequency of clones in mixed infections predicts transmission success in this system (Taylor and Read 1998; de Roode et al. 2005b), and given the normal provisos about extrapolating from animal models to field conditions (reviewed in this particular context by Råberg et al. 2006), our results suggest that immunization has negligible effects on the potent selection on parasite traits coming from competition within hosts. Most importantly from a practical perspective, there is no evidence that enhancing standing immunity by vaccination will increase the within-host selection that favors more virulent parasites: the relative disadvantage of the focal clone was maintained irrespective of the immune status of the host. Other interventions that alter host immune status by reducing the force of infection, and hence rate of acquisition of naturally acquired immunity, should similarly have negligible impact on this source of selection on pathogens. These other interventions include bednets, improved housing, drainage, mass chemotherapy, and transmission- and infection-blocking vaccines. Thus, we expect none of these interventions to alter the strength of selection within mixed infections in favor of virulence.

Our finding that immunity does not affect the selective consequences of the parasites' social environment supports at least one simplification in this area of evolutionary epidemiology, an area renown for complexity. For instance, it justifies the assumptions of some models of population-level processes that host immunity does not affect the within-host selection occurring in mixed infections (e.g., Hastings and D'Alessandro 2000; Gandon et al. 2001a; Porco et al. 2005; Hastings 2006). In the context of drug resistance evolution, for example, the useful life span of a drug is determined by the rate of spread of resistance; this in turn depends on the fitness costs and benefits of resistance, which are substantially affected by within-host interactions. Costs of resistance are exacerbated in mixed infections if resistance is associated with reduce competitive ability; drug treatment greatly enhances the relative fitness of resistance strains if removal of susceptible strains lead to competitive release within hosts (Hastings 2006). Our experiments suggest that immunization will not interact with at least these particular components of selection on resistance.

It is unclear why immunization had little impact on competitive suppression. Råberg et al. (2006) compared the extent of competition in immunodeficient mice and immunocompetent mice. Immunodeficient mice were "nude" mice that have a recessive mutation that blocks the development of the thymus. These mice have no mature T-cells and are unable to mount cell-mediated immune responses or form most types of antibodies. The immunocompetent animals used for comparison were nude animals experimentally reconstituted with T-cells. These experiments showed immune-mediated competitive suppression between days 10 and 12 postinfection. However, in more recent experiments, there was no evidence of immune-mediated competition in normal animals rendered CD4+ T-cell deficient by antibody depletion (Barclay et al. 2008). This could mean that CD8+ T-cells are involved, but the experiments we report here, which involve comparisons of naive and immune-enhanced animals also showed no evidence of immune-mediated competition; it may be that the relatively small immune-mediated component to competition revealed in Råberg et al.'s experiments is swamped out in intact animals.

Alternatively, the result we report here could be a Type II error. Mackinnon and Read (2004) found that virulence evolution proceeded more rapidly when a single clone of *P. chabaudi* was serially passaged through live-parasite immunized hosts compared with hosts that had not previously seen malaria parasites. A leading explanation for this observation is that more virulent mutants were more strongly favored in immunized hosts because they have a fitness advantage in the face of immune-mediated competition. A small advantage could be difficult to detect in single generation experiments like those reported here and by Barclay et al. (2008), yet over the course of several serial passages, the advantage could cumulate to the point of detectability. It is very difficult to rule this possibility out. A quantitative estimate of the magnitude of any such advantage that would be consistent with the null results reported here would require an estimation of confidence intervals for an effect size for the competition × immunity interaction term summed over our three experiments. We nonetheless doubt

that our conclusion is a false negative: the three experiments here together contained more than twice the number of mice used by Raberg et al. (2006), and no trend is visually detected in any of the three experiments for either total parasite densities, or relative frequencies (Figs. 1–5). Perhaps immune-mediated competition is more critical when antigenically very similar parasites are competing, as will be the case for any mutants that arise de novo in clonal infections like those studied by Mackinnon and Read (2004).

Even so, it remains unclear as to why immunization—which reduced parasite densities—did not detectably alter competition in the experiments reported here. Perhaps resource-based competition is only loosely density-dependent, or that there is immune-mediated competition, but its importance relative to resource-based competition is altered by immunization in such a way that the two effects more or less cancel out, resulting in little change in competitive suppression.

Immunity against malaria parasites has strain-transcending and strain-specific components (e.g., Martinelli et al. 2005). Strain-specific immunity (SSI) by definition acts more strongly against homologous challenge than heterologous challenge, and thus has the potential to relieve competitive suppression when directed against a competitor. During the acute phase of primary infections in naïve animals, it is generally assumed that the immune response is largely nonspecific, involving a general cellular immune response (Taylor-Robinson 1995; Li et al. 2001). In contrast, during the chronic phase of the infection it is thought that strain-specific mechanisms become important, principally antibody-mediated responses (Jarra and Brown 1985; Phillips et al. 1997; Mota et al. 1998; Buckling and Read 2001). This is seen as reduced competition in chronic phases in *P. chabaudi* (Bell et al. 2006). In experiment 3, our choice of clones with high sequence homology at the immune target of the recombinant antigen was designed to minimize SSI as a complexity. Our other two experiments were not designed to test for SSI, but there were some hints that it occurred. In experiment 1, AS was excluded during the chronic phase in homologously but not heterologously immunized mice. In experiment 2, only one of the nine immunized hosts infected with a mixed infection was parasite positive. This host had been immunized with the parasite clone CB and was positive for the parasite clone AS, suggestive of CB-specific immunity and in agreement with many previous observations (Jarra and Brown 1989; Snounou et al. 1992; Martinelli et al. 2005; Cheesman et al. 2006). If our live parasite immunizations were indeed generating a component of SSI, this was insufficient to detectably relieve competition. If vaccination or naturally acquired immunity in the field has an SSI component, this will tend to alleviate competition, further reinforcing our conclusion that vaccination will not strengthen within-host selection for virulence arising from parasite competition.

Our data support both theory and experimental data that mixed genotype infections select for increased virulence, and show that this process works similarly in both naïve and immune environments. Nonetheless, widespread vaccination could affect the social evolution of malaria parasites in other ways. Importantly, vaccination and a range of other interventions can reduce transmission. This will decrease the number of clones per host and thus the potential for within-host competition. However, our results suggest that when coinfection does occur, immune-induced changes in the strength of competition will not be an overwhelming complexity in the analysis of the social evolution that will subsequently take place (Adler and Mosquera Losanda 2002; Galvani 2003).

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