

Published in final edited form as:

*Parasite Immunol.* 2011 January ; 33(1): 73–78.

## Strain-specific immunity induced by immunization with pre-erythrocytic stages of *Plasmodium chabaudi*

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### SUMMARY

One of the most promising approaches in the efforts to produce a malaria vaccine involves the use of attenuated whole sporozoite immunizations. Attenuation may be achieved by the use of genetic modification, irradiation, chemical attenuation, or by the contemporaneous administration of antimalarial drugs that target only the erythrocytic stages of the parasite. Most research to date has focused on the efficacy of these approaches upon challenge with parasites homologous to those used for the initial immunizations. We, as have others, have previously shown that a component of the immunity achieved against the erythrocytic stages of the rodent malaria parasite *Plasmodium chabaudi chabaudi* is strain-specific, with a stronger immune response targeting the immunizing strain than genetically distinct strains. Here, we show that the immunity induced by infection with the pre-erythrocytic stages of these parasites, achieved via inoculation of sporozoites contemporaneously with mefloquine, also has a strain-specific component.

### Keywords

*Plasmodium chabaudi*; rodent malaria parasites; sporozoites; strain-specific immunity

### INTRODUCTION

Antigenic variation between strains of malaria parasites has been shown to lead to the phenomenon of 'strain-specific immunity' in which the protective effect of exposure-induced immunity, both in the laboratory (1) and in the field (2), is stronger against a homologous strain than against genetically distinct heterologous strains. One of the most comprehensive studies of this phenomenon to date was conducted using the rodent malaria parasite *Plasmodium chabaudi chabaudi*, for which it was shown that the major genetic determinant of the strain-specificity of the immunity achieved *via* immunization with blood-stage parasites is the merozoite surface protein 1 gene (*mSP1*) (3). Natural malaria infections of both rodents and humans are initiated by the bite of malaria parasite-infected *Anopheles* mosquitoes, which inoculate sporozoites into the skin during blood feeding. Very effective

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Disclosure: None

protective immunity against malaria can be achieved by immunization with sporozoites that have been attenuated by irradiation (4). More recently, other methods of sporozoite attenuation such as genetic modification (5) and chemical attenuation (6) have also been shown to confer protective immunity against re-infection. A similar approach in which live sporozoites are inoculated contemporaneously with anti-erythrocytic stage drugs such as chloroquine (CQ) has recently been shown to confer sterile protective immunity against *Plasmodium falciparum* in human volunteers (7). The protective efficacies of these vaccine strategies have, most commonly, been assessed using parasites homologous to the vaccinating strain. Those few studies which have assessed the level of protection against heterologous challenge have almost exclusively assessed the degree of cross-protection between malaria parasite species (8-15) and are generally inconsistent in their conclusions. Should it occur, parasite strain-specificity to the induction of immunity by live sporozoites of *P. falciparum* will need to be understood if such vaccination is to be used effectively. Here, we present the results of experiments to test for and determine the degree of cross protection between strains of *Plasmodium chabaudi* immunized by inoculation of live sporozoites in conjunction with mefloquine (MF) treatment.

## MATERIALS AND METHODS

All experiments were carried out in compliance with the British Home Office Animals (Scientific Procedures) Act 1986. For sporozoite immunizations, two groups of 20 inbred female CBA/Ca mice (6 weeks old at the time of first immunization) were inoculated *via* intraperitoneal (IP) injection with known numbers of sporozoites of *P. c. chabaudi* clones AJ or CB diluted in a 50 : 50 mixture of Foetal Calf Serum (FCS) and Ringer's solution contemporaneously with oral MF treatment (20 mg/kg/day for 5 days). Immunizations were performed twice with an interval of 3 weeks between inoculations. Each mouse received an inoculation of ~400 sporozoites of each strain in the first immunization, and ~2000 in the second. Twenty control mice were inoculated with 50 : 50 FCS: Ringer's solution only, and also drug treated. Five weeks following the second immunization, mice were each challenged IP with 2400 sporozoites of either strain, or with  $1 \times 10^6$  parasite-infected red blood cells (iRBCs). Immunized and mock-immunized control mice were separated into groups of five and received either a homologous or heterologous challenge strain of sporozoites or iRBCs. The mock-immunized group that received an AJ challenge were reduced to two mice in the group because of a technical error during challenge. The resulting blood-stage infections were followed by microscopic examination of Giemsa's solution-stained thin blood smears taken daily using venous blood from the tail. In order to determine the day at which parasites first became detectable in the blood, at least 10 000 red blood cells were examined per smear. For the generation of sporozoites, *Anopheles stephensi* mosquitoes were allowed to feed on anaesthetized mice that had been inoculated with  $1 \times 10^6$  iRBCs IP 6 days previously. Prior to feeding, mouse blood was checked for the presence of gametocytes, and their viability assessed by the observation of exflagellation of microgametocytes in fresh blood preparations. Seven to 10 days post-feed, mosquito mid-guts were dissected and the presence of oocysts confirmed. Sixteen days post-feed, mosquito salivary glands were dissected into a 50 : 50 solution of FCS and Ringer's solution, crushed in a glass and Teflon tissue homogeniser, and the numbers of sporozoites in the homogenate assessed by counting with a haemocytometer. In order to assess sporozoite viability, only those sporozoites displaying circular gliding motility were considered viable. There were no discernable differences in the viability of CB and AJ sporozoites, and sporozoites of both strains were handled in exactly the same manner prior to immunization and challenge inoculation. All mice were kept on 0.05% para-aminobenzoic acid (PABA)-supplemented water *ad libitum* and were housed at 21°C on a 12 h-light-dark cycle. *Anopheles stephensi* mosquitoes were fed with 0.05% PABA-supplemented 10% glucose solution and were housed at 27°C and 70% humidity on a 12-h light-dark cycle. We used R version 2.7.0; The

R Foundation for Statistical Computing; <http://www.R-project.org>) for data analysis. To analyse patterns of parasitaemia during infections, we used mixed effects models because, by treating each infection as a ‘random’ effect, we can account for repeated measures from each infection and overcome pseudoreplication problems associated with such data. These models were fitted with Poisson error distributions and minimized following stepwise deletion of the least significant term, using log-likelihood ratio tests to evaluate the change in model deviance, until only significant terms remained. We present  $F$ -ratios for fixed effects remaining in minimal models. Mann–Whitney tests were used to compare patency data. Cumulative, summary data were analysed with linear models, using  $F_{ANOVA}$  ( $F$  ratios) to evaluate significance of terms.

## RESULTS

The days on which parasites became detectable by microscopy (patent) in the blood of mice subjected to various immunization and challenge regimens are shown in Table 1. In mice that had been immunized with the CB strain by inoculation with live sporozoites contemporaneously with MF administration, the onset of patent parasitaemia following homologous strain (CB) sporozoite challenge was delayed by around 2 days relative to an equivalent CB sporozoite challenge in mock immunized mice ( $P = 0.017$ ) (Table 1, cf rows 1 and 3). The prepatency period for CB immunized mice was significantly greater in the CB sporozoite-challenged group compared to AJ sporozoite-challenged group ( $P = 0.010$ ) (Table 1, cf rows 1 and 2). This suggests that live sporozoite immunization under MF drug cover with the CB strain induced a strain-specific, anti-parasitic immunity against homologous CB sporozoite-induced infection, and that this anti-parasitic immunity was already acting before the appearance of a patent blood infection.

In mice immunized with AJ strain sporozoites using the same protocol as described earlier and subsequently challenged with sporozoites of either CB or AJ, blood-stage parasites of both strains tended to appear even earlier following equivalent challenge in naïve mice (Table 1, cf rows 5 and 3, and cf rows 6 and 4), although this effect was not statistically significant (homologous (AJ sporozoite) challenge vs. mock immunized,  $P = 0.143$ ; homologous (AJ sporozoite) challenge vs. heterologous (CB sporozoite) challenge,  $P = 0.403$ ).

The course of blood infections in both sporozoite-induced and blood-stage parasite-induced infections in sporozoite-immunized and in mock-immunized control mice are shown in Figure 1. The infection dynamics reveal that sporozoite challenges result in significantly lower parasitaemias than blood-stage challenges ( $F_{1,50} = 21.96$ ;  $P = 0.0001$ ). Immunization with CB reduced parasitaemias of challenge infections significantly more than AJ immunization for both challenge strains ( $F_{2,50} = 29.28$ ;  $P = 0.0001$ ). Moreover, the reduction in parasitaemias following CB immunization were greater for homologous challenges ( $F_{2,50} = 6.05$ ;  $P = 0.004$ ), but this was not the case following immunization with AJ.

Specific comparisons of the cumulative proportion of parasitized red blood cells for each type of challenge with its mock-immunized control group supported these findings for the effects of immunization. For CB sporozoite challenge, CB immunization strongly reduced parasitaemias but those achieved following AJ immunization were not significantly different to mock-immunized control infections (Figure 1a;  $F_{2,11} = 8.69$ ;  $P = 0.005$ ). For AJ sporozoite challenge, there was a similar trend in which the lowest parasitaemias were reached after CB immunization (Figure 1b;  $F_{2,8} = 0.01$ ;  $P = 0.009$ ). For CB blood-stage challenge, CB immunization strongly reduced parasitaemia and a slight reduction was achieved following AJ immunization (Figure 1c;  $F_{2,12} = 70.57$ ;  $P = 0.0001$ ). For AJ blood-

stage challenge, CB immunization strongly reduced parasitaemia relative to that in the mock immunised mice, but parasitaemias achieved following AJ immunization were not significantly different to mock-immunized control infections (Figure 1d;  $F_{2,12} = 10.82$ ;  $P = 0.002$ ).

## DISCUSSION

Immunization of female CBA mice by infection with live sporozoites of a single strain, CB or AJ, of the malaria parasite *P. c. chabaudi*, under the cover of the anti-bloodstage antimalarial drug, MF, induced responses that were variously effective before and/or during patent blood infection following challenge with either sporozoites or blood-stage parasites of one or the other of these two strains of parasite. The effects of immunization with live sporozoites under MF cover included strain-specific suppression of pre-patent parasite growth (CB sporozoite-immunization suppressed pre-patent parasite growth in CB sporozoite-induced infections but not in those of AJ sporozoite-induced infections); strain-specific suppression of patent erythrocytic parasite growth (CB sporozoite-immunisation suppressed blood-parasite growth in sporozoite- and blood parasite-induced infections of CB more than it did to growth of blood parasites in corresponding AJ infections; AJ sporozoite-immunized mice partially suppressed growth of AJ blood parasites in sporozoite- and blood parasite-induced infections but did not suppress growth of CB blood parasites); pan-strain suppression of patent erythrocytic parasite growth (CB sporozoite-immunization suppressed growth of erythrocytic parasites in sporozoite- and blood parasite-induced infections of both AJ and CB).

It should also be noted that the parasites showed strain-specificity, or its absence, in their immunological properties not only as targets of immunity but also as inducers of immunity. While both AJ and CB were involved in the induction of strain-specific immunity against the blood-stage parasites, only CB, and not AJ, live sporozoite immunization induced powerful pan-strain effects in suppressing blood-stage parasites. Such strain-specific properties of the induction of immunity against blood-stage parasites have been recorded previously among strains of *P. c. chabaudi* (1). The two strains differed also in the immunity they induced against the parasites pre-blood patency. Experiments testing whether strains such as CB induce pan-strain immunity through broader antigen repertoire and whether this is linked to lower parasite densities in control infections are now required. Quantifying variation in strain-specificity and explaining the underlying mechanisms are central to predicting the success of interventions that work by inducing immunity.

It is conceivable that differences in the viabilities of CB and AJ sporozoites may have contributed to some of the effects observed in this study, as this would result in the development of differing numbers of exo-erythrocytic stage parasites for each strain during the immunization procedure. However, we found no evidence for any differences in viabilities when assessing sporozoite motility prior to inoculation. Nevertheless, as viabilities were not directly assessed, differences between strains remain possible.

The most significant findings of this study are, we suggest, the following. We show that immunity induced by sporozoites under a drug cover that should prevent development of the parasites in the blood, has a marked strain-specific component against both sporozoite and blood-stage parasite challenge. The strain-specific effect appears to apply to the parasites in sporozoite-induced infections at a stage before they are detectable in the blood by conventional blood smear microscopy. This could be because there is strain-specific anti parasite immunity acting against the parasites in the liver. However, our results also clearly show that strain-specific immunity is acting against the blood stage parasites themselves. We suggest that this anti-blood stage immunity arises either through the expression of

antigens common to blood-stage parasites during exo-erythrocytic schizont development, as was shown previously for MSP1 (16), or by the exposure to the immune system of the exo-erythrocytic merozoites which are released, and invade red blood cells, before being killed by the effect of MF in our immunization protocol. Each *P. chabaudi* liver schizont is believed to release in the order of 20 000 merozoites (17). As the immunizing inocula in the present experiments probably delivered many tens, at least, of sporozoites successfully to the liver (based on an evaluation of the IP route for sporozoite inoculation, Inoue & Culleton, unpublished data), each sporozoite immunization under MF would probably have resulted in the release of the order of at least  $10^5$  blood stage merozoites. This, we suggest, is a likely basis for the induction of the immunity, both pan- and strain-specific, that we observed against the blood-stage parasites in these experiments.

The protective immunity achieved *via* immunization with both irradiation attenuated and genetically attenuated sporozoites is thought to be mediated mainly through CD8<sup>+</sup> T-cell responses, at least for the *Plasmodium berghei* and *Plasmodium yoelii* parasites (18). There is also evidence for the involvement of other immune mechanisms in these systems, including those involving B cells, CD4<sup>+</sup> T-cells and NK cells (19–21). When immunizations are performed with live sporozoites under the cover of anti-blood stage chemoprophylaxis, as in our study, it appears that both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells are involved in the protective effect achieved, and there is little evidence for the role of antibodies (22). However, these experiments were performed with *P. yoelii* parasites, and it is possible that protective mechanisms differ between parasite species (10,23). The two *P. c. chabaudi* strains used in this study, AJ and CB, have previously been shown to differ considerably at the nucleotide level (24). This genetic diversity incorporates known antigen genes, such as MSP1 (25), which elicit strongly strain-specific immune responses (3). It is, therefore, highly likely that antigenic proteins expressed during the pre-erythrocytic stages of these parasite strains also differ significantly and may explain the strain-specific effects observed in this study. For example, there is a clear size polymorphism in the gene encoding the major sporozoite surface antigen circumsporozoite protein (CSP), which indicates sequence variation between these strains (data not shown). CSP contains both T-cell and B-cell epitopes (26,27), and differences between strains at these domains could result in the strain-specific effects we have observed. Interestingly, it has recently been shown that CSP plays only a minimal role in the protection obtained with live sporozoites under anti-blood stage chemoprophylaxis, indicating the involvement of other, as yet uncharacterized major antigens (28). A future direction of this work is to utilize the strain-specificity of pre-erythrocytic stage immunity apparent between strains of *P. c. chabaudi* in genetic linkage analyses, including Linkage Group Selection (29), in order to identify these antigens.

## Acknowledgments

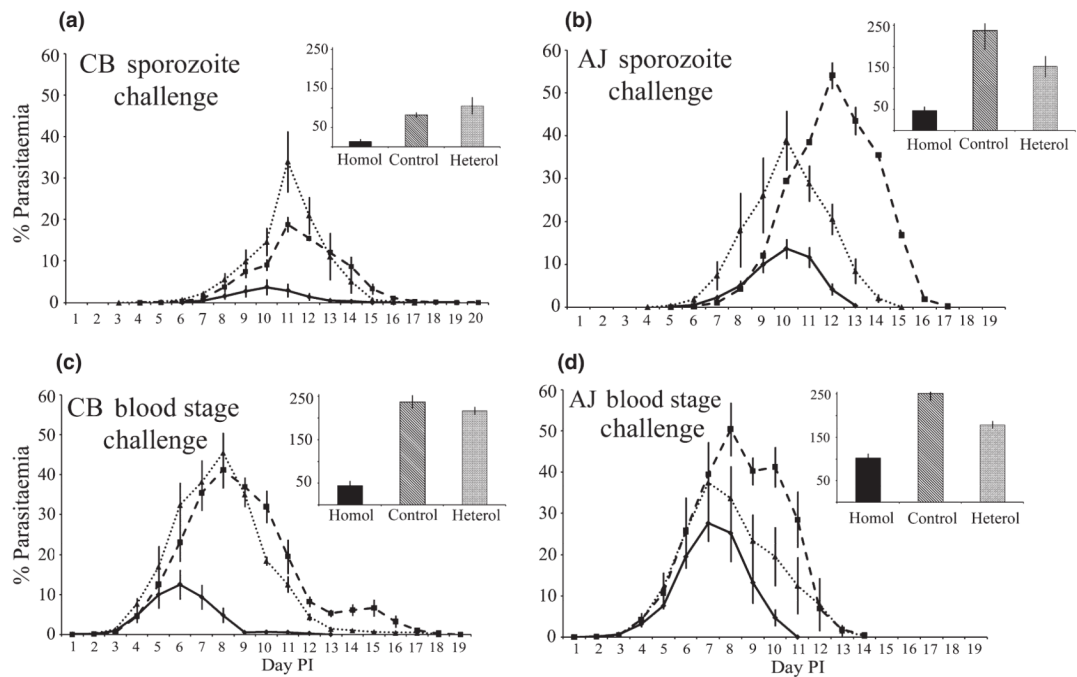
We thank Les Steven for technical assistance and Sofia Trindade Borges for discussion. This work was supported by The Cunningham Trust of the UK (to R.C), A Royal Society Bilateral Grant for Co-operative Research (to R.C and R.L.C) and a Sasakawa Foundation Butterfield Award (to R.L.C).

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**Figure 1.**

Course of parasitaemia in challenge infections with *Plasmodium chabaudi chabaudi* in groups of mice twice immunized by sporozoite infection under a mefloquine cover with CB (solid lines) or AJ (light dotted lines) sporozoites of *P. c. chabaudi* and in mock-immunized control mice (heavy dashed lines). Cumulative proportion of parasitized red blood cells throughout the infections is shown in the bar chart insets. The challenge infections were with sporozoites of CB (Panel a) and AJ (b), and with blood-stage parasites of CB (c) and AJ (d). Error bars indicate one standard error above and below the mean.



**Table 1**

Day of patency of blood-stage parasites following sporozoite challenge (number of mice positive/total number in group)

Immunizing strain	Challenge strain	Day post-challenge				
		3	4	5	6	7
CB	CB	0/5	0/5	0/5	2/5	4/5
CB	AJ	0/5	0/5	5/5	5/5	5/5
Mock-immunized	CB	0/5	2/5	5/5	5/5	5/5
Mock-immunized	AJ	0/2	0/2	1/2	2/2	2/2
AJ	CB	1/5	4/5	5/5	5/5	5/5
AJ	AJ	0/5	3/5	5/5	5/5	5/5