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Chemically attenuated *Plasmodium* sporozoites induce specific immune responses, sterile immunity, and cross-protection against heterologous challenge

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Abstract

Vaccination with *Plasmodium* sporozoites attenuated by irradiation or genetic manipulation induces a protective immune response in rodent malaria models. Recently, vaccination with chemically attenuated *P. berghei* sporozoites (CAS) has also been shown to elicit sterile immunity in mice. Here we show that vaccination with CAS of *P. yoelii* also protects against homologous infection and that a *P. berghei* CAS vaccine cross protects against heterologous challenge with *P. yoelii* sporozoites. Vaccination with *P. yoelii* or *P. berghei* CAS induced parasite-specific antibodies and IFN-γ-producing $CD8^+$ T cells at levels not significantly different from radiation-attenuated sporozoites. Our findings provide an initial characterization of the immune response generated by CAS vaccination and suggest that this attenuation process could be used in the production of an effective cross-protective liver stage vaccine for malaria.

Keywords

chemically attenuated sporozoites (CAS); $CD8^+$ T cells; malaria

1. Introduction

The only experimental vaccine to confer complete protection against malaria in humans utilizes attenuated *Plasmodium* sporozoites. Radiation-attenuated sporozoites (RAS) have been used both in rodent models and human volunteers as vaccines to elicit protective immune responses [1,2]. Presumably, the attenuation of irradiated sporozoites occurs due to a set of random double-strand breaks in the parasite DNA that lead to a block in liver stage development [3]. However, with this method, the issue of adequate irradiation dosage is a concern since

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suboptimal attenuation could result in breakthrough infections [1,4]. As an alternative, vaccines using genetically attenuated parasites (GAS) have been generated in which genes that are essential for liver-stage development are deleted. This vaccine strategy has been validated in the rodent malaria models *Plasmodium berghei* and *Plasmodium yoelii* [5-10], although the effectiveness of GAS has not been experimentally determined in humans.

Many studies have shown that infection with attenuated sporozoites induces similar immune responses using either the irradiated or genetically attenuated models. Protection induced by vaccination with both RAS and GAS sporozoites is essentially mediated by interferon (IFN)-γ-producing $CD8^+$ T cells [8,11,12]. Antibodies generated in response to attenuated parasite vaccines also contribute to protection, but $CD8^+$ T cells are believed to play the major protective role [12,13].

Recently, we have shown that sporozoites can also be attenuated using the DNA-binding drug, centanamycin [14]. These chemically-attenuated sporozoites (CAS) are generated by treatment of sporozoites *in vitro* with centanamycin and vaccination with CAS protects two mouse strains against homologous challenge with *P. berghei* [14]. RAS and GAS vaccines from both *P. berghei* and *P. yoelii* have been shown to induce protection in mice [2,5-7,10,15,16]. Here, we report that CAS vaccines are also protective using a homologous prime-boost schedule with *P. yoelii*, and that this strategy also cross-protects mice when immunized with *P. berghei* and challenged with *P. yoelii* 10 days after the final immunization. Heterologous protection was not seen, however when the challenge was delayed to 21 days. We also show that high levels of $CD8^+$ T cells and antibodies are generated in response to immunization with CAS, suggesting that the immune effector mechanisms induced by CAS vaccination are similar to those induced by RAS and GAS vaccines.

2. Materials and Methods

2.1. Attenuation of sporozoites

Anopheles stephensii mosquitoes were maintained as described [17] and infected with *P. berghei* ANKA $PbGFP_{CON}$ [18] or *P. yoelii* (17XNL) as indicated. Salivary glands of mosquitoes infected with *P. berghei* were dissected at or about day 18 post-feeding (p.f.) and kept on ice. *Plasmodium yoelii* were dissected at day 14 p.f. and kept at room temperature. Sporozoites were quantified using a hemocytometer. Centanamycin (2M) was prepared in a PET (polyethylene glycol 400, ethanol, Tween 80)/glucose solution [19]. CAS were generated by treatment with 2mM centanamycin diluted in DMEM, while control sporozoites were treated with the same volume of vehicle. Sporozoites were incubated with centanamycin or vehicle for 30 min at room temperature for all immunizations, or 30, 60, or 90 min at room temperature for the membrane integrity assay, centrifuged at 21,000 × *g* for 7 min and resuspended in DMEM. RAS were generated by exposure of dissected sporozoites to a γ-irradiator (MDS Nordion Gammacell 1000 Elite) at a dose of 120 Gy. For each experiment, control, CAS and RAS sporozoites were always generated from the same initial pool of sporozoites.

2.2. Membrane integrity

Assessment of membrane integrity in *P. yoelii* 17NXL sporozoites was completed as described [14] except that after incubation, 200 sporozoites were counted per group, per experiment.

2.3. Immunization and challenge

Procedures for all animal experiments were approved by New York School of Medicine Institutional Animal Care and Use Committee. Eight-week old female BALB/c mice were initially immunized with 2×10^4 or 5×10^4 *P. yoelii* or 5×10^4 *P. berghei* RAS or CAS, as

indicated, and in the case of multiple immunizations, were then boosted two additional times at 7 to 9 day intervals with 2×10^4 *P.yoelii* or *P. berghei* RAS or CAS, as indicated. Challenges were completed with 100 *P. yoelii* untreated sporozoites 10 or 21 days after the final boost, as indicated. At each immunization and challenge, 2 to 5 age-matched, naïve mice were injected with control sporozoites from the same mosquito batch as the experimental groups. Parasitemia was evaluated from day 2 p.i. onwards by Giemsa-stained thin blood smears. Protected mice were followed for at least 28 days, while control mice were followed until the parasitemia peaked (at about 30% for *P. yoelii* or 80% for *P. berghei*) and were euthanized.

2.4 Enzyme-linked immunospot assay (ELISPOT)

An additional experiment was completed using the *P. yoelii* and *P. berghei* multiple immunization strategy. Ten days after the final boost, the mice immunized with CAS and RAS were sacrificed and the spleen mononuclear cells were isolated by disruption in a 100μm cell strainer using the end of a sterile 3 mL syringe plunger. Age-matched naïve control mice and mice that received control sporozoites were also included. Cells were washed and resuspended in ACK (ammonium-chloride-potassium) lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). The spleen cells were then counted on a hemocytometer and 2-fold dilutions of the cells were plated in triplicate. A20 cells (ATCC TIB-208) were used as antigen-presenting cells. The cells were coated with a H-$2K^d$-specific $CD8^+$ peptide from the *P. yoelii* CSP (SYVPSAEQI), or the *P. berghei* CSP (SYIPSAEKI) and incubated with splenocytes for 48 h. Epitope-specific $CD8^+$ T cells were quantified using an IFN-γELISPOT assay as described [20]. Purified anti-mouse IFN-γ (R4) and biotinylated anti-mouse IFN-γ (XMG1.2) were obtained from BD Pharmingen. Plates were processed and dried at room temperature, and the spot forming cells were counted on a CTL ImmunoSpot Plate Reader (Series 3) using default settings. Assays were performed in triplicate for each dilution of spleen cells from each mouse using antigen-presenting cells that were either unprimed (to show background) or primed with the peptide.

2.5 Immunofluorescence titration

For titration of *P. yoelii*- and *P. berghei*-specific antibody levels, salivary-gland sporozoites were air-dried on glass Multiwell IFA slides. Mouse serum from the same animals as processed for the ELISPOT assay was titrated and primary antibody bound to sporozoites was detected using FITC-labeled anti-mouse IgG. A monoclonal CSP-specific antibody (2F6) was used as a positive control. Each titration was completed in triplicate for each dilution and each mouse.

2.6 Statistical analysis

Statistical analysis was completed using Prism (v. 4.0a). Normality was tested in the ELISPOT assay using the Kolmogorov-Smirnov Goodness-of-Fit Test. Data with a p value > 0.10 were considered normal. The differences were then tested using an ANOVA with a Tukey's Multiple Comparison post-hoc test. Significant differences between groups are indicated. Fisher exact test was used to calculate p values in protection experiments (Table 1).

3. Results

3.1 Immunization of mice with CAS protects against homologous and heterologous challenge

We evaluated the ability of CAS of *P. yoelii* to produce sterile protection in the mouse malaria model. First, we tested the membrane integrity of sporozoites treated with centanamycin using propidium iodide uptake experiments to ensure that centanamycin treatment did not result in decreased viability of sporozoites relative to controls, as observed with centanamycin-treated *P. berghei* sporozoites [14]. Similar low level labeling with propidium iodide was observed in

control and centanamycin-treated sporozoites, suggesting that centanamycin did not affect membrane integrity (Fig. 1). A higher percentage of *P. yoelii* 17XNL sporozoites remained viable after 90 minutes at room temperature (94.8 % ± 1.77 SD in the control group and 96.8 % ± 1.77 SD in the centanamycin-treated group; Fig 1) compared with the viability of *P. berghei* ANKA sporozoites (65.5 % ± 0.95 SD in the vehicle-treated group and 62.0 % ± 1.42 SD in the centanamycin-treated group) as described previously [14].

Since *P. yoelii* sporozoites were viable after treatment with centanamycin, they were evaluated as a homologous CAS vaccine. Groups of mice (BALB/c) were given an initial immunization dose of 5×10^4 CAS or RAS (as a positive control), and two subsequent doses of 2×10^4 CAS or RAS, for a total of 3 immunizing doses, at 7 day intervals (Group 1, Table 1). Mice were challenged with 100 untreated, wild-type *P. yoelii* sporozoites 10 days after the final immunizing dose. All mice that received either the RAS or CAS were fully protected against homologous *P yoelii* challenge (Group 1, Table 1). Immunization with one dose of either CAS or RAS was not protective with homologous *P. yoelii* challenge (Group 2, Table 1), but resulted in a 1.5-day delay in patent parasitemia for both CAS- and RAS-immunized mice. To evaluate the ability of a heterologous CAS vaccine to induce cross protection against challenge with different parasite species, mice were immunized with *P. berghei* CAS (or RAS as a positive control) using the same schedule, followed by heterologous challenge with P. *yoelii* sporozoites: this protocol also resulted in complete protection in all animals (Group 3, Table 1) only when challenge was completed 10 days after the final immunization. If the challenge was delayed to 21 days, neither RAS- or CAS-immunized mice were protected (Group 4, Table 1).

3.2 Immunization of mice with CAS induces antigen-specific IFN-γ producing $CD8^+$ T cells

Since RAS and GAS vaccines induce antigen-specific IFN-γ producing $CD8^+$ T cells, which mediate protection against sporozoite challenge [8,11,12] we evaluated the ability of a *P. yoelii* and *P. berghei* CAS vaccine to induce $CD8^+$ T cell responses to a species-specific CSP epitope. We immunized BALB/c mice with three doses (5×10^4, 2×10^4, and 2×10^4 at 7-day intervals) of CAS and RAS (as a positive control). Ten days after the final immunization, we measured IFN-γ secretion by splenic T cells specific for a H-$2K^d$-specific $CD8^+$ peptide from the *P. yoelii* CSP (SYVPSAEQI) [21] or *P. berghei* CSP (SYIPSAEKI) [22] using an ELISPOT. Parasite-induced IFN-γ secretion by $CD8^+$ T cell responses in both *P. yoelii* and *P. berghei* CAS and RAS were significantly higher (p=0.0031, ANOVA, n=5 and p=0.0088, ANOVA, n=3) than background responses in unstimulated control cells (Fig. 2A and B) and splenic cells from naïve mice (data not shown). Interestingly, there was no significant difference between the number of spot forming cells in *P. yoelii* or *P. berghei* CAS (152.6 ± 34.8 SEM and 44.6 ± 6.2 SEM) and RAS (132.9 ± 26.4 SEM and 169.3 ± 55.0 SEM).

3.3 Immunization of mice with CAS induces sporozoite-specific antibodies

Antibodies recognizing *Plasmodium* sporozoites are produced in animals immunized with both RAS and GAS, which contribute to the generation of protective immunity [11-13]. We tested whether immunization with *P. yoelii* or *P. berghei* CAS induces parasite-specific antibodies using serum harvested from the same animals used in the ELISPOT assay. Animals immunized with *P.yoelii* or *P. berghei* RAS and CAS showed similar high antibody titers to whole sporozoites of the same species, in contrast to naïve mice (Fig. 3 A and B). Antibody titers were also examined in heterologous sporozoites. We found that sera from mice immunized with *P. yoelii* did not react with *P. berghei* sporozoites. When the reverse experiment was performed, only sera from some *P. berghei* immunized mice reacted at very high concentrations (1:100) with *P. yoelii* sporozoites (data not shown).

4. Discussion

This study shows that vaccination with *P. yoelii* CAS produce sterile protection in BALB/c mice, and that three doses of *P. berghei* CAS were sufficient to cross protect against heterologous challenge with *P. yoelii* sporozoites if challenged within 10 days. However, the cross-protecting immunity was short lived and was not observed after 21 days. Vaccination with CAS was as effective as RAS at producing sterile immunity against homologous and heterologous challenge with *P. yoelii*: heterologous protection has been described between *P. berghei* and *P. yoelii* RAS and GAS [23]. The generation of heterologous protection is important when considering the potential development of an attenuated sporozoite vaccine since due to the high multiplicity of infection in the field, protection needs to be generated against multiple strains of *P. falciparum* [24].

This study is the first characterization of the basic immune effector mechanisms induced by vaccination with centanamycin-attenuated *Plasmodium* parasites. Previous studies in BALB/c mice have identified H-2K^d-restricted major histocompatibility complex class I epitopes derived from CSP as important targets of protective immunity in mice vaccinated with RAS from both *P. yoelii* and *P. berghei* [12,21,25-27]. RAS and GAS generate immunity to sporozoite challenge, which is to a large extent mediated by IFN-γ producing CD8$^+$ T cells [8,11,12]. Our results show that a similar CD8$^+$ T cell response is induced by vaccination with CAS which, in addition induced an antibody response to whole sporozoites similar to that induced by RAS [28] and GAS [11,12].

Interestingly, the compound centanamycin used in the chemical attenuation process may provide some advantages over other attenuation approaches. Attenuation of RAS presumably occurs due to double-strand breaks in the DNA that leads to a block in liver stage development [3]. Sporozoites treated with centanamycin, however, would contain a set of adducts covalently bound to adenine nucleotides [19] and the number of adducts can be defined bioinformatically [29,30]. Given that over- and under-irradiation of sporozoites could impair the immunogenicity of RAS, CAS may have the advantage that the attenuation process can be strictly controlled to achieve saturation of the potential DNA binding sites. Free compound can be washed from the treated sporozoites before vaccination to minimize the risk of toxicity. In addition, a parasite that is attenuated by irradiation or non-saturating doses of centanamycin may produce sporozoites that arrest at various stages of development, producing a potentially more diverse immune response before the parasites are cleared from the liver. In fact, it was recently shown that the transcript and protein expression profiles of the *Plasmodium* liver stage change significantly during late development [31]. Genetic attenuation is likely to produce parasites that arrest at the same stage of development, when the function of an essential gene product is required.

In summary, the results reported here and those observed with CAS of *P. berghei* [14] demonstrate that chemical attenuation could complement other strategies for the generation of highly effective attenuated sporozoite vaccines. This study provides the basis on which to further explore the immune mechanisms induced by a CAS vaccine that protect the host from malaria.

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Abbreviations

p.f., post-feeding; i.v., intravenous; CAS, chemically attenuated sporozoites; RAS, radiation-attenuated sporozoites; p.i., post-infection; SFC, spot-forming cells; CSP, circumsporozoite protein.

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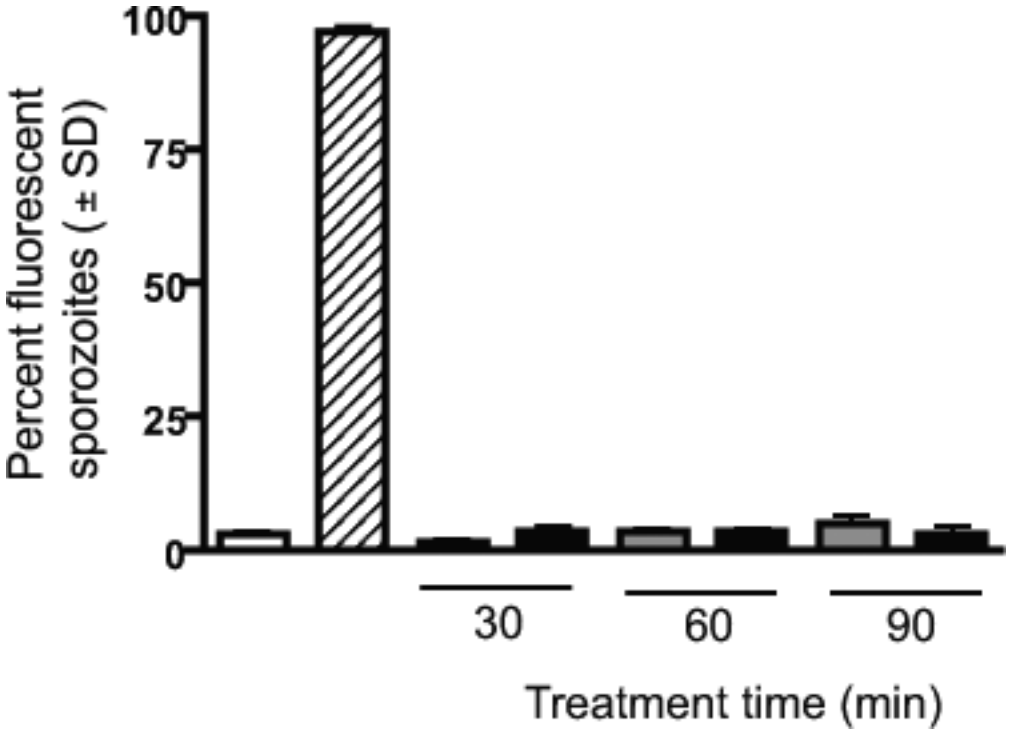

Figure 1. Treatment of P. yoelii 17 XNL sporozoites in vitro does not affect sporozoite membrane integrity

Sporozoites were incubated with vehicle (gray bars) or 2mM centanamycin (black bars) for 30, 60, or 90 min before addition of propidium iodide. Control sporozoites were either tested immediately following dissection (open bar) or heat killed (striped bar) for 15 min at 65°C before counting. For each sample, 200 sporozoites were counted in two separate experiments, and the average percentage of staining with propidium iodide is shown.

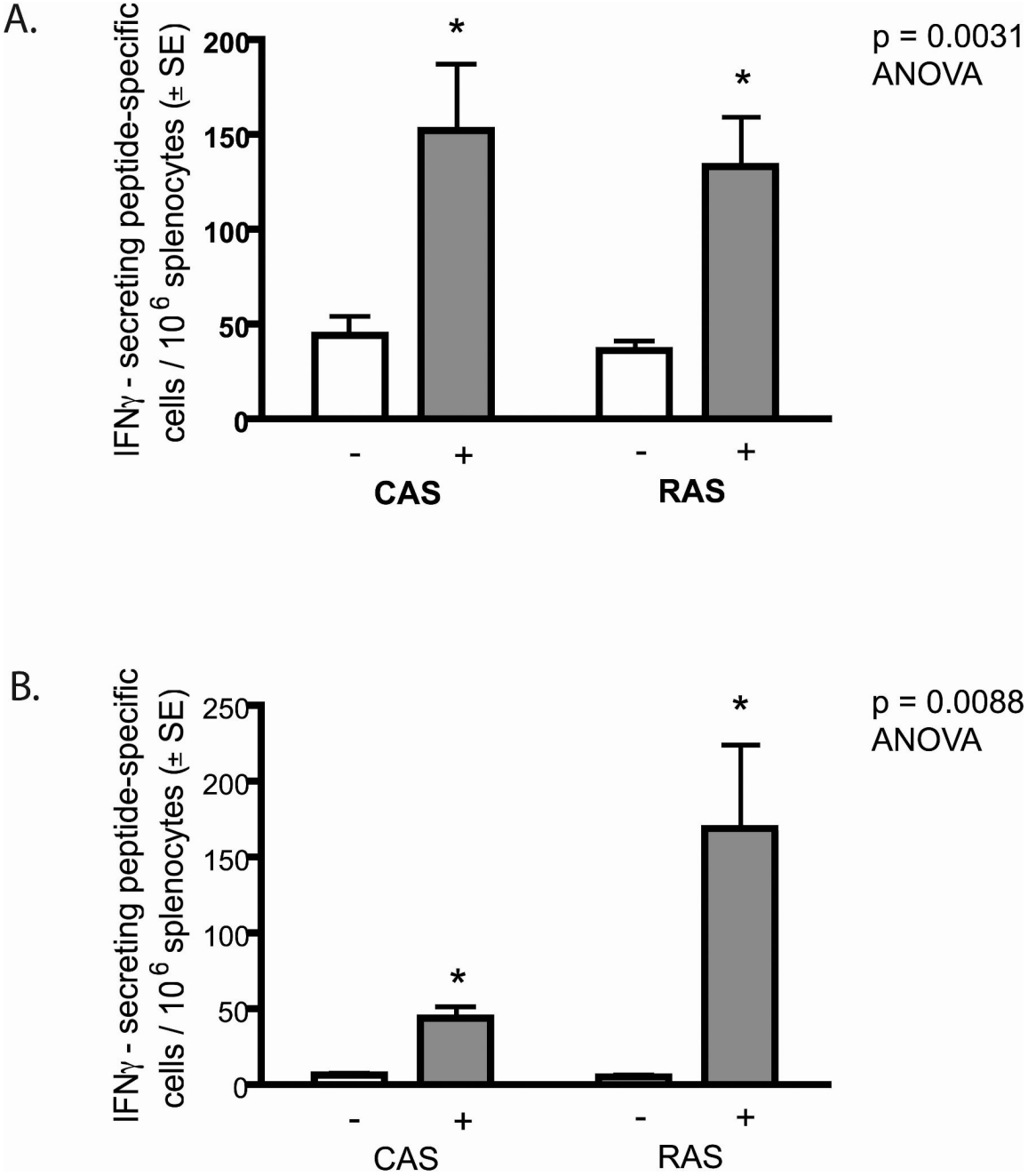

Figure 2. Antigen-specific IFN-γ producing CD8$^+$ T cell responses in mice following a prime-boost vaccination with P. yoelii 17 XNL or P. berghei ANKA CAS or RAS

The CD8$^+$ T-cell response to a H-2K^d-specific CD8$^+$ peptide (SYVPSAEQI) from the *P. yoelii* CSP (A) or *P. berghei* CSP (SYIPSAEKI) (B) was measured by an IFN-γ ELISPOT assay of BALB/c mouse splenocytes. Grey bars represent the number of spot-forming cells per 10^6 splenocytes incubated with antigen-presenting cells loaded with CSP peptide. White bars represent the background control splenocytes incubated with unprimed antigen-presenting cells. Splenocytes were harvested from five (A) or three (B) mice and assayed individually. The experiment was completed in triplicate for each animal and the mean of all animals is shown for both groups. *Indicates significant difference: p=0.0031, ANOVA, n=5 (A) or

p=0.0088, ANOVA, n=3 (B). There is no significant difference between the number of spot-forming cells in RAS and CAS in (A) or (B).

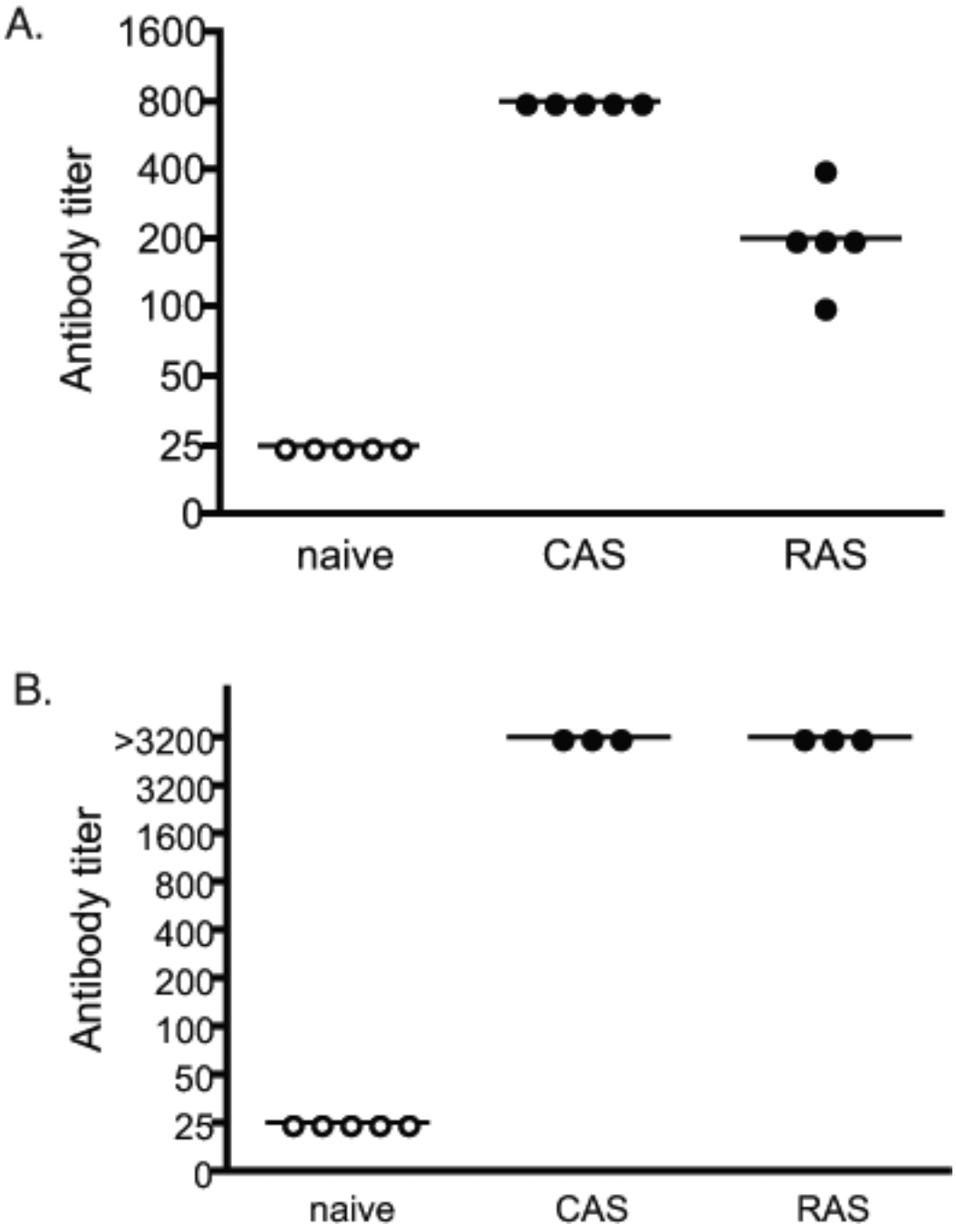

Figure 3. Specific antibody titers in mice to P. yoelii and P. berghei sporozoites following a prime-boost vaccination regimen with CAS or RAS

Sera from animals immunized with *P. yoelii* CAS or RAS was obtained and titers were determined by immunofluorescence using whole sporozoites from *P. yoelii* (A) or sera from animals immunized with *P. berghei* was used to determine titers and *P. berghei* sporozoites (B). Each circle represents data from one mouse. Black circles represent data where sporozoites were florescent, while open circles represent the lowest dilution of serum that did not produce florescent sporozoites. The experiment was completed in triplicate at each concentration for sera from each animal.

Table 1

Protection of mice immunized with CAS or RAS against challenge with wild-type sporozoites

Group	Mouse strain	Immunization[a] RAS/ CAS × 10^3	Challenge[b]	Day of challenge	Sterile immunity?	Prepatent period (days)	No. protected (no. challenged)		
							Control[c]	CAS[d]	RAS
1	BALB/c	50/20/20 (P.y.)	100 (P.y.)	10	yes	NA[e]	0 (8)	4 (4)	4 (4)
2	BALB/c	20 (P.y.)	100 (P.y.)	21	no	1.5[f]	0 (5)	0 (5)	0 (5)
3	BALB/c	50/20/20 (P.b.)	100 (P.y.)	10	yes	NA	0 (8)	4 (4)	4 (4)
4	BALB/c	50/20/20 (P.b.)	100 (P.y.)	21	no	<2[g]	0 (11)	0 (8)	0 (8)

[a] Groups of mice were immunized with *P. berghei* ANKA (P.b.) or *P. yoelii* 17XNL (P.y.) control (vehicle-treated) sporozoites, CAS, or RAS as indicated, isolated from the same mosquito batches and were immunized at 7 day intervals.

[b] Groups of mice were challenged with *P. berghei* ANKA or *P. yoelii* 17XNL wild-type sporozoites

[c] Naïve, age-matched mice were used at the time of all immunizations and challenges.

[d] Statistical analysis using Fisher exact test for comparison of Control and CAS groups gave $p<0.002$ for groups 1 and 3.

[e] NA, not applicable.

[f] Naïve, age-matched mice developed patent parasitemia 1.5 days p.i. in both the CAS and RAS groups.

[g] All mice showed patent parasitemia 2 days p.i.