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14. ABSTRACT: Because resistance to current antimalarials is widespread, new targets for malaria chemotherapy are needed to protect military personnel stationed in developing countries. Malaria parasites cannot make purines needed for RNA and DNA and must salvage purines from their host. Our preliminary studies reveal purine salvage is unique in malaria parasites. We would like to determine whether the unique activities of the malaria enzymes can be exploited to develop specific treatments for malaria that will be effective but not toxic. While study of drug targets in vivo is critical for all infectious diseases, evaluation in an animal model is especially critical for evaluation of purine salvage as a drug target. We perform our studies in Plasmodium yoelii, a rodent malaria whose genome has been sequenced and for which there are techniques for genetic manipulation. We have refined techniques for transformation of this rodent malaria species and have developed GFP reporter parasite lines. Using this system we have genetically disrupted purine salvage enzyme purine nucleoside phosphorylase (PNP) and are attempting to disrupt adenosine deaminase (ADA). We are testing the effects of malaria-specific PNP inhibitors on malaria infection in mice. These novel drugs will be tested in combination with other antimalarials and will also be evaluated for efficacy against exoerythrocytic malaria forms. We hope these experiments will lead to the development of new effective and nontoxic agents that can protect our military personnel from the lethal effects of malaria infection.					
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Evaluation of Purine Salvage as a Chemotherapeutic Target in the *Plasmodium yoelii* Rodent Model

W81XWH-05-2-0025

Kami Kim, MD

Introduction:

Because resistance to current antimalarials is widespread, new targets for malaria chemotherapy are needed to protect military personnel stationed in developing countries. Malaria parasites cannot make purines needed for RNA and DNA and must salvage purines from their host. Our preliminary studies reveal purine salvage is unique in malaria parasites. We would like to determine whether the unique activities of the malaria enzymes can be exploited to develop specific treatments for malaria that will be effective but not toxic. While study of drug targets in vivo is critical for all infectious diseases, evaluation in an animal model is especially critical for evaluation of purine salvage as a drug target. Malaria parasites are routinely maintained in the laboratory with high concentrations of purines, but levels of purines in mammalian blood are tightly regulated and 100-fold less than typical culture conditions. Therefore the efficacy of purine salvage inhibition and importance of purine salvage enzymes must be examined under physiological conditions that cannot be replicated during in vitro culture conditions. We plan to perform our studies in *Plasmodium yoelii*, a rodent malaria whose genome has been sequenced and for which there are techniques for genetic manipulation. Using this system we will genetically disrupt purine salvage genes and test their importance to the parasite. We will test the effects of malaria-specific purine salvage inhibitors on malaria infection in mice. These novel drugs will be tested in combination with other antimalarials and will also be evaluated for efficacy against exoerythrocytic malaria forms. We hope these experiments will lead to the development of new effective and nontoxic agents that can protect our military personnel from the lethal effects of malaria infection.

Task 1 PNP gene disruption in *Plasmodium yoelii*.

1. Make double cross-over constructs for PyPNP including one that replaces *P. falciparum* PNP for PyPNP. Months 1-3, year 1
2. Transfect, select and clone disruptants in mice. Months 4-12 year 1.
3. Analyze phenotype of disruptants and PfPNP replacements for mouse virulence, ability to complete the full life cycle, ability to transport and incorporate purines. Year 1 and year 2.

We have continued to refine and improve protocols for *Plasmodium yoelii* transfection. We have now shown that TgDHFR (DHFR from *Toxoplasma gondii*) and hDHFR (human DHFR) can be used as additional markers in *P. yoelii*. A manuscript describing our techniques is in press in Molecular and Biochemical Parasitology (Jongco *et al* Improved transfection and new selectable markers for the rodent malaria parasite *Plasmodium yoelii*. Mol Biochem Parasitol. 2006 Apr;146(2):242-250. Epub 2006 Jan 17). We have also made stable clones that express GFP (green fluorescent protein) under the EF1 α promoter. In collaboration with Photini Sinnis, we have demonstrated that these GFP clones are able to transit the complete life-cycle and retain

GFP expression. We plan to investigate whether these cell lines can be adapted for studies of drug susceptibility in the exoerythrocytic forms (EEF).

We have made double cross over constructs for PyPNP. We have also made constructs for the single crossover integration into the PyPNP that does not disrupt the gene. We have also made single and double crossover constructs for the disruption of CSP, the gene encoding the circumsporozoite protein, the major surface protein of sporozoites. These constructs are useful transfection controls as the CSP gene is not essential in blood stages.

We have performed transfections with CSP and PNP constructs. Single cross-over PNP constructs continue to yield disruptants that delete the transfection construct. As reported earlier, these disruptants grow more slowly and appear to be attenuated in mice, but have been difficult to analyze due to the reversion. Other PNP constructs appear to work judged on PCR analysis of transfections, but the fraction of integrants has been low. Thus Southern blots have been negative or show very weak bands for the expected integration events. Repeat experiments have been performed with further optimization of transfection conditions, including rounds of FACS sorting of positive cells based upon expression of the DHFR-GFP fusion used for selection. These experiments appear very promising as the proportion of true positives appears higher. We are in the process of performing Southern blots to confirm PCR analysis and will clone parasites when Southern blots confirm a significant fraction of parasites bear a gene disruption.

As reported in *P. berghei*, double crossover gene disruption appears to be less efficient. We have been able to detect double crossover events by PCR but not by Southern. Our most recent experiment using the FACS enrichment protocol appears promising and analysis is ongoing. The parasites that have emerged from this transfection grow more slowly, mimicking the phenotype of single cross-over *PyPNP* disruptants.

Task 2 ADA disruption in *Plasmodium yoelii*.

1. Make single and double cross-over disruption constructs. Months 10-12, year 1
2. Transfect, select and clone disruptants. Months 1-6, year 2.
3. Analyze phenotype of disruptants for mouse virulence, ability to complete the full life cycle, ability to transport and incorporate purines. Months 7-12 year 2. Continue year 3

We have made single cross-over disruption constructs for PyADA. We are making double cross-over constructs but this effort has been hampered by cloning difficulties due to the AT richness of the genome, particularly in noncoding regions. We are making constructs for the single crossover integration into the locus that does not disrupt the gene.

The ADA single cross-over construct has been transfected into *P. yoelii*. After selection we can detect PCR bands consistent with successful disruption of the ADA gene. The population appears to be quite low and disappears with time. This is what we initially observed with our single crossover PNP disruptants before we optimized the harvesting protocols. We are repeating experiments with the goal of earlier harvest and analysis. We predict that ADA disruption will be as or more deleterious than *PNP* disruption. Therefore, although technically difficult, these initial transfection results are consistent with our hypothesis that ADA is important for *P. yoelii* survival.

Task 3 Test the effect of immucillins on the *P. yoelii* rodent malaria model.

1. Test whether the immucillins with best in vitro activity or best malaria-specific activity can cure mice with malaria. Year 1-2
2. Test whether effective immucillins are effective against transgenic *P. yoelii* that carry PfPNP. Year 3
3. Compare effects of malaria specific immucillins to immucillins that inhibit only mouse PNP or inhibit both PNPs in wild-type and parasites with disruption in purine salvage genes. Year 3

We have tested Immucillin H, DADMe Immucillin H and MT-Immucillin H against *P. yoelii*. Unfortunately, none are able to cure mice. MT-ImmH, an inhibitor with 112-fold specificity for *P. falciparum* PNP, gave the best results with delay to death in some experiments. We are in the process of using cloned recombinant PyPNP to thoroughly compare its enzyme kinetics and inhibition profile to that of PfPNP. Initial studies have shown that PyPNP is similar to PfPNP and metabolizes both inosine and methylthioinosine.

Task 4 Test immucillins in combination with antimalarials

1. Establish dose response of agents (DMFO, deoxycoformycin, atovaquone, mycophenolic acid) against *P. yoelii*. Years 2-3
2. Test additive and synergistic effects of agents (particularly polyamine and ADA inhibitors) with best immucillin(s). Year 3-4
3. Test best drug combination for “causal” prophylaxis and cure after sporozoite challenge using *P. yoelii* sporozoites. Year 4

Not yet initiated. Task was not due to be initiated until later in the project.

Key Research Accomplishments:

1. Optimization of *P. yoelii* transfection conditions
2. Stable GFP reporter line able to complete entire malaria life cycle
3. Disruption of PyPNP

Reportable Outcomes:

Accepted manuscript:

Jongco *et al* Improved transfection and new selectable markers for the rodent malaria parasite *Plasmodium yoelii*. Mol Biochem Parasitol. 2006 Apr;146(2):242-250. Epub 2006 Jan 17

Conclusions:

The project is progressing well. Transfection studies for disruption of PNP and ADA appear promising. In addition we have developed techniques and created cell lines that are likely to prove useful for others using the *P. yoelii* malaria model. Testing of PNP inhibitors will proceed in the next year.

References: N/A

Appendices:

Jongco *et al* Improved transfection and new selectable markers for the rodent malaria parasite *Plasmodium yoelii*. Mol Biochem Parasitol. 2006 Apr;146(2):242-250. Epub 2006 Jan 17

Improved transfection and new selectable markers for the rodent malaria parasite *Plasmodium yoelii*[☆]

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Abstract

The rodent malaria *Plasmodium yoelii* is a useful model to study protective immunity to pre-erythrocytic stages of infection, pathogenesis of erythrocytic stages, and vaccine development. However, the utility of the *P. yoelii* model system has not been fully realized because transfection and genetic manipulation methodologies for this rodent species are less developed than that of another rodent species *Plasmodium berghei*. Here we report improved transfection efficiency using the AMAXA nucleofector[®] system compared to conventional transfection methodologies. We also show that heterologous promoters from *P. berghei* can be used to drive expression of a green fluorescent protein (GFP) reporter protein in *P. yoelii*. In an effort to develop additional selectable markers for this parasite, we also tested positive selectable markers that have been used successfully in *P. falciparum* and *P. berghei*. Human dihydrofolate reductase (*dhfr*) and *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase (*Tgdhfr-ts*) conferred drug resistance to WR99210 and pyrimethamine, respectively, when introduced as episomes. These improvements should make genetic manipulation of *P. yoelii* more amenable and facilitate further studies of host–parasite interactions using this attractive rodent model.

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Keywords: *Plasmodium yoelii*; Transfection; Nucleofector[®]; GFP; FACS

1. Introduction

Malaria, caused by *Plasmodium* spp., continues to be an important public health problem. Animal models and genetic manipulation methodologies provide useful opportunities to study the biology of the parasite and host–parasite interactions, which facilitate the rational design of more effective drugs and vaccines.

Stable transformation and gene targeting are well established for the rodent malaria *Plasmodium berghei* [1–3]. Although *P. berghei* is a useful model, other rodent species may be better models for human infection, pathology, and immunity. For example, the sporozoite inoculum needed to produce successful blood stage infection is more similar between *P. yoelii* and *P. falciparum*, whereas at least tenfold more *P. berghei* sporozoites are needed [4–6]. Compared to *P. berghei*, *P. yoelii* also appears to more closely resemble *P. falciparum* with respect to the kinetics of the ensuing protective immunity to pre-erythrocytic stages [7,8]. Moreover, *P. yoelii* parasite lines with distinct virulence characteristics and a completed genome of the nonlethal 17× strain are available. However, the potential of *P. yoelii* has not been realized because of limited genetic manipulation methodologies and reagents to further dissect parasite biology [9].

Here we report improvements upon current electroporation protocols and feasibility of using the AMAXA nucleofector[®] system (Amaza GmbH) for *P. yoelii*. We also demonstrate that heterologous promoters from *P. berghei* can be used to drive

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DHFR-TS, dihydrofolate reductase-thymidylate synthase; EF-1α, elongation factor-1α; GFP, green fluorescent protein; WT, wild type; dpi, days post-infection; hpi, hours post-infection

[☆] Data in this paper are from a thesis to be submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University.

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expression of a GFP reporter, and that introduction of *hDHFR* and *TgDHFR-TS* allows for selection using WR99210 and pyrimethamine, respectively. These improvements should make genetic manipulation of *P. yoelii* more amenable and facilitate further studies of host–parasite interactions using this attractive rodent model.

2. Materials and methods

2.1. Experimental animals and parasites

BALB/C female mice of 4–6 weeks weighing 18–20 g were used. Lethal YM *P. yoelii* lines were obtained as cloned lines from the WHO Registry Standard Malaria Parasites, University of Edinburgh. *P. yoelii* YM parasites were used for pyrimethamine and WR99210 sensitivity assays as well as to assess the feasibility of using the AMAXA nucleofector® system. Parasite reference populations (stabilates) were prepared from BALB/C mice infected by bite of *Anopheles stephensi* mosquitoes. Cryopreserved parasite stabilates (corresponding to $\sim 3 \times 10^7$ parasites/mouse) were thawed at room temperature and promptly syringe passaged into 18–20 g BALB/C mice intraperitoneally (i.p.). Parasites were then used directly from these mice after sufficient parasitemia for transfection had been reached. In order to obtain a preparation containing >90–95% schizonts for transfection, blood from mice exhibiting 20–50% total parasitemia was collected by eye vein puncture. The collected blood was initially placed in 10 mL of complete culture medium (RPMI 1640 medium containing 20% heat inactivated fetal calf serum, 50 IU mL⁻¹ neomycin and 25 mM Hepes), containing 40 IU of heparin, and washed once at $300 \times g$ for 8 min. The isolated blood sample was subsequently incubated in 150 mL of complete culture medium at 10% O₂, 5% CO₂, 85% N₂ gas mixture at 37 °C, and shaken at 77 rpm. 1 mL aliquots were taken hourly, smeared, and fixed to monitor the percentage of schizonts. When culture reached at least 50% schizont, each 35 mL of culture suspension was layered on top of 10 mL of 60% Accudenz (Sigma)/phosphate buffered saline (PBS) gradient and centrifuged for 25 min at $200 \times g$ at room temperature without brakes setting. The schizonts were collected on the interface and washed once for 10 min at $200 \times g$ at room temperature with culture medium. The schizont pellet was resuspended, counted on a hemocytometer, aliquoted to 5×10^7 parasites/transfection, and washed again as above.

2.2. Bio-Rad transfection and selection of transformants

For each electroporation using the Bio-Rad GenePulser II, 30 µg of circular plasmid DNA was diluted in PBS or cytomix [10] and then added to 5×10^7 schizonts in a 0.4 cm electroporation cuvette and electroporated as previously described [9]. Cytomix was “incomplete” (not supplemented with glutathione or ATP) and consisted of 120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄ pH 7.6, 25 mM HEPES pH 7.6, 2 mM EGTA pH 7.6, 5 mM MgCl₂ [10]. PBS was added to 400 µL, then 200 µL each was injected into tail vein intravenously (i.v.) into two 18–20 g mice. Starting at 24 h post-infection, mice

were treated i.p. with 1 mg kg⁻¹ pyrimethamine (Sigma) daily. With treatment, parasitemia decreased to undetectable levels on smears (<0.001%). When the parasites re-emerged and increased to detectable levels under continuous drug selection, resistant parasites were transferred to new mice via i.v. injection (tail vein). These mice were treated with 1 mg kg⁻¹ pyrimethamine daily until parasite numbers were sufficient for parasite DNA isolation and analysis. Drug was administered to mice daily until they were sacrificed.

2.3. AMAXA transfection and selection for transformants

For each transformation, 100 µL of AMAXA nucleofector® solution or cytomix was added to 5 µg of circular plasmid DNA. The DNA solution was then added to 5×10^7 schizonts, mixed well, transferred to the AMAXA cuvette, then nucleofected using various pre-programmed settings on the AMAXA nucleofector®. Each condition was tested at least twice. PBS was added to 400 µL, then 200 µL each was injected i.v. (tail vein) into two 18–20 g mice. Starting at 24 h post-infection, mice were treated i.p. with 1 mg kg⁻¹ pyrimethamine daily. Parasitemia initially decreased to undetectable levels. When the parasitemia increased to detectable levels under continuous drug selection, resistant parasites were transferred to new mice via i.v. injection (tail vein). These mice were treated with 1 mg kg⁻¹ pyrimethamine daily until parasite numbers were sufficient for parasite DNA isolation and analysis. Drug was administered to mice daily until they were sacrificed.

For parasites transfected with *hDHFR* plasmid, the mice were treated daily i.p. with 1 mg kg⁻¹ pyrimethamine starting at 24 h post-infection. Mice were treated daily until parasites were no longer detectable. When the parasitemia increased to detectable levels under continuous drug selection, resistant parasites were transferred to new mice via i.v. injection (tail vein) to increase parasite numbers sufficient for parasite DNA isolation and analysis. One of the second generation mice was treated i.p. with 1 mg kg⁻¹ WR99210 (Jacobus Pharmaceuticals, Princeton, NJ) while two were treated i.p. with 1 mg kg⁻¹ pyrimethamine. For all regimens, drug was administered to mice daily until they were sacrificed.

2.4. Flow-activated cell sorting analysis of resistant parasite populations and fluorescence microscopy

Blood from mice infected with resistant *P. yoelii* populations was collected by tail bleeding, and diluted to approximately 10⁶ parasites mL⁻¹ in PBS, then pelleted for 5 min at $700 \times g$ at room temperature. Samples were then analyzed in a Becton-Dickinson FACScan equipped with a 488 nm Argon laser at the Albert Einstein College of Medicine Fluorescence Activated Cell Sorting Facility. Erythrocytes were selected on size by gating forward/side-light scatter to exclude platelets and debris. Ten thousand events were collected per sample. Data analysis was performed using CellQuest software (Becton-Dickinson, CA) and FlowJo version 6.2.1 (Tree Star Inc., Ashland, OR). Five microlitres of sample was then dotted onto a slide, air dried,

and fixed in 90% acetone/10% methanol for 5 min. After fixation, slides were incubated with 1 $\mu\text{g mL}^{-1}$ DAPI (Molecular Probes, Eugene, OR) in PBS for 5 min then washed with ddH₂O, air dried, and covered with a cover slip. Slides were then viewed under Zeiss Axiophot microscope at the Albert Einstein College of Medicine Analytical Imaging Facility.

2.5. DNA analysis of resistant parasite populations

Blood from mice harboring resistant parasites was collected by eye vein puncture, and then depleted of leukocytes by passage through a CF11 cellulose column previously washed with 5–10 mL of PBS. The blood was then washed once with PBS by centrifugation at $750 \times g$ for 5 min. The erythrocyte pellet was resuspended in approximately five volumes of PBS and erythrocytes lysed with 0.2% saponin (w/v) in PBS. To wash the parasites, the sample was centrifuged for 10 min at $15,000 \times g$, and the dark pellet was washed once with PBS. Total DNA was obtained using QIAMP blood kit (QIAGEN, USA) according to manufacturer's directions. The resulting DNA was then used for PCR-amplification reactions.

2.6. *P. yoelii* pyrimethamine and WR99210 sensitivity

To test the sensitivity of YM parasites to pyrimethamine and WR99210, experiments were performed in which groups of three *P. yoelii* infected mice were treated i.p. for different periods of time with different concentrations of drug. To assess the level of sensitivity to the drug, parasitemia was monitored by staining blood smears with 10% Giemsa stain after methanol fixation.

2.7. Plasmid constructs used

In order to assess the feasibility of using *TgDHFR-TS* and *hDHFR* as positive selectable markers for transformation of *P. yoelii*, we used several plasmids (gift of Chris Janse and Andy Waters) encoding a GFP-reporter under the control of different promoters that also contained different selectable markers (Table 1). Detailed descriptions of these plasmids can be found at Leiden University Medical Center Malaria Research Group website (<http://www.lumc.nl1040/research/malaria/malaria.html>). The pMD205GFP plasmid has been described previously [9].

3. Results

3.1. *P. yoelii* YM sensitivity to pyrimethamine and WR99210

The pyrimethamine-resistant allele of the *P. bergheii* bifunctional enzyme DHFR-TS has successfully been used as a selectable marker for the transformation of *Plasmodium* spp. asexual blood stages, and is currently the only available marker for *P. yoelii* [9]. Although *P. yoelii* 17 $\times$ has been shown to be sensitive to pyrimethamine [9], we wanted to evaluate the sensitivity of the lethal YM strain to the drug. Furthermore, we sought to determine if the human DHFR (*hDHFR*) could be used as a new selectable marker for *P. yoelii*, using the antifolate drug WR99210 to select for transformants. In order to study the in vivo sensitivity of *P. yoelii* YM to these drugs, groups of 3 BALB/C mice infected with *P. yoelii* YM were treated with different concentrations of the drug and their parasitemia followed for 6 days post-treatment. As shown in Fig. 1A, *P. yoelii* YM is highly sensitive to pyrimethamine at doses of 0.6–5 mg kg⁻¹, and two consecutive days of treatment were sufficient to eradicate the infection. At 0.2 and 0.4 mg kg⁻¹, treatment had to be prolonged for two to three more days. A dose of 1 mg kg⁻¹ bodyweight was used for subsequent experiments.

As shown in Fig. 1B, *P. yoelii* YM is sensitive to WR99210, and all mice can be cured at doses higher than 0.5 mg kg⁻¹ bodyweight. At doses of 2–4 mg kg⁻¹, three consecutive treatments were sufficient to clear parasitemia, but treatment was prolonged for one more day when 1 mg kg⁻¹ was used. These results demonstrated that WR99210 could also potentially be used in vivo to select WR99210-resistant parasites expressing *hDHFR*. A dose of 1 mg kg⁻¹ bodyweight was used for subsequent experiments.

3.2. Use of cytomix versus PBS improves transfection efficiency via electroporation

Available methodologies for *P. yoelii* transformation by electroporation are not optimized, and few laboratories currently perform *P. yoelii* transfections regularly and reproducibly. Many current published electroporation protocols for Apicomplexa use cytomix instead of PBS, which was used originally for *P. yoelii* [9]. Fig. 2A depicts a schematic of *P. yoelii* transfection using the Bio-Rad GenePulser II. In two independent experiments, the use of cytomix when electroporating the

Table 1
Plasmids used in this study

Plasmid	GFP		Selection cassette		
	Promoter	3' UTR	Selectable marker	Promoter	3' UTR
pEFhDHFR-HPEA	NA	NA	hDHFR	PbEf-1 α	PbDHFR-TS
pPbGFP _{con}	PbEf-1 α	PbDHFR-TS	TgDHFR	PbDHFR-TS	PbDHFR-TS
pDEFGFP5UTR21-3UTRPbDHFR	Pbs21	PbDHFR-TS	TgDHFR	PbDHFR-TS	PbDHFR-TS
pMD205GFP	PbDHFR-TS	PbDHFR-TS	PbDHFRGFP	PbDHFR-TS	PbDHFR-TS

NA: not applicable.

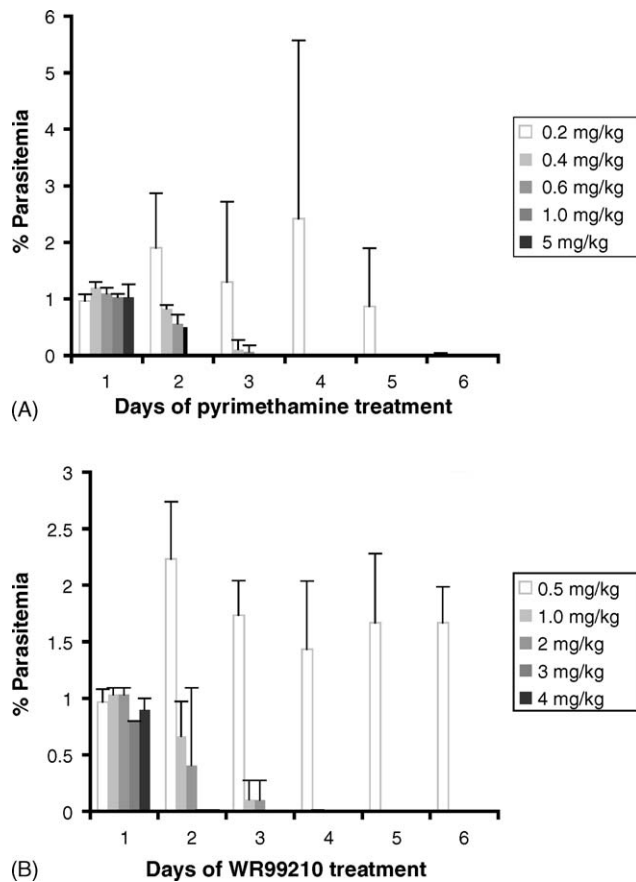


Fig. 1. Sensitivity of *P. yoelii* YM blood stage parasites to pyrimethamine (A) and WR99210 (B). Groups of three BALB/C mice were treated with different concentrations of pyrimethamine (0.2–5 mg kg⁻¹ bodyweight) or WR99210 (0.5–4 mg kg⁻¹ bodyweight), respectively. All data are expressed as the arithmetic means of the three mice in each group.

pMD205GFP plasmid consistently resulted in the emergence of a pyrimethamine-resistant fluorescent parasite population sufficient for subsequent passage to a new set of mice by 8 days post-infection (dpi) (Table 2). Under continuous drug administration, drug-resistant fluorescent parasites could be expanded in these second generation mice to sufficient levels for subsequent applications such as FACS and DNA extraction for PCR-amplification reactions. In our hands, the use of PBS as electroporation buffer does not always result in the emergence of a pyrimethamine-resistant parasite population in mice infected with transfected parasites (data not shown).

3.3. AMAXA transfection system can be used to transform *P. yoelii*

Recently, it has been shown that AMAXA nucleofection technology can be used to successfully transform *P. berghei* schizonts when using the U33 or V13 programs with human T cell solution [11]. The main advantages of the AMAXA system include the need for less DNA (only 5 µg), and higher transfection efficiency (10⁻² to 10⁻³ for linear DNA; 10⁻³ to 10⁻⁴ for circular DNA) [11]. The availability of pre-made solutions and pre-programmed settings on the nucleofection apparatus facilitates optimization of transformation conditions. In order to evaluate the feasibility of using the AMAXA system with *P. yoelii*, we tested various combinations of solutions and programs for nucleofection of GFP-expression plasmids. Fig. 2A depicts a schematic of *P. yoelii* nucleofection using the AMAXA nucleofector®. Initial experiments utilized pMD205GFP vector, and the various combinations of programs T01, T16 or U33 with cytomix or AMAXA solutions human T cell or 8851 or 8841. Under all conditions tested, parasites could be detected in mice 24 h post-infection, suggesting that the nucleofection did not kill the parasites. 1 mg kg⁻¹ pyrimethamine i.p. treatment was begun 24 h post-infection and administered daily,

Table 2
Summary of transfection conditions

Transfection condition	Emergence of resistance (dpi)	GFP-expression	Emergence of resistance in second generation (dpi)	GFP-expression in second generation (dpi)
GenePulser II				
0.8 kV; 25 µF/cytomix	7–8	Yes	2–3	2–3
Nucleofector®				
T01/8841	6–7	No	NA	NA
T01/8851	6–7	No	NA	NA
T01/T cell	6–7	Yes	1	1
T01/cytomix	7–8	Yes	1	1
T16/8841	6–7	No	NA	NA
T16/8851	5–6	Yes	1	1
T16/T cell	7–8	No	NA	NA
T16/cytomix	5–6	Yes	1	1
U33/8841	6–7	Yes	1	1
U33/8851	6–7	Yes	1	1
U33/T cell	5–6	Yes	1	1
U33/cytomix ^a	5–6	Yes	1	1

NA: not applicable.

^a This was a valid combination in initial trials, but generated an error message in subsequent experiments and could not be used further.

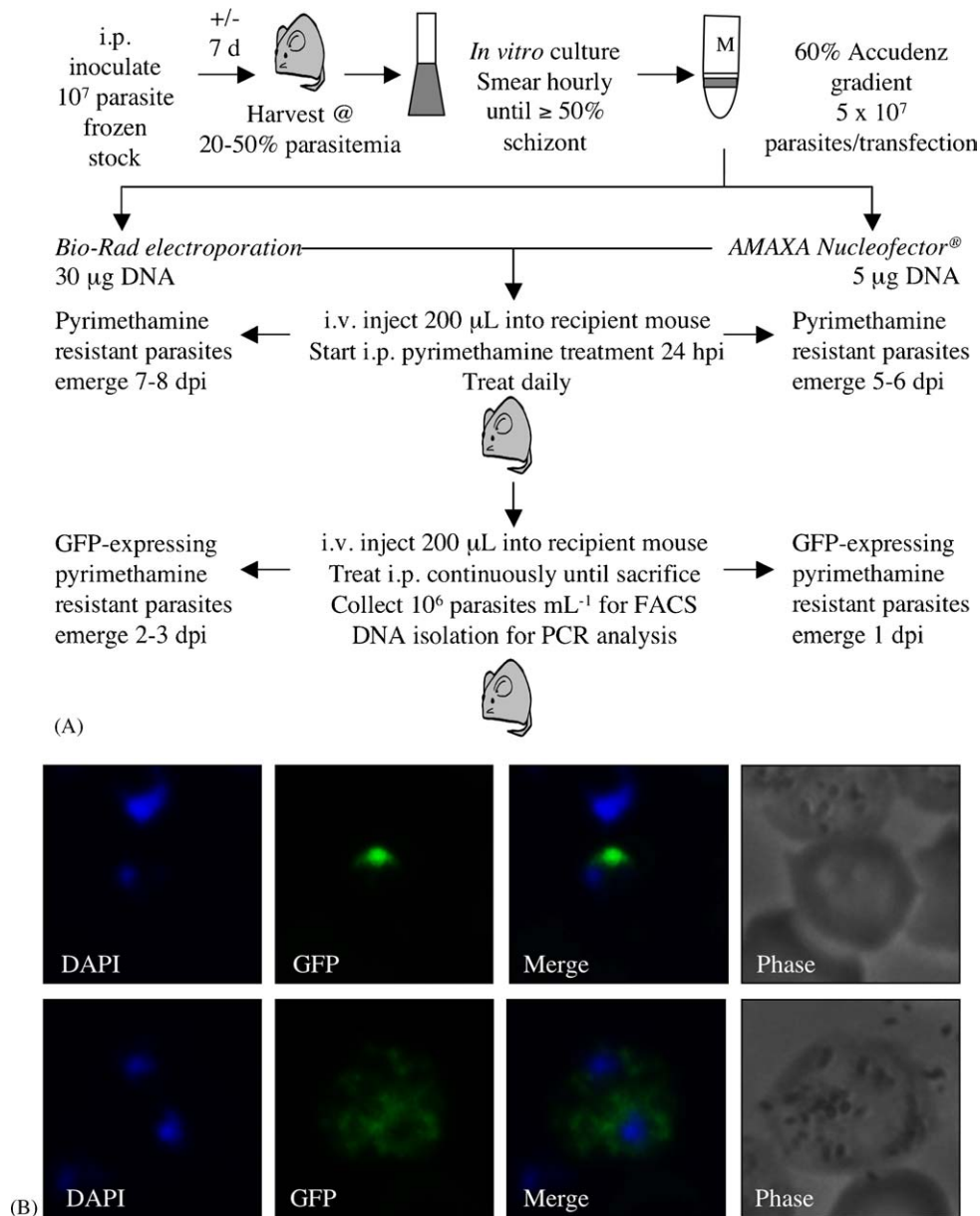


Fig. 2. Schematic representation of *P. yoelii* transfection using Bio-Rad GenePulser II electroporation and AMAXA nucleofector® system (A). A 1×10^7 parasites from frozen stock were i.p. inoculated into BALB/C mice. Parasitemia was monitored daily by Giemsa stained thin smears. When parasitemia reached 20–50% in approximately 7 days, parasites were collected by eye vein puncture. Parasites were cultured in vitro in complete media at 10% O₂, 5% CO₂, 85% N₂ gas mixture, and shaken at 77 rpm at 37 °C. 1 mL aliquots were taken hourly and smeared until $\geq 50\%$ schizont was attained. Cultures were then subjected to 60% Accudenz gradient. Media (M) fraction formed on top, non-schizont infected erythrocytes collected on the bottom of the Accudenz/PBS fraction, while schizont pellet (gray band) formed below the interface. Schizont pellet was collected, resuspended, counted on hemocytometer, and aliquoted to 5×10^7 parasites/transfection. Bio-Rad electroporation protocol is summarized on the left, while AMAXA nucleofection® is shown on the right. For Bio-Rad electroporation, 30 μ g of circular plasmid was resuspended in cytomix and electroporated using 0.8 kV and 25 μ F. For AMAXA nucleofection®, 5 μ g of circular plasmid was resuspended in cytomix or AMAXA 8851 or human T cell solutions, and nucleofected using programs T16 or U33. For both protocols, PBS was added to 400, and 200 μ L of resuspended transfected parasite was i.v. injected into recipient mice tail vein. Pyrimethamine was administered i.p. at 24 hpi, and mice were treated daily. Pyrimethamine-resistant parasites emerged at around 7–8 dpi using Bio-Rad electroporation, but emerged at around 5–6 dpi using AMAXA. Resistant parasites were collected by eye vein puncture, and 200 μ L was i.v. injected into tail vein of recipient second generation mice. Second generation mice were treated with pyrimethamine i.p. until sacrifice. GFP-expressing pyrimethamine-resistant parasites emerged 2–3 dpi with Bio-Rad electroporation, but emerged 1 dpi with AMAXA. 1×10^6 parasites mL⁻¹ was collected by tail bleed from mice for FACS analysis, and DNA was isolated at sacrifice for PCR analysis. Fluorescent microscopic analysis of GFP-positive infected erythrocytes stained with DAPI expressing pMD205GFP under continuous 1 mg kg⁻¹ pyrimethamine (B). Top panel depicts ring stage parasite, while bottom panel depicts more mature stage.

such that parasitemias were undetectable by 4 dpi. Depending on the condition, a drug-resistant fluorescent population had emerged under continuous drug pressure between 5 and 7 dpi, and transferred to new mice. 1 mg kg⁻¹ pyrimethamine

was continually administered i.p. to the second generation mice since it has been shown previously that autonomously replicating plasmids are stable only under continued drug pressure in *P. yoelii* [9].

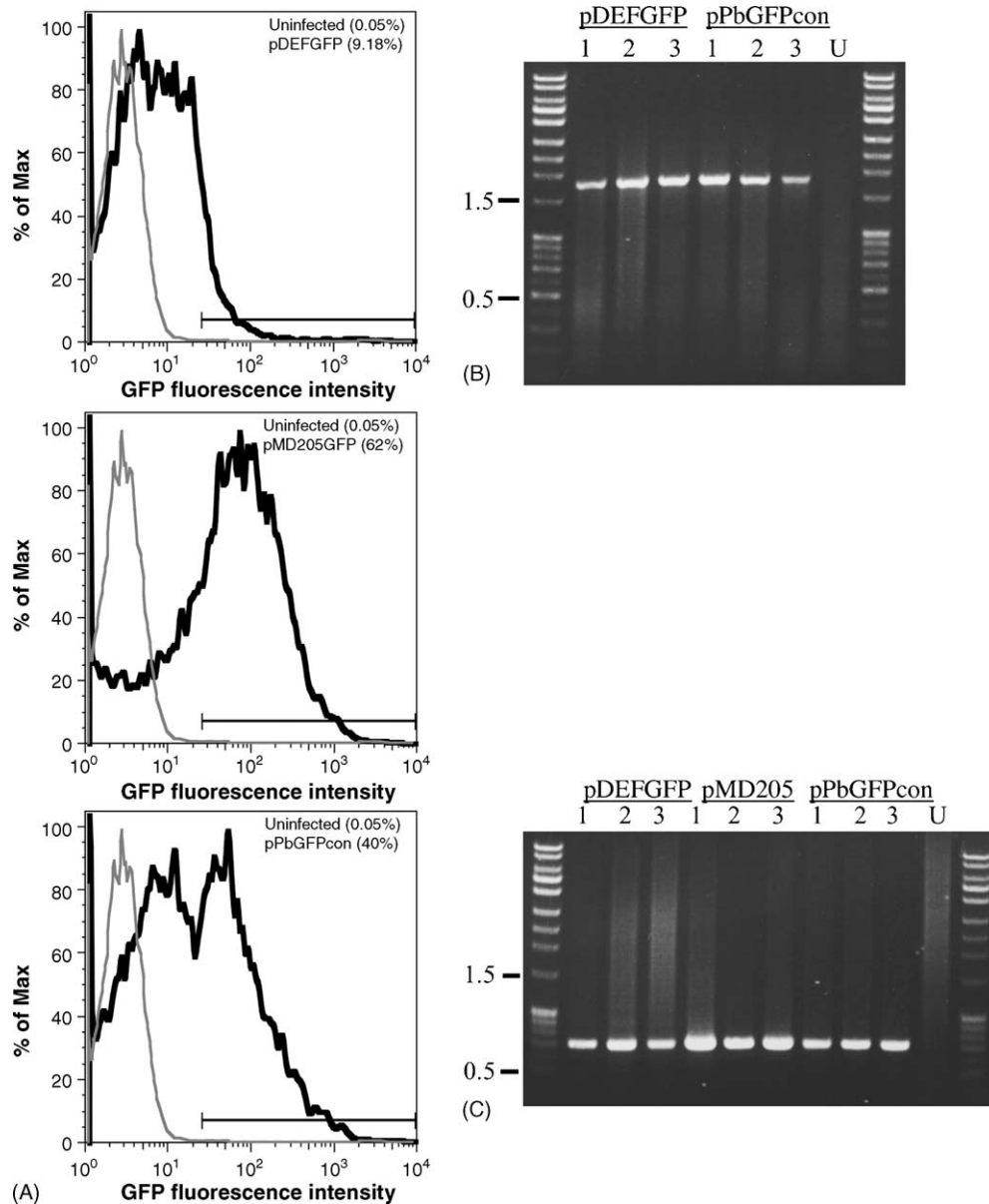


Fig. 3. Transformation of *P. yoelii* YM blood stages with different constructs using the AMAXA nucleofector[®] system. Flow cytometric analysis of GFP fluorescence intensity taken at 4 dpi from a representative second generation mouse harboring pDEFGFP or pMD205GFP or pPbGFP_{con} parasites under continuous 1 mg kg⁻¹ pyrimethamine (A). Gray line represents uninfected and black line represents nucleofected sample. Percentage of GFP-positive is indicated in parentheses. Horizontal bar represents gate for GFP-positive. PCR analysis to demonstrate presence of *TgDHFR* (B) or *gfp* (C) gene in nucleofected *P. yoelii* YM blood stages of second generation mice. Each lane represents total DNA from an individual second generation mouse sacrificed at 5 dpi. U signifies untransformed WT parasite DNA. A 1 kb plus molecular ladder is provided on the left.

Fluorescence microscopy was used to verify GFP fluorescence (Fig. 2B). Regardless of the conditions used, ring-infected erythrocytes routinely exhibited weak GFP fluorescence compared to erythrocytes infected with more mature stages. Flow cytometry was used to quantitate the percentage of GFP-positive infected erythrocytes. These initial trials suggested that highest levels of parasitemia and percentage of GFP-positive infected erythrocytes could be attained using the following conditions: U33/T cell, U33/cytomix, T16/cytomix, or T16/8851 (Table 2). These four conditions appeared to lead to the emergence of a drug-resistant population 1–2 days earlier, as well as yield

higher parasitemia and percentage of GFP-positive infected erythrocytes when compared to traditional electroporation protocol with cytomix. Although U33/cytomix was a valid combination in initial trials, in subsequent experiments, this condition resulted in an error message and could no longer be used. Although parasitemia increased, there were no observable GFP-positive populations for T01/8841, T01/8851, T16/8841, or T16/T cell.

Parasitemias were 0.025–0.075% 1 day after transfection. The number of GFP-positive parasites was low. Due to the low parasitemia, the GFP-positive cells could not be reliably discriminated from false positives (in the range of 0.05%; see Fig. 3A).

The low initial parasitemia and the low fluorescence of rings made direct estimates of transfection efficiency via flow cytometry impossible. Using formulas established for *P. berghei* [11], we estimated, based upon parasitemias the day after transfection and the rate of emergence of pyrimethamine-resistant parasites, that transfection efficiency of the circular plasmids was in the range of 10^{-4} to 10^{-5} , approximately a 100-fold improvement over the Bio-Rad GenePulser II method. This estimate assumes a replication rate of 8- to 10-fold/day for parasites and that transfected and untransfected parasites replicate at a similar rate.

Once drug-resistant parasites had re-emerged, parasites transfected with a GFP construct could be readily sorted by FACS. Nearly all parasites sorted were GFP-positive when examined by fluorescence microscopy, and parasites that appeared negative by microscopy were usually immature ring parasites that express less GFP. Although currently not feasible, further improvements in transfection efficiency may also permit direct sorting of GFP-positive parasites without selection as reported for *P. berghei* [11].

3.4. *TgDHFR* can be used to select for pyrimethamine-resistant *P. yoelii*

We also wanted to determine if *P. berghei* expression vectors harboring modified *TgDHFR* could be used to select for pyrimethamine-resistant *P. yoelii*. The pPbGFP_{con} and pDEFGFP5UTR21-3UTRPbDHFR (pDEFGFP) plasmids (gift of C. Janse and A. Waters) were transformed into *P. yoelii* using T16/cytomix. In both vectors, *TgDHFR* is driven by PbDHFR promoter, but the GFP reporter is driven by different promoters (see Table 1). By 6 dpi under continuous 1 mg kg^{-1} pyrimethamine i.p. treatment, both these vectors yielded pyrimethamine-resistant fluorescent parasites. Drug-resistant parasites were i.v. injected (tail vein) to new mice to expand the population under 1 mg kg^{-1} pyrimethamine daily. Parasitemia continued to increase in the second generation mice under this drug regimen as monitored by Giemsa stained thin smears. Further, the percentage of GFP-positive infected erythrocytes continued to increase in the second generation mice as assessed by flow cytometry. The observed fluorescence intensities differed among the various constructs, as shown in representative histograms from individual mouse samples taken at 4 dpi (Fig. 3A). All three constructs produced GFP-positive populations with heterogeneous fluorescence intensities. However, pMD205GFP and pPbGFP_{con} appeared to yield greater proportions of GFP expressing infected erythrocytes that were more intensely fluorescent than pDEFGFP. The weaker fluorescence of pDEFGFP-transfected constructs led to underestimation of parasitemia when GFP fluorescence was used to monitor parasitemia (data not shown).

The second generation mice were sacrificed at 5 dpi, DNA was obtained, and primers specific for *TgDHFR* and *gfp* were used to test for the presence of the selectable marker and reporter gene via PCR amplification. As shown in Fig. 3B and C, bands of expected size corresponding to *TgDHFR* and *gfp* were seen in the transformed parasites but not in untransfected wild type

YM parasites, confirming the presence of the plasmid in the transformed parasites.

3.5. *hDHFR* can be used to select for WR99210-resistant *P. yoelii*

Since we had shown that *P. yoelii* YM parasites are sensitive to WR99210, we wanted to determine if we could successfully select for WR99210-resistant parasites after transformation with *hDHFR* containing plasmid. The pEFhDHFR plasmid (gift of C. Janse and A. Waters) was transformed into *P. yoelii* using T16/cytomix. In this vector, *hDHFR* is driven by PbEf-1 α promoter. This vector yielded pyrimethamine-resistant parasites by 6 dpi under daily 1 mg kg^{-1} pyrimethamine i.p. administration. Parasites were transferred via i.v. injection (tail vein) to new mice for expansion of the resistant population under 1 mg kg^{-1} WR99210 or 1 mg kg^{-1} pyrimethamine daily. As shown in Fig. 4A, parasitemia continued to increase in the second generation mice under both drug regimens as monitored by Giemsa stained thin smears. The mice were sacrificed at 5 dpi, DNA was obtained, and primers specific for *hDHFR* were used to test for the presence of the selectable marker via PCR amplification. As shown in Fig. 4B bands of expected size corresponding to *hDHFR* were seen in the transformed parasites but not in untransfected wild type YM parasites, confirming the presence of the plasmid in the transformed parasites.

4. Discussion

Although *P. yoelii* is an attractive model for human malaria, the lack of genetic tools and methodologies for this rodent malaria have contributed to its under utilization. *P. yoelii* is less robust than *P. berghei* but appears to mimic *P. falciparum* more closely. Because it is related to *P. berghei*, little work has been done to show formally that reagents and methodologies developed for *P. berghei* also work for *P. yoelii*. We sought to determine whether selectable markers currently available for *P. berghei* could be used for *P. yoelii*. We also compared various conditions for *P. yoelii* transformation.

Here we report improvements upon the current transformation methodologies. The use of cytomix increases reproducibility as compared to published protocols that utilize PBS [9]. Moreover, the AMAXA nucleofactor[®] system can be used to transform *P. yoelii*, improving transfection efficiency and enabling transfection with only $5 \mu\text{g}$ of DNA. The availability of pre-programmed settings on the apparatus and pre-made solutions facilitates optimization and is convenient. On average, drug-resistant parasites emerge approximately 2 days earlier and higher levels of parasitemia and GFP-expression are reached, when compared to traditional electroporation with cytomix. Interestingly, the optimal solution/program conditions we identified include combinations other than the recommended solution/program for *P. berghei* (U33 or V13/Tcell). This may reflect biological differences between the two species. We did not try the V13 program in combination with any of the solutions because optimal settings for *P. berghei* were not accessible at the time of our trials.

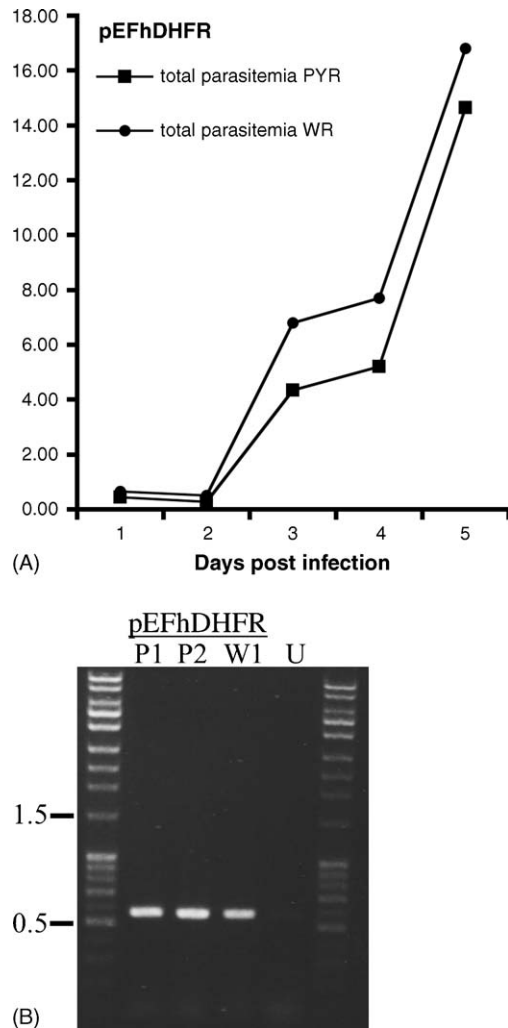


Fig. 4. Transformation of *P. yoelii* YM blood stages with *hDHFR* using the AMAXA nucleofector® system. Expansion of parasitemia in parasites harboring pEFhDHFR plasmid in second generation mice under 1 mg kg⁻¹ pyrimethamine (square) or 1 mg kg⁻¹ WR99210 (circle) selection (A). PCR analysis to demonstrate presence *hDHFR* gene in nucleofected *P. yoelii* YM blood stages of second generation mice (B). P1 and P2 represent total DNA from individual pyrimethamine-treated second generation mice while W1 represents total DNA from WR99210-treated second generation mouse, sacrificed at 5 dpi. U signifies untransformed WT parasite DNA. A 1 kb plus molecular ladder is provided on the left.

Ring-infected erythrocytes demonstrated much weaker GFP fluorescence compared to erythrocytes infected with more mature stages as assessed by microscopy. It is possible that flow cytometry may not be able to detect all the infected from the uninfected cells based on GFP-fluorescence intensity, thus leading to an underestimate of the percentage of GFP-positive infected erythrocytes. Co-labeling with the DNA-specific dye Hoechst 33258, which will improve detection of infected erythrocytes, can enhance the sensitivity of this approach [12,13]. Furthermore, this may be compensated for by enriching for more mature stages prior to sorting, or adjusting for the percentage of rings versus more mature stages in the sample being analyzed. In *P. berghei*, a stably-transfected reporter parasite PbGFP_{con} has been generated that expresses GFP at a high level throughout the complete life cycle [14]. In PbGFP_{con} parasites, the weak GFP-

expression in rings is the result of a integration of a single copy of *gfp* [14]. However, FACS can still be used to sort these reporter parasites by life stage, regardless of co-staining with Hoechst 33258 [14]. In the current work, we have not determined if the decrease in the percentage of GFP-positive parasites is a result of integration of the reporter gene into the genome or expression from a single episomal copy of the gene or an increase in the proportion of ring stage parasites versus mature stages at the time of FACS analysis.

Using existing *P. berghei* vectors, we have demonstrated that a modified version of *TgDHFR* can be used as a positive selectable marker to select for pyrimethamine-resistant transformed *P. yoelii* YM parasites. We have also shown *hDHFR* can be used as a selectable marker to select for WR99210-resistant transformed parasites. Parasites transformed with *PbDHFR* and initially selected with pyrimethamine remain sensitive to WR99210 (data not shown), suggesting that pyrimethamine selection of *TgDHFR* or *PbDHFR* can be followed by WR99210 selection for *hDHFR* transformants. Thus, more complex genetic studies such as targeted disruption of a gene and complementation of the knock out is now feasible.

We have also shown that *P. berghei* promoters can be used to drive expression of reporter genes in *P. yoelii*. We did not explicitly determine whether timing of expression of the promoters is stage-specific as in *P. berghei*. Thus, the variation in the observed fluorescence intensities among the various constructs, in part, may be due to different optimal peak expression times of the different promoters and the proportion of that particular life stage at the time of FACS analysis. It has been shown that the PbEF-1 α promoter is constitutively on throughout blood stage development [15], while *PbDHFR-TS* promoter is on during late trophozoite/young schizogony [16], and the *Pbs21* promoter is on during female gametocyte development [17,18]. In most species, homologous recombination is favored by use of syngeneic DNA. Thus, use of *P. berghei* cassettes should also favor targeting to the desired *P. yoelii* locus.

Better tools and protocols for genetic manipulation of *P. yoelii* should enable us to exploit the utility of this model system in understanding aspects of malaria biology. With the annotated genome sequence for the 17 $\times$ line, we can undertake functional analysis of genes. The *P. yoelii* model has been used for vaccine studies and is thought to mimic more faithfully *P. falciparum* invasion and development in the liver. The similarities between *P. yoelii* and human malarias can now be dissected more rigorously and will hopefully facilitate the identification and development of new targets for chemotherapeutics and vaccines.

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