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Protection against malaria by immunization with non-attenuated sporozoites under single-dose piperaquine-tetraphosphate chemoprophylaxis

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ABSTRACT

Experimental whole-parasite immunization through concurrent administration of infectious *Plasmodium* sporozoites with drugs that prevent pathogenic blood-stage infection represents the current benchmark in malaria vaccine development. Key questions concerning translation remain, including the requirement for single-dose drug regimens that can reliably prevent breakthrough infections. We assessed the feasibility and efficacy of immunization with single-dose piperaquine chemoprophylaxis and concurrent sporozoite administration (PPQ-CPS) in the murine *P. berghei* ANKA/C57BL/6 infection model. We demonstrate that PPQ-CPS is protective with an efficacy comparable to previous findings using whole-parasite immunization under chloroquine chemoprophylaxis. PPQ-CPS immunization resulted in an expansion of intrahepatic and intrasplenic effector memory $CD8^+$ T cells. In summary, PPQ-CPS appears to be a safe and efficacious immunization regimen in the rodent malaria model and may thus become an important improvement regarding the translation of whole-parasite immunization toward a human malaria vaccine.

1. Introduction

Despite a rapid scaling up of malaria control interventions, malaria remains a significant cause of morbidity and mortality, particularly in young children in Sub-Saharan Africa [1]. An effective malaria vaccine could become an important preventive tool, and could thereby lower the burden of malaria worldwide. In recent years, several vaccination strategies based either on whole parasite or subunit approaches have been assessed in clinical trials [2].

The current gold standard for malaria vaccination is the administration of live-attenuated sporozoites, which remains the only vaccination approach resulting in complete protection against malaria infection [3]. Several methods of attenuation have been described, including gamma-irradiation [4], genetic modification [5], and co-administration of blood-stage suppressing chemoprophylactic drugs (chemoprophylaxis with sporozoites, CPS) [6]. Regarding the latter approach, most animal studies [7–10] and the only published trial on immunization by CPS in humans applied chloroquine as the chemoprophylactic agent [11]. More recently, the finding that CPS results in complete protection in malaria has been supplemented by animal studies on alternative drugs, including pyrimethamine [12] as well as clindamycine and azithromycin [13].

Regarding the translation of CPS immunization from experimental studies toward routine vaccination, the safety of vaccination with live parasites under protective drug cover critically

Abbreviations: CPS, chemoprophylaxis with sporozoites; CQ, chloroquine; CQ-CPS, chloroquine chemoprophylaxis with sporozoites; DHA-PPQ, co-formulation of dihydroartemisinin and piperaquine-tetraphosphate; GMP, good manufacturing practice; PPQ, piperaquine-tetraphosphate; PPQ-DW, drinking water supplied with piperaquine-tetraphosphate; PPQ-CPS, single dose piperaquine-tetraphosphate chemoprophylaxis with sporozoites; SPZ, *P. berghei* ANKA sporozoites.

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depends on reliable and sufficient uptake of potent antimalarial drugs. Hence, a simplified chemoprophylactic regimen, limited to as few doses as possible and given prior to administration of infectious SPZ, would be an important improvement to this vaccination strategy.

Piperaquine-tetraphosphate (PPQ) is a 4-aminoquinoline which is structurally related to chloroquine [14]. PPQ has a prolonged elimination half-life of about 14 days in rodents, and 14 and 22 days in children and adults, respectively [15,16]. Due to its long elimination half-life and favorable safety profile, we hypothesized that PPQ might be an interesting drug to apply as single-dose chemoprophylaxis in whole-SPZ immunization. By using the rodent *P. berghei* ANKA/C57BL/6 infection model, our study evaluated the safety and efficacy of single-dose PPQ administration as a simplified CPS immunization regimen.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed according to FELASA category B and GV-SOLAS standard guidelines. Animal experiments were approved by German authorities (Regierungspräsidium Karlsruhe, Germany, §8 Abs. 1 Tierschutzgesetz (TierSchG)).

2.2. Data assessment and statistical analysis

All data assessment and statistical analysis was performed using Prism Version 5.0b for Mac OS X (GraphPad Software Inc., La Jolla, CA, USA) and Stata/IC 13.1 for Windows (StataCorp LP, College Station, TX, USA). Groups of immunized and non-immunized mice were compared using Students-*t* test or Mann–Whitney-*U*-test, as appropriate. Log rank-test was applied to compare survival distributions.Drug administration, animals and immunization procedure

All experiments were carried out with the *Plasmodium berghei* ANKA (*Pb* ANKA) parasite in inbred 6–8 weeks old female C57BL/6J mice (Janvier Labs, France). *Pb* ANKA sporozoites (SPZ) were isolated by dissection of salivary glands from female *Anopheles stephensi* mosquitoes at day 17–25 post infection. Immunizations were performed by intravenous injections of 10^4 or 5×10^4 SPZ at the beginning of the 24 h continuous drug supply period, or concomitant with oral or intraperitoneal drug administration, respectively.

Following the administration of SPZ at day 0, daily blood smears were obtained from day 3 to day 14. Blood smears were assessed by light microscopy of 25 optical fields, and blood-stage infection (pre-patency) was reported when a single parasite was detected on at least one occasion.

For an initial assessment of the safety and efficacy of oral PPQ administration in mice, we continuously supplied PPQ (Sigma-Tau, Rome, Italy) in the drinking water for 24 h following intravenous *Plasmodium* sporozoite inoculation. Following the previous description of continuous oral CQ administration [17], drinking water solutions were prepared containing a PPQ concentration of 1.88 mg/ml and were adjusted to a pH of 4. Glucose powder (5 vol%) was dissolved in the drug solution (hereafter referred to as PPQ-DW) to make it more palatable to the animals. Solutions were administered in tinted light-resistant bottles, and no other liquid source was available. For oral or intraperitoneal drug administration, PPQ or dihydroartemisinin/piperaquine-tetraphosphate (DHA-PPQ, Eurartesim®, Sigma-Tau Arzneimittel GmbH, Germany) was dissolved in sterile phosphate-buffered saline (PBS, Gibco) at concentrations varying from 10 to 37.5 mg/ml PPQ. To allow for optimal solubility of the drug, pH was adjusted to 4–5. A total volume of 0.2 ml was administered by intraperitoneal injection or orally through a gavage tube, resulting in an absolute dosage ranging from 2 mg to 7.5 mg PPQ per mouse.

2.3. Assessment of protection against SPZ challenge

To assess the protective capacity of single-dose PPQ administration in whole-sporozoite immunization (single dose piperaquine in chemoprophylaxis with sporozoites PPQ-CPS), we vaccinated mice by either a single PPQ-CPS or a prime one-boost PPQ-CPS regimen. Following PPQ-CPS immunization, mice were subjected to an intravenous challenge with 10^4 SPZ. Due to the long half-life of PPQ, the intravenous SPZ challenge was performed 10–17 weeks after the immunization procedure (for immunization and challenge regimen see Figure S1 A (single PPQ-CPS regimen) and B (prime one-boost PPQ CPS regimen)). Following the challenge with 10^4 SPZ, daily blood smears were obtained from days 3 to 7 and on days 10 and 14 after challenge.

2.4. Whole-body imaging

The transgenic *P. berghei* line 676m1cl1 (*Pb* GFP-Luc_{con}) [18] was used for real-time in vivo imaging of liver-stage development. *Pb* GFP-Luc_{con} SPZ were injected into the tail vein of C57BL/6J mice. Bioluminescence measurement was performed as previously described [19]. Luciferase activity was visualized immediately after the administration of D-Luciferin through whole-body imaging using an in vivo Imaging System (IVIS 100; Caliper Life Sciences, USA). Bioluminescence was acquired with an exposure time of 180 s and analyzed using Living Image 2.50.1 (Xenogen Corp., Hopkinton, MA, USA).

2.5. Cell preparation, cell staining and flow cytometry

The phenotype (activation status) and polyfunctionality of $CD4^+$ and $CD8^+$ T-lymphocytes from PPQ-CPS vaccinated mice was determined in two independent experiments comparing either mice immunized by a prime-one boost PPQ-CPS ($n = 4$) regimen versus naïve mice ($n = 4$) or mice immunized by a single PPQ-CPS ($n = 5$) regimen versus control mice receiving only PPQ ($n = 4$, immunization and challenge regimen see Figure S1 C). All mice were challenged with 10^4 SPZ i.v. and euthanized 72 h post-challenge. Following intracardial perfusion with 10 ml of PBS, the spleen and liver were removed and prepared as single cell suspensions by homogenization through either a 70 μm cell strainer or a metal sieve in RPMI complete medium (10% FCS, 5 ml 100× MEM NEAA solution (Gibco), 5 ml 100 mM sodium pyruvate solution (Gibco), 5 ml penicillin/streptomycin (Gibco), 10 μl heparin). Liver-derived lymphocytes were isolated by re-suspension in Easycoll (Biochrom) solution (1.6 ml 10× PBS, 29.1 ml HBSS, 14.2 ml Easycoll 1.124 g/ml density, 10 μl Heparin), and the resulting pellet after centrifugation at 2000 rpm was collected. The respective cell suspensions were subjected to erythrocyte lysis (0.037 g EDTA, 1 g $KHCO_3$, 8.26 g NH_4Cl in 1 L ddH_2O, pH 7.4) and subsequently washed and counted in a hemocytometer.

To determine the activation status of the T-lymphocyte population, cells were stained and analyzed with antibodies obtained from eBiosciences for expression of CD8a (clone: 53–6.7/PerCP-Cy5.5), CD4 (clone: RM4-4/FITC), CD62L (clone: MEL-14/PE) and CD44 (clone: IM7/APC) surface markers. Staining was performed for 20 min on ice in the presence of mouse anti-CD16/CD32 (clone 93) antibody to block Fc-receptors. Washed and pelleted cells were re-suspended in 1% PFA in PBS and stored at 4 °C until data acquisition. To assess T-cell polyfunctionality, cells were again harvested at 72 h post-challenge and lymphocyte populations were incubated without additional re-stimulation in the presence of 10 μg/ml Brefeldin A (Sigma–Aldrich) for 4 h at 37 °C. Subsequently, cells

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