International Journal for Parasitology 43 (2013) 869–874

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Succinctus

Immunisation against a serine protease inhibitor reduces intensity of *Plasmodium berghei* infection in mosquitoes

Andrew R. Williams [a,*], Sara E. Zakutansky [a], Kazutoyo Miura [b], Matthew D.J. Dicks [a], Thomas S. Churcher [c], Kerry E. Jewell [a], Aisling M. Vaughan [a], Alison V. Turner [a], Melissa C. Kapulu [a], Kristin Michel [d], Carole A. Long [b], Robert E. Sinden [a,e], Adrian V.S. Hill [a], Simon J. Draper [a], Sumi Biswas [a]

[a] *The Jenner Institute, University of Oxford, Roosevelt Drive, Oxford OX3 7DQ, UK*
[b] *Laboratory of Malaria and Vector Research, NIAID, NIH, Rockville, MD 20852, USA*
[c] *Department of Infectious Disease Epidemiology, Imperial College London, London, UK*
[d] *Kansas State University, Division of Biology, Manhattan, KS 66506, USA*
[e] *Division of Cell and Molecular Biology, Imperial College London, London, UK*

ARTICLE INFO

Article history:
Received 1 April 2013
Received in revised form 6 June 2013
Accepted 12 June 2013
Available online 18 July 2013

Keywords:
Malaria
Vaccine
Mosquito
Antibodies
Serpins

ABSTRACT

The mosquito innate immune response is able to clear the majority of *Plasmodium* parasites. This immune clearance is controlled by a number of regulatory molecules including serine protease inhibitors (serpins). To determine whether such molecules could represent a novel target for a malaria transmission-blocking vaccine, we vaccinated mice with *Anopheles gambiae* serpin-2. Antibodies against *Anopheles gambiae* serpin-2 significantly reduced the infection of a heterologous *Anopheles* species (*Anopheles stephensi*) by *Plasmodium berghei*, however this effect was not observed with *Plasmodium falciparum*. Therefore, this approach of targeting regulatory molecules of the mosquito immune system may represent a novel approach to transmission-blocking malaria vaccines.

Malaria, caused by protozoan parasites of the genus *Plasmodium*, is one of the world's most devastating infectious diseases. It is estimated that more than 500 million people per year develop malaria, with close to 1 million deaths (Murray et al., 2012). Eradication of malaria has been proposed, but for this goal to be achieved a highly effective vaccine will be necessary. *Plasmodium* has a complex lifecycle that involves asexual replication in both the liver and red blood cells of the vertebrate host and sexual reproduction within the midgut of the *Anopheles* mosquito vector. Vaccines can target the parasite at any one of three different stages; the pre-erythrocytic stage (either the sporozoite or the infected hepatocyte), the erythrocytic stage or within the mosquito. Vaccines that target the parasite at the mosquito stage prevent transmission of the parasite through the vector – hence, they are known as transmission-blocking vaccines (TBVs). TBVs aim to induce herd immunity amongst a community and their development is likely to be essential for the eradication of malaria (The malERA Consultative Group on Vaccines, 2011). Several TBV candidate antigens have been studied, with the majority consisting of proteins on the surface of gametocytes, gametes or the ookinete, and antibodies against these can substantially reduce transmission in pre-clinical models (Sinden, 2010; The malERA Consultative Group on Vaccines, 2011).

In addition to targeting antigens on the parasite, antigens located in the mosquito (which are essential for parasite development) have also shown potential as TBV candidates. For example, it has been shown that immunising mice with either an *Anopheles* aminopeptidase (APN1) (Dinglasan et al., 2007), or carboxypeptidase (CPBAg1) (Lavazec et al., 2007), can raise antibodies that block the transmission of *Plasmodium* parasites, suggesting an important role of specific mosquito molecules in parasite invasion of the mosquito midgut. In recent years, much attention has also been focused on understanding the mechanisms behind mosquito innate immunity. After taking a blood meal, mosquitoes mount a potent, non-specific innate immune response that is thought to protect against establishment of bacteria in the midgut as a result of blood-feeding (Dong et al., 2009). This innate immunity can also act against *Plasmodium* parasites and in fact the mosquito immune response normally clears the vast majority of the invading parasites (Alavi et al., 2003). It is therefore possible that this natural resistance may be exploited to prevent the transmission of malaria.

* Corresponding author. Current address: Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 15, DK-1870 Frederiksberg C, Denmark. Tel.: +45 353 32797.
E-mail address: arw@sund.ku.dk (A.R. Williams).

0020-7519/$36.00
http://dx.doi.org/10.1016/j.ijpara.2013.06.004

Innate parasite rejection is mediated by lysis and melanin neutralisation (Blandin et al., 2004). It is apparent that this process is controlled by a number of regulatory molecules that prevent the immune response from over-activation. For example, serpins, a group of serine protease inhibitors present in all eukaryotes, negatively regulate insect immune responses to bacteria and protozoan parasites (Ligoxygakis et al., 2002; Michel et al., 2005). The importance of serpins in controlling the mosquito innate immune response has been demonstrated by RNA interference (RNAi) silencing of the serpin-2 (*SRPN2*) gene in *Anopheles gambiae*, leading to massive formation of pseudotumors and mosquito death (Michel et al., 2005; An et al., 2011). Strikingly, knockdown of SRPN2 also dramatically reduced the numbers of oocysts during infection with the rodent malaria parasite *Plasmodium berghei* (Michel et al., 2005). Consequently, some groups have proposed the idea of genetically modifying mosquitoes to either over-express genes involved in parasite killing or under-express genes involved in regulation of these highly potent immune mechanisms (Dong et al., 2011), thus rendering them refractory to *Plasmodium* infection and unable to transmit the parasite to humans.

Here we explored whether molecules that regulate the innate immune response within the mosquito could also be candidate antigens for a malaria TBV. We hypothesised that antibodies against these molecules would inhibit their function, result in increased activation of the mosquito innate immune response and reduce transmission of the parasite. We report that immunisation of mice with *A. gambiae* SRPN2 (AgSRPN2) raises antibodies that significantly reduce the intensity of *P. berghei* infection in *Anopheles stephensi*, suggesting that this approach warrants further investigation as a novel strategy for the development of malaria TBVs.

Recombinant adenoviral and poxviral vectors expressing the full-length AgSRPN2 protein, excluding the signal peptide (amino acids, aa1-21) were generated. The AgSRPN2 gene (AGAP006911-PA/XP_308845), aa 22–409 was codon optimised for expression in humans and synthesised by GeneArt GmbH. A BLAST search indicated that AgSRPN2 had a maximum of 33% identity with murine proteins, the closest match being a serpin B11 (AAH10313.1), with other serpins having around 25–26% sequence identity. This gene was cloned into ChAd63 and MVA shuttle vectors, downstream of the human tissue plasminogen leader sequence in order to aid secretion of the antigen from virally infected/immunised cells, and the recombinant viruses were generated as previously described for other antigen inserts (Douglas et al., 2011; Goodman et al., 2011). Mice were then immunised in a heterologous prime-boost regime which has previously been shown to induce antibodies in both pre-clinical studies and in Phase I/IIa clinical trials of blood-stage malaria vaccine candidates (Draper et al., 2008; Goodman et al., 2011; Sheehy et al., 2012). Six week old female BALB/c mice were obtained from Harlan, UK. Mice were first primed with i.m. injection of 1×10^8 infectious units (iu) of ChAd63 and then 8 weeks later were boosted i.m. with 1×10^7 plaque forming units (pfu) of MVA. Control mice were immunised with vectors expressing an irrelevant antigen (GFP).

Immunogenicity of the vectors was confirmed by ELISA using recombinant AgSRPN2 protein as antigen. Nunc Maxisorp plates (Fisher Scientific, UK) were coated at 100 ng per well with recombinant active AgSRPN2 (produced as described by An et al. (2011)) and left overnight. Plates were washed six times the next day with PBS containing 0.05% Tween 20 (PBS/T) and blocked with 10% skimmed milk in PBS/T for 1 h. Sera were added and incubated for 2 h. Alkaline phosphatase-conjugated goat anti-mouse IgG at 1:5000 dilution was used for detection. Plates were washed and bound antibodies were detected by adding p-nitrophenylphosphate substrate (pNPP, Sigma, UK) diluted in diethanolamine buffer (Fisher Scientific, UK). OD 405 nm (OD_{405}) was read using an ELx800 microplate reader (BioTek, UK). End-point titers were taken as the *x*-axis intercept of the dilution curve at an absorbance value three S.D.s. greater than the OD_{405} for naïve mouse serum.

Anti-AgSRPN2 antibody titres were below the assay detection limit in mice immunised with GFP (data not shown). In contrast, anti-AgSRPN2IgG antibodies were induced by the priming AgSRPN2 immunisation, with titers significantly higher at day 55 than at day 14 ($P < 0.05$ by repeated-measures ANOVA), and these were boosted further by the MVA immunisation (Fig. 1). We thus confirmed that vectors expressing a component of the mosquito immune system are immunogenic in mammals, using a vaccine delivery platform that is safe and induces antibodies in humans (Sheehy et al., 2012).

Next, the functional transmission-blocking activity of the vaccine-induced antibodies was tested. First, we used an ex vivo direct membrane feeding assay (DMFA) using the rodent malaria parasite *P. berghei*. Two weeks after the final immunisation, mice were bled by cardiac puncture under terminal anaesthesia to harvest serum. In parallel, the *P. berghei* ANKA strain (clone 2.34) was maintained by serial passage in TO mice. For membrane feeding assays, hyperreticulocytosis was induced in mice by injection of 6 mg/mL of phenylhydrazine 3 days prior to infection with 10^7 parasitised red blood cells (pRBCs). Three days after infection, exflagellation of male gametocytes was checked as described previously (Blagborough and Sinden, 2009). Blood containing infectious *P. berghei* gametocytes was then collected by cardiac puncture, split into two equal aliquots and mixed with pooled serum from mice immunised with either AgSRPN2 or GFP (each $n = 8$) at a dilution of 1:5 serum:blood and fed to *A. stephensi* mosquitoes through plastic membrane feeders. Unfed mosquitoes were removed the next day and the infected mosquitoes were then maintained at 19 °C and 70% relative humidity. Midguts were dissected 11 days after infection and stained with 0.05% mercurochrome for enumeration of oocysts. DMFAs were performed on three separate occasions. Parasite burdens were significantly reduced in mosquitoes that were fed with blood containing anti-AgSRPN2 antibodies compared with serum from GFP-immunised controls, with an average reduction in oocyst intensity (i.e. the number of oocysts per mosquito) of 54% (95% confidence interval, (CI) 34–67%; $P < 0.0002$ using a generalised-linear mixed model, GLMM (Churcher et al., 2012); Fig. 2). There was only a modest reduction of 10% (CI –0.3–30%) in oocyst prevalence (the number of infected mosquitoes, i.e. those with at least one oocyst), which was not significant ($P = 0.055$ using a GLMM with binomial error structure).

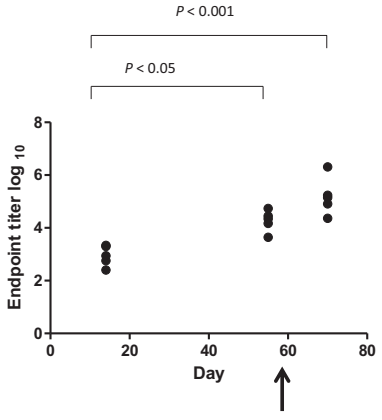

Fig. 1. *Anopheles gambiae* serpin-2-specific total IgG responses following immunisation. BALB/c mice ($n = 5$) were immunised with Ad-MVA *Anopheles gambiae* serpin-2 (ChAd63AgSRPN2 prime, MVA *Anopheles gambiae* serpin-2 boost). Total IgG responses against recombinant *Anopheles gambiae* serpin-2 protein were measured by ELISA in the serum of mice taken at the number of days following first immunisation as shown. The arrow indicates the day of the boosting immunisation.