Inquiro, the UAB undergraduate science research journal

Volume 2008 | Number 2 Article 16

2008

Utilizing a Unique Study in the Peruvian Amazon to Assess the Malaria Vaccine Candidate Antigen Plasmodium falciparum Merozoite Surface Protein 6

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short report

"Utilizing a Unique Study in the Peruvian Amazon to Assess the Malaria Vaccine Candidate Antigen Plasmodium falciparum Merozoite Surface Protein 6":

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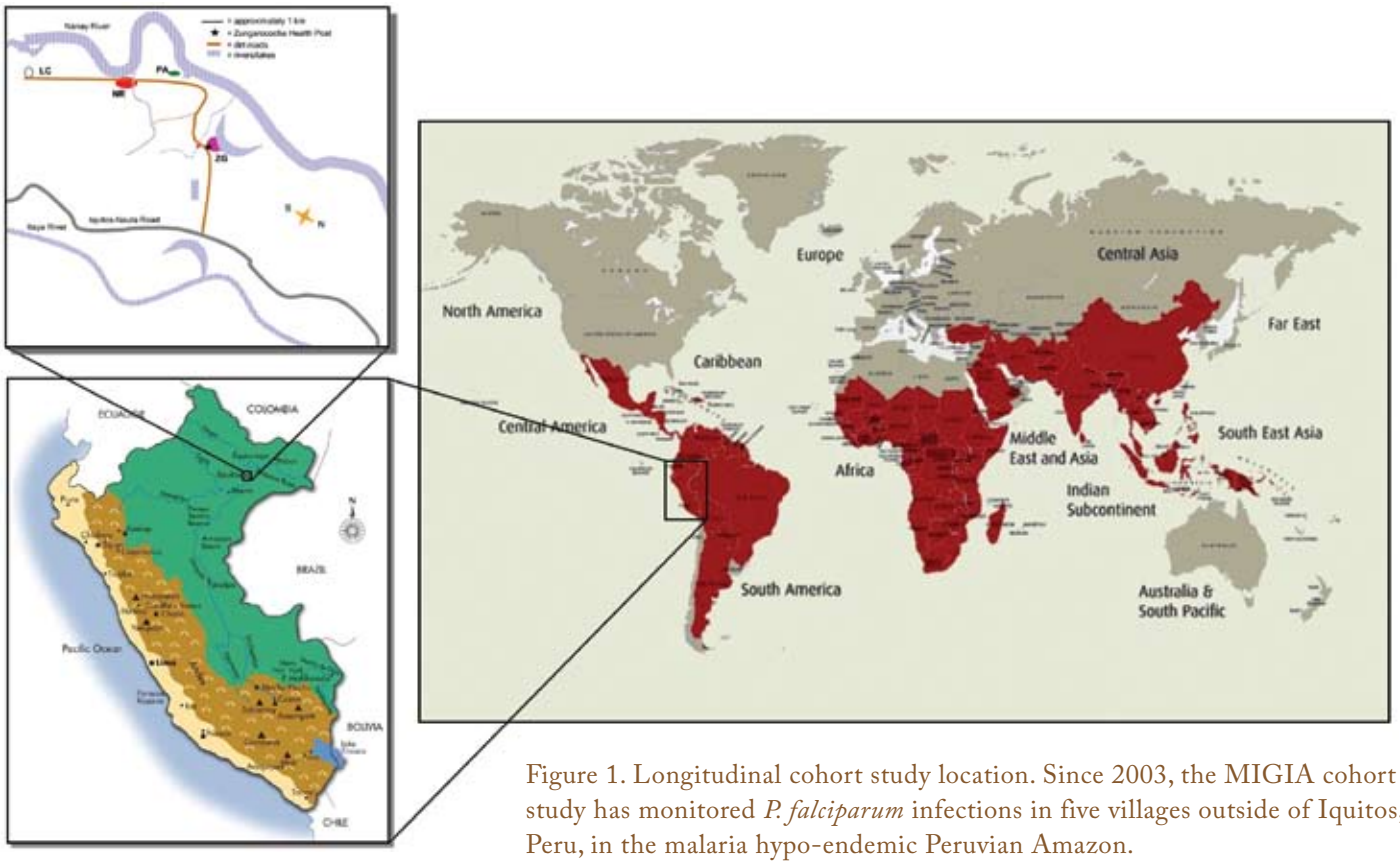


Figure 1. Longitudinal cohort study location. Since 2003, the MIGIA cohort study has monitored *P. falciparum* infections in five villages outside of Iquitos, Peru, in the malaria hypo-endemic Peruvian Amazon.

Abstract

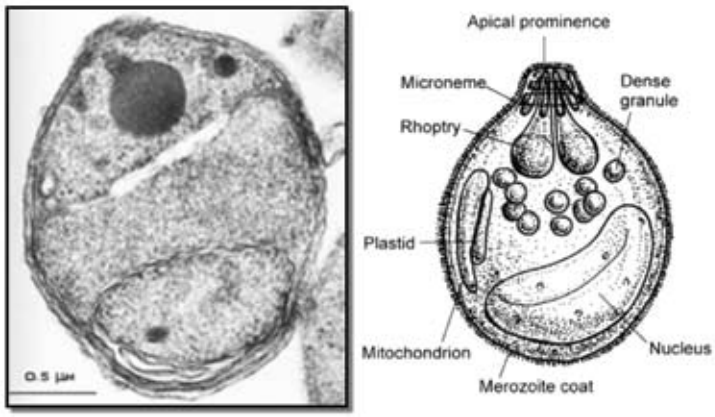
Malaria is responsible for 1-3 million deaths annually, mostly in children under age five. Almost all malaria deaths are due to infection by the protozoan parasite *Plasmodium falciparum*, but no effective vaccine exists to prevent *P. falciparum* malaria. This ongoing study focuses on *P. falciparum* Merozoite Surface Protein 6 (PfMSP6), a dimorphic antigen present on the surface of the *P. falciparum* parasite during erythrocyte invasion. Although PfMSP6 is a promising vaccine candidate, there currently exists no data to establish which PfMSP6 domain(s) should be included in a vaccine. To provide this data, we utilized samples from a unique longitudinal cohort study in the hypo-endemic Peruvian Amazon in which blood and serum samples from *P. falciparum* infected patients have been collected over four years. Using these serum samples, indirect ELISAs were conducted in Iquitos, Peru, to establish which sub-domain(s) of PfMSP6 are targeted by naturally generated antibodies. Serum from 243 patients, separated into time of infection samples and post-infection samples, was tested against the N-terminus of the PfMSP6 allele types HB3 and Dd2. For the time of infection samples, serum from 243 patients was tested, resulting in 79 positive responders, 49 of which responded against both allele types. For the post-infection samples, serum from 56 patients was tested, resulting in 5 positive responders. Though this data is very preliminary, the results indicate that PfMSP6 generates a lower level of positive responders than is seen with other Merozoite Surface Proteins. However, when anti-PfMSP6 antibodies are generated, they appear to cross-protect between allele types, a rarity among *P. falciparum* vaccine candidates. This initial exploration of the immunogenicity of PfMSP6 will provide a foundation for later studies to establish which PfMSP6 sub-domain(s) and/or allele(s) should be included in a malaria vaccine.

Introduction

Mosquito-borne *Plasmodium* parasites cause 300-500 million cases of malaria each year, resulting in a devastating 1-3 million deaths worldwide, mostly in children under the age of five (Breman, 2001). Of the four protozoan parasites that

Figure 2. (left) Conducting indirect ELISAs in Peru. All indirect ELISAs were conducted on-site in Iquitos, Peru, in the labs of the MIGIA cohort study.

Figure 3. (below) SEM image and schematic diagram of *P. falciparum* merozoite. Merozoites are the invasive form of *P. falciparum* during the blood stages. PfMSP6, a component of the surface coat, is exposed to the antibody-mediated immune system.



cause the disease, *Plasmodium falciparum* is responsible for the majority of the infections and almost all of the deaths, making it the most aggressively-combated target. The rapid spread of drug resistance amongst the parasite has further increased the need for an effective *P. falciparum* vaccine.

Of particular interest in vaccine research is the merozoite, the invasive form of the parasite during the blood stages (See Figure 3). Prior to erythrocyte entry, the merozoite is exposed to the antibody-mediated immune system, a critical event for vaccine success. Discovered in early 2001, the vaccine candidate PfMSP6, a 36 kDa secreted antigen associated with the surface of *P. falciparum* merozoites (Trucco *et al.*, 2001), has shown promise but lacks significant data. The antigen consists of a highly-conserved C-terminal domain and a variable N-terminal domain that gives rise to two distinct allele classes, HB3 and Dd2 (Pearce *et al.*, 2004) (See Figure 4). Though individuals protected against *P. falciparum* malaria have a strong antibody response to PfMSP6 (Singh *et al.*, 2005), it is unknown whether individuals develop antibodies against one or both domains, or against one or both allele classes.

The purpose of this project was to collect preliminary data on the anti-PfMSP6 antibody responses of patients in a malaria-endemic environment. To accomplish this, we utilized a unique longitudinal cohort study in the hypo-endemic Peruvian Amazon. (See Figure 1). Since 2003, the Malaria Immunology and Genetics in the Amazon (MIGIA) Project has documented and collected blood samples from natives in communities outside of Iquitos, Peru. The low transmission rate of 0.4215 infections per person per year in these villages has allowed us to examine patient samples both at the time of *P. falciparum* infection and up to two months after the same infection (Branch, *et al.*).

Materials and Methods

Human Serum Samples

P. falciparum-infected patient serum samples were obtained through the MIGIA cohort study. When patient blood is collected, it is centrifuged to separate the serum from the erythrocytes. While the erythrocyte fraction will contain any *P. falciparum* merozoites, the serum fraction will contain any human antibodies. Use of the serum samples was approved by the UAB Institutional Review Board for Human Use (IRB protocol number X080403004).

Recombinant Antigen Generation

We generated two histidine-tagged recombinant PfMSP6 antigens consisting of the N-terminal domain of the HB3 allele and the N-terminal domain of the Dd2 allele. Expression of the C-terminal domain of PfMSP6 was attempted, but failed. Efforts to express this antigen are ongoing. The antigen constructs were made by PCR from Peruvian *P. falciparum* genomic DNA that was genotyped and sequenced to ensure that the recombinant antigens were consistent with the genotypes of the study population. The PCR amplicons were cloned into pET 15-b (EMD Biosciences) and sequenced. The recombinant antigens were then expressed in Rosetta strain *E. coli*. From the soluble fraction of a bacterial lysate, the recombinant antigens were purified using

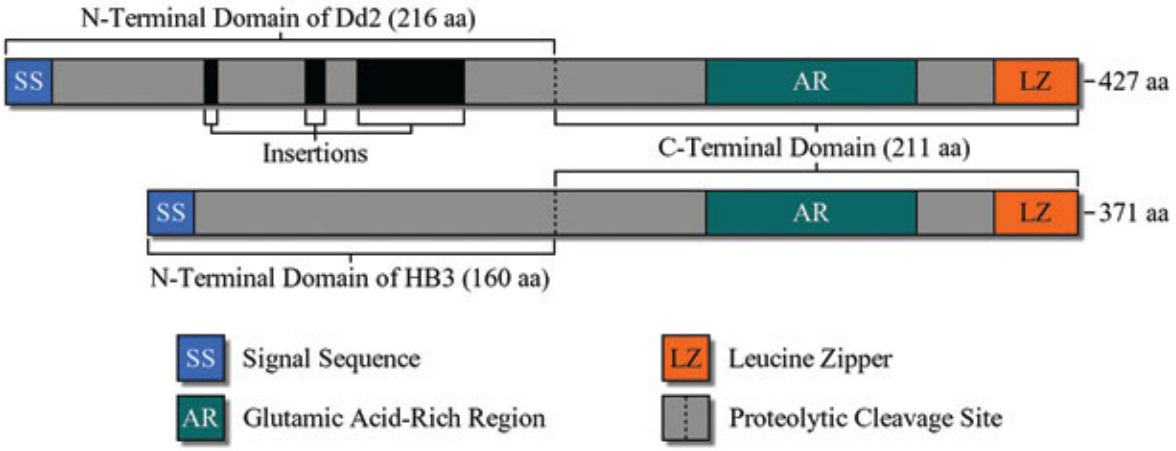


Figure 4. Schematic diagram of PfMSP6 domain structure and alleles. PfMSP6 is a dimorphic antigen consisting of the allele classes HB3 and Dd2, which differ at several polymorphisms in the N-terminal domain.

affinity chromatography (nickel column), followed by anion-exchange chromatography (MonoQ column). After purification, antigen folding was verified by circular dichroism spectroscopy.

Indirect ELISAs

Indirect ELISAs were used to detect any naturally generated anti-PfMSP6 antibodies. All ELISAs were conducted in the research labs of the MIGIA cohort study in Iquitos, Peru (See Figure 2). 96-well plates were coated at a recombinant antigen concentration of 50 ng/well, using only one recombinant antigen in each experiment. 100µL of sera diluted 1:100 in phosphate buffer was added to each well, followed by multiple washes and the addition of horse-radish peroxidase (HRP) linked mouse anti-human secondary antibodies. After the addition of HRP substrate, each reaction was stopped and each plate was read at 450nm using an ELISA plate reader. An optical density (OD) was obtained for each reaction. Each serum sample was tested against both antigens and was run in duplicate, using the average of the OD values for analysis.

Negative controls consisted of serum from patients living in the city of Iquitos who have not traveled out of the city and have no record or recollection of having *P. falciparum* malaria. Positive controls were serial dilutions of a positive pool consisting of individuals known to have strong anti-*P. falciparum* antibody responses. Unfortunately, the positive pool that had been established for the antigen PfMSP3 did not function for PfMSP6. As a result, antibody levels in patient serum samples could not be quantified. Samples were instead assigned values of "positive" if the average OD value was greater than the OD values of the negative controls or "negative" if the average OD value was less than the OD values of the negative controls.

Results and Discussion

Serum from a total of 243 Peruvian patients was tested against the N-terminus of the PfMSP6 allele types HB3 and Dd2. Of these 243 patients, all had serum extracted during *P. falciparum* infection. Figure 1 indicates the results of ELISAs using time of infection serum samples. A total of 79 patients responded as positive, constituting 32.5% of the patients tested. Compared to other MSPs, particularly PfMSP3, this response appears low. However, unlike other MSPs, anti-PfMSP6 antibodies seem to cross-protect against allele types. Of the 79 positive responders, 49 showed a positive response against both allele classes, even though only one infection occurred. The potential of cross-protection among anti-PfMSP6 antibodies warrants further investigation of PfMSP6 as a malaria vaccine candidate.

Of the 79 positive responders, 56 patients had blood drawn at regular intervals after *P. falciparum* infection. These intervals consisted of one week, two weeks, three weeks, one month, and two months post-infection. Figure 2 indicates the results of ELISAs using post-infection serum samples. Of the 56 patients tested, 5 showed a positive response at least one week after infection. The longest positive response detected lasted one month post-infection. The low longevity of anti-PfMSP6 antibodies was not entirely unexpected since this is commonly seen with antibodies generated against other MSPs.

The results of this preliminary study suggest that PfMSP6 is not very immunogenic. Despite this, when antibodies are generated, they appear to cross-protect between allele types. Since cross-protection is uncommon among antibodies generated against MSPs, PfMSP6 should not be overlooked as a vaccine candidate. The authors intend to elaborate on this research in the future to provide sufficient information

regarding the immunogenicity and vaccine potential of PfMSP6.

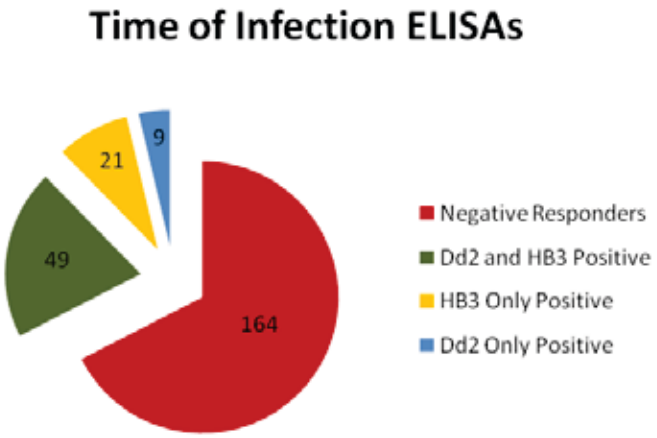


Figure 5. Results of time of infection ELISAs. Serum extracted from *P. falciparum* infected patients during infection was tested against recombinant PfMSP6 N-terminal domains of allele classes HB3 and Dd2.

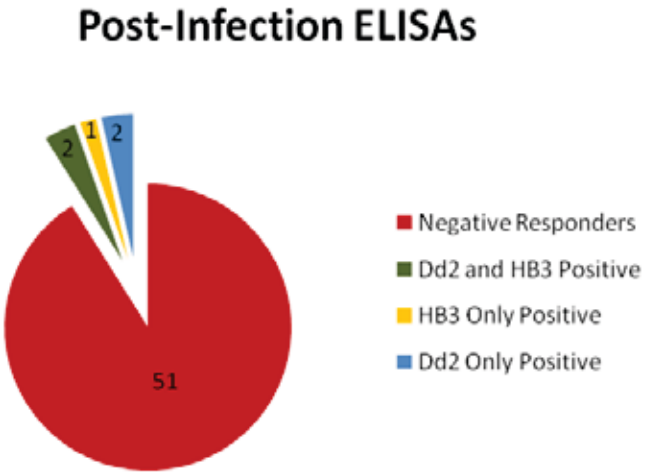


Figure 6. Results of post-infection ELISAs. Serum extracted from *P. falciparum* infected patients post-infection was tested against recombinant PfMSP6 N-terminal domains of allele classes HB3 and Dd2.

Acknowledgements

The authors would like to thank the Sparkman Center for Global Health and the UAB Framework Program for Global Health. Thanks are also due to Eva Clark, the MIGIA project team, and the participants within the MIGIA cohort study.

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