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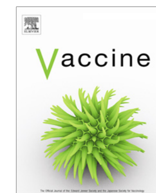

Comparison of functional assays used in the clinical development of a placental malaria vaccine

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ABSTRACT

Background: Malaria in pregnancy is associated with significant morbidity in pregnant women and their offspring. *Plasmodium falciparum* infected erythrocytes (IE) express VAR2CSA that mediates binding to chondroitin sulphate A (CSA) in the placenta. Two VAR2CSA-based vaccines for placental malaria are in clinical development. The purpose of this study was to evaluate the robustness and comparability of binding inhibition assays used in the clinical development of placental malaria vaccines.
Methods: The ability of sera from animals immunised with different VAR2CSA constructs to inhibit IE binding to CSA was investigated in three *in vitro* assays using 96-well plates, petri dishes, capillary flow and an *ex vivo* placental perfusion assay.
Results: The inter-assay variation was not uniform between assays and ranged from above ten-fold in the flow assay to two-fold in the perfusion assay. The intra-assay variation was highest in the petri dish assay. A positive correlation between IE binding avidity and the level of binding after antibody inhibition in the petri dish assay indicate that high avidity IE binding is more difficult to inhibit. The highest binding inhibition sensitivity was found in the 96-well and petri dish assays compared to the flow and perfusion assays where binding inhibition required higher antibody titers.
Conclusions: The inhibitory capacity of antibodies is not easily translated between assays and the high sensitivity of the 96-well and petri dish assays stresses the need for comparing serial dilutions of serum. Furthermore, IE binding avidity must be in the same range when comparing data from different days. There was an overall concordance in the capacity of antibody-mediated inhibition, when comparing the *in vitro* assays with the perfusion assay, which more closely represents *in vivo* conditions. Importantly the ID1-ID2a protein in a liposomal formulation, currently in a phase I trial, effectively induced antibodies that inhibited IE adhesion in placental tissue.

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1. Introduction

Infection with *Plasmodium falciparum* in pregnancy is associated with maternal anemia, low birth weight and stillbirths [1,2]. The infected erythrocytes (IE) adhere to chondroitin sulphate A (CSA) in the placenta [3]. The binding is mediated by the *P. falciparum* erythrocyte membrane protein 1 VAR2CSA [4]. After exposure to *P. falciparum* during pregnancy, women develop VAR2CSA specific antibodies protecting them during subsequent pregnancies [5,6]. Substantial evidence suggests that antibodies inhibiting the VAR2CSA-CSA binding convey protection against placental malaria [7,8]. Although opsonising antibodies might contribute to immunity [9], activated monocytes and macrophages might cause pathology [10]. Therefore, the induction of anti-adhesive VAR2CSA IgG is the focus for vaccine design. Two VAR2CSA based vaccines are currently in phase I clinical trials [11,12]. The large full-length VAR2CSA protein may not be feasible as a vaccine, and identification of a construct that elicits protective antibodies has been a

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central goal for vaccine development. The ability of VAR2CSA constructs to elicit anti-adhesive antibodies in animals has been evaluated using different binding inhibition assays. Commonly used binding inhibition assays in vaccine development are: (i) a high-throughput 96-well assay [13], (ii) a petri dish assay [14] and (iii) a flow assay [15]. The 96-well and petri dish assays are static assays where IEs bind to chondroitin sulphate proteoglycan (CSPG) coated onto plastic surfaces, where after non-adherent cells are washed off. The flow assay use channels or chambers coated with CSA [15], CSA-expressing cells such as BeWo [16] or primary trophoblast [17], or placental cryosections [18]. A weakness of *in vitro* models is the difficulty of translating IE binding inhibition to the *in vivo* situation and to which degree they represent correlates of immunity.

We recently described adhesion of *P. falciparum* IEs in a more biologically relevant placental perfusion model, where IE circulate through the intervillous space of a non-fixed intact human placental cotyledon [19].

It has not been established to which degree results from binding inhibition studies can be translated between assays. Laboratories involved in vaccine development have used different assays complicating interpretation and comparison of vaccine candidate performance. Identification of potential surrogate measures of protection could reduce duration of clinical trials to measure the efficacy of a placental malaria vaccine. Therefore, an understanding of performance of the different assays will be important for the further clinical development of VAR2CSA based placental malaria vaccines. The objective of this study was to evaluate the robustness and read-outs of binding inhibition assays used for clinical development of placental malaria vaccines.

2. Materials and methods

2.1. Parasite cultures

Parasite isolates were cultured *in vitro* as previously described [20,21]. FCR3 parasite cultures were selected for binding to BeWo cells (purchased from American Type Culture Collection (http://www.lgcpromochem-atcc.com) [22,23], producing an isolate exclusively expressing VAR2CSA [20]. Cultures were regularly analysed for mycoplasma infection and genetic identity verified by PCR [24,25].

2.2. Protein production

The recombinant full-length VAR2CSA antigen and VAR2CSA constructs were produced as described (Table 1) [26–28]. All proteins were quality controlled by reduced and non-reduced SDS page, ELISA and western blot [27,29].

2.3. Immunizations

Rat (Wistar (inbred), Taconic, Denmark) antisera were produced as previously described [30]. Rabbit (New Zealand White (outbred), HB Lidköping Kaninfarm, Sweden) FV2 antisera were produced by subcutaneous injection and collected 10–14 days after the final boosting injection. The proteins for rat and rabbit immunisations were adjuvanted with Freund's adjuvant (Sigma-Aldrich) or Alhydrogel (Statens Serum Institut, Denmark). Mice ID1-ID2a antisera were produced in adult mice (C57bl/6 (inbred), Taconic, Denmark) by intra-muscular injection. The protein was adjuvanted with a liposomal formulation (Infectious Disease Research Insititute, Seattle, USA) [31]. Groups of 8 mice were immunized and sera were collected 7 days after the final boosting injection and pooled.

An overview of constructs and immunizations is shown in Table 1.

2.4. Binding inhibition assays

2.4.1. 96-well assay

A high-throughput binding assay was performed as described previously [13]. Briefly, 2×10^5 tritium-labelled late-stage IE and immune-sera or control pre-immunization serum from each species at 1:10, 1:100, 1:1000 and 1:2000 in were added in triplicates to wells coated with 2 µg/ml Decorin (D8428; Sigma-Aldrich). Background binding was evaluated in BSA coated wells. After incubation for 90 min at 37 °C, unbound IE were washed off by resuspension. The number of adhering IE was determined by liquid scintillation counting. Three individual experiments were performed.

A binding ratio was obtained by dividing the median number bound IE in each sample by the median number bound IE in wells with IE incubated with pre-immunization sera from the same species and at the same concentration.

The inter-assay variation of FCR3-CSA binding was determined using the mean binding on each day to calculate a coefficient of variation (CV-standard deviation of the day means/mean of the day means).

The proportion of IE binding in a well was estimated after determining the counts per minute/IE in wells with a known number of IE.

2.4.2. Petri dish assay

The assay was performed as described by Saveria et al. [14] with some modifications. 22 spots in a Petri dish (Falcon 351029) were

Table 1
Protein production and immunisations.

Sera	VAR2CSA construct	Expression system	Species	Adjuvant	96 well	Petri dish	Flow	Perfusion
1	FV2[d]	Baculo[c]	Rabbit	Freund's[a]	X	X	X	X
2	ID1-DBL4	Baculo[c]	Rabbit	Freund's[a]	X	X	X	X
3	ID1-ID2a	S2[b]	Rabbit	Freund's[a]	X	X	X	X
4	ID1-ID2a	S2[b]	Rabbit	Alhydrogel	X	X	X	X
5	ID1-ID2a	S2[b]	Mouse	IDRI LS-127	X	X	X	X
6	FV2[d]	Baculo[e]	Rat	Freund's[a]	X	X	Not done	X
7	ID1-ID2a	S2[b]	Rat	Alhydrogel	X	X	Not done	X
8	NTS-ID2a	E. coli	Rat	Alhydrogel	X	X	Not done	X
9	DBL3-4	Baculo[c]	Rat	Freund's[a]	X	X	Not done	X
10	DBL2-3	Baculo[c]	Rat	Freund's[a]	X	Not done	Not done	X

[a] Primary dose in Freund's complete adjuvant (Sigma-Aldrich) followed by booster injections in Freund's incomplete adjuvant.
[b] Expression using the pExpreS2-1 plasmid (Expres2ion) in *Droshophila* Schneider 2 cells (Expres2ion).
[c] Expression using the baculovirus vector pAcGP67-A (BD Bioscience) in High-Five insect cells (BD Biosciences).
[d] FV2: Full-length VAR2CSA.

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