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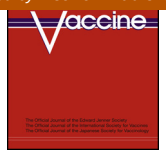

Differential adhesion-inhibitory patterns of antibodies raised against two major variants of the NTS-DBL2X region of VAR2CSA

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ABSTRACT

Background: VAR2CSA is a large polymorphic Plasmodium falciparum protein expressed on infected erythrocytes (IE) that allows their binding in the placenta, thus precipitating placental malaria (PM). The N-terminal part of VAR2CSA that contains the binding site to placental chondroitin sulfate A (CSA) is currently recognized as the most attractive region for vaccine development. An ultimate challenge is to define epitopes in this region that induce a broad cross-reactive adhesion inhibitory antibody response.
Methods: Based on phylogenetic data that identified a dimorphic sequence motif in the VAR2CSA DBL2X, we raised antibodies against the NTS-DBL2X constructs containing one sequence or the other (3D7 and FCR3) and tested their functional properties on P. falciparum isolates from pregnant women and on laboratory-adapted strains.
Results: The CSA binding inhibitory capacity of the antibodies induced varied from one parasite isolate to another (range, 10%–100%), but the combined analysis of individual activity highlighted a broader functionality that increased the total number of isolates inhibited. Interestingly, the differential inhibitory effect of the antibodies observed on field isolates resulted in significant inhibition of all field isolates tested, suggesting that optimal inhibitory spectrum on field isolates from pregnant women might be achieved with antibodies targeting limited variants of the N-terminal VAR2CSA.
Conclusions: Our findings indicate that the NTS-DBL2X region of VAR2CSA can elicit strain-transcending anti-adhesion antibodies and suggest that the combination of the two major variants used here could represent the basis for an effective bivalent VAR2CSA-based vaccine.

1. Introduction

The pathogenesis of malaria during pregnancy results from the selective accumulation of *Plasmodium falciparum* infected erythrocytes (IE) in the intervillous spaces of the placenta [1,2]. This placental sequestration of IE severely increases the risks of miscarriage, maternal anemia and low birth weight [3]. A member of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) family, named VAR2CSA, is the parasite ligand for chondroitin sulfate A (CSA) [4], a glycosaminoglycan present in the placenta. CSA is considered to be the principal or only placental receptor for IE [5], and selective disruption of the *var2csa* gene abrogates the binding ability of IE to CSA *in vitro* [6]. VAR2CSA has been shown to be predominantly expressed by placental isolates [7–9], and plasma from individuals in malaria-endemic regions recognizes VAR2CSA-expressing isolates in a sex-specific and parity-dependent manner [10,11]. High plasma levels of anti-VAR2CSA antibodies are associated with improved pregnancy outcomes in areas where women are exposed to *P. falciparum* infections [10–12]. Recombinant proteins reproducing selected extra-cellular domains of VAR2CSA

Abbreviations: PM, placental malaria; IE, Pfalciparum-infected erythrocytes; *Pf*EMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; CSA, chondroitin sulfate A; CSPG, chondroitin sulfate proteoglycan; DBL, Duffy binding-like; Id, inter-domain; NTS, N-terminal sequence; DSM, dimorphic sequence motif; PBS, phosphate buffered saline; BSA, bovine serum albumin; MFI, median fluorescence intensity; IPTp, intermittent preventive treatment during pregnancy.

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are able to induce antibodies that efficiently inhibit CSA-binding of IE [13–16]. The combination of these observations clearly identifies VAR2CSA as the main target of acquired protective immunity to placental malaria (PM), and it is thus a candidate for the development of a vaccine against this clinical malaria syndrome.

VAR2CSA is a large protein with a molecular weight of ~350 kDa formed by six Duffy binding-like domains (DBL1-6) and four inter-domains (Id1-4) [17–19]. One of the major challenges in vaccine development is to identify a minimal region that induces strain-transcending inhibitory antibodies. We recently showed that antibodies to the N-terminal region of VAR2CSA display broad and strong adhesion-blocking capacity on field isolates [20,21]. The identification of the minimal CSA-binding in this region [22–24], suggests that efficient anti-adhesion and very likely naturally-acquired protective antibodies bind to this part of VAR2CSA.

Another challenge to vaccine development is the sequence polymorphism present in VAR2CSA [8,18]. Antibodies raised against a single FCR3 variant of NTS-DBL2X inhibited CSA-binding of field isolates with an average inhibition level of 65% (range, 32–95%) [20], suggesting that antibodies against more than one variant would be needed for optimal protection. We have previously identified a dimorphic sequence motif (DSM) in the DBL2X domain of VAR2CSA [25] with two distinct phylogenetic groups of FCR3 and 3D7 types. In this study, we examined the prevalence of each DSM type among *P. falciparum* parasites isolated from Beninese pregnant women. Furthermore, we examined the adhesion inhibitory capacity of antibodies raised against each of the NTS-DBL2X variants on field and laboratory-maintained isolates.

2. Materials and methods

2.1. Ethics statement

Pregnant women were included in this study after written informed consent was obtained. The study was approved by the ethical committee of the Faculty of Health Science (University of Abomey-Calavi, Benin). Animal immunization followed the FELASA (Federation of Laboratory Animal Science Associations) guidelines and was approved by the ethical committee affiliated to the University Paris Descartes.

2.2. P. falciparum isolates

P. falciparum IE were obtained from pregnant women at Suru Léré maternity clinic, Cotonou, Benin. The study site is characterized by hyper-endemic malaria and high transmission with two peaks during the two rainy seasons [26]. Pregnant women attending antenatal visit or admitted for delivery were screened for malaria using a rapid diagnostic test Parascreen™ (Zephyr Biomedicals Goa, India). Eight milliliters of venous blood were collected from women with plasmodial infection, in vacutainers with citrate phosphate dextrose adenine anticoagulant. Two hundred μl of erythrocyte pellets were stored at −20 °C for subsequent DNA extraction or homogenized in 10 volumes of TRIzol reagent (Invitrogen) and stored at −80 °C until RNA extraction. IE pellets were immediately cultured *in vitro* to trophozoite-stage as described [27]. Briefly, isolates were grown in RPMI 1640 supplemented with Hepes and L-glutamine (Lonza Biowhittaker), 0.3 g/L L-glutamine, 0.05 g/L gentamicin, 5 g/L albumax. Cultures were grown for no more than 48 h before testing. Laboratory-adapted parasite strains FCR3, HB3 and NF54 were also grown and selected following several panning on the choriocarcinoma cell line BeWo, as described [28].

2.3. DNA extraction and msp genotyping

DNA was extracted from 100 μl of blood pellet using GeneJet Genomic Purification Kit (Fermentas) as recommended by the manufacturer. *Msp1* and *msp2* genes were amplified by nested PCR using specific primers [29]. Multiplicity of infection (MOI) was determined as the average number of distinct fragments detected in each sample.

2.4. RNA extraction, cDNA synthesis and DBL2X genotyping

Total RNA was extracted from samples stored in TRIzol reagent (Invitrogen), as recommended by the manufacturer. To remove potential contamination by genomic DNA (gDNA), RNA samples were treated with DNase I (Invitrogen) for 15 min at room temperature (RT). Absence of gDNA in RNA samples was confirmed by no amplification in real-time PCR using primers targeting the housekeeping gene *seryl-tRNA synthetase* [4]. Reverse transcription was performed using Thermoscript (Invitrogen) with random hexamer primers, as recommended.

To type the previously described DSM in the VAR2CSA DBL2X domain of *P. falciparum* isolates [25], the DBL2X gene sequence was selectively amplified from cDNA of each isolate using high fidelity Fusion Taq Polymerase. Primers forward 5′-TTAYCCCC-AAGAACACA-3′ and reverse 5′-TTTTAAATTTTTTCCATGAA-3′ were used under the cycling conditions 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 50 s, with final extension at 68 °C for 10 min. PCR products were digested with restriction enzymes *BstCI* (cuts the FCR3 DSM-type) and *Hppy188I* (cuts the 3D7 DSM-type) for 1 h at 50 °C and 37 °C, respectively.

2.5. Antibody production and IgG preparation

The NTS-DBL2X fragments and the full-length sequence of the optimized *var2csa* gene from the FCR3 and 3D7 parasite lines were used to produce specific anti-VAR2CSA IgG, by DNA vaccination as described [20]. Briefly, DNA sequences were cloned into a pVax1 vector derivative and fused to the mEPO signal sequence as described [30]. Anaesthetized New-Zealand rabbits (Janvier-France) were immunized by injection followed by application of transcutaneous electric pulses in 5 sites of each *longissimus dorsi* muscle. Animals were immunized at days 0, 30 and 60, and antisera were collected at day 0 and 75 days. Total IgG were purified from non-immunized (day 0) and immune rabbit (day 75) sera on a Hi-Trap Protein G HP column, according to the manufacturer's recommendations (GE-Healthcare).

3. Flow cytometry assay

The reactivity of IgGs to the surface of *P. falciparum* IEs was analyzed by flow cytometry (FACS Calibur) as described [31]. Briefly, CSA-adhering lines (FCR3-Bewo and HB3-Bewo) and field parasite isolates were enriched by exposure to a strong magnetic field (VarioMACS and CS columns, Miltenyi). For each test 2×10^5 IE were labeled with ethidium bromide, sequentially exposed to rabbit IgG (final concentration approximately 10 μg/ml) for 30 min, and to FITC-conjugated anti-rabbit for 30 min (Invitrogen). Data were acquired, analyzed and the median fluorescence intensity (MFI) was determined. Labeling was defined positive with MFI ratio >1.2 (MFI with post-immunization IgG at day 75/MFI with pre-immunization IgG (negative control at day 0), as described [31].

3.1. Inhibition of binding assay (IBA)

Antibodies' capacity to inhibit IE binding to chondroitin sulfate proteoglycan (CSPG) was explored using a static Petri dish assay, as

Table 1
Characteristics of the study population.

Characteristics	All women ($n=123$)	Samples in Flow analysis ($n=47$)	Samples selected for IBA[b] ($n=18$)
Age, years median (IQR[a])	24 (20–30)	25 (21–30)	24 (21–30.5)
Median of parity (IQR)	2 (1–4)	2 (1–4)	2.5 (1–4)
Gestational Age, median (IQR)	20 (15–28)	23 (12–29)	25.5 (17.5–31.3)
MOI, median (IQR)	3 (2–4)	3 (1–4)	4 (2–4.5)
Parasitemia (IQR), parasites/μl	1638.5 (311.5–18,177.5)	11340 (2937.3–54,996.5)	23,200 (3049–81300)

[a] Interquartile range.
[b] Inhibition binding assay.

described [20]. Briefly, 20 μl of 5 μg/ml CSPG-Decorin (Sigma) or 10 μg/ml bovine serum albumin (BSA) diluted in PBS were coated as spots in a 100 mm × 15 mm Petri dish (Falcon 351029). Spots were incubated overnight at 4 °C in a humid chamber and blocked with 3% BSA. Late-stage IE with a parasitemia adjusted to 20% in 1×10^5 cells were incubated with purified IgG (250 μg/ml) or 500 μg/ml of soluble CSA (Sigma). Cells were allowed to bind to coated-plates for 15 min at RT. Non-adherent cells were removed by an automated washing system. Spots were fixed with 1.5% glutaraldehyde, stained with Giemsa and adherents IE were quantified by microscopy.

4. Statistical analysis

All experiments were performed in duplicate. Kruskall–Wallis, Mann–Whitney or Wilcoxon rank sum tests were used to compare continuous variables while categorical variables were compared using the Fisher exact test. Prism software (version 4; Graphpad) was used to plot data and statistical analyses were performed by using STATA 12 software. Significance was defined at P values ≤ 0.05.

5. Results

5.1. Determination of multiplicity of infection and molecular typing of var2csa among parasites isolated from pregnant women

A total of 1538 pregnant women were screened by RDT, from May through August 2012. Active *P. falciparum* infection was confirmed by microscopy on 123 women (8% prevalence). Characteristics of the women enrolled in the study are presented in Table 1. The mean MOI of these isolates was 3.1 (range, 1–7). cDNA was obtained from 122 isolates. A PCR–RFLP was performed on all field isolates to investigate the distribution of the DBL2X DSM. A restriction enzyme-based analysis of this dimorphic region revealed the presence of at least one of the dimorphic variants in transcripts from all parasites [25]. Fifty-four isolates (44%) transcribed only the 3D7 DSM-type while the FCR3 DSM-type was the only genotype detected in the transcripts of 47 isolates (39%). Both types of transcripts were detected in 21 isolates (17%), suggesting a mixture of genotypes (Fig. 1).

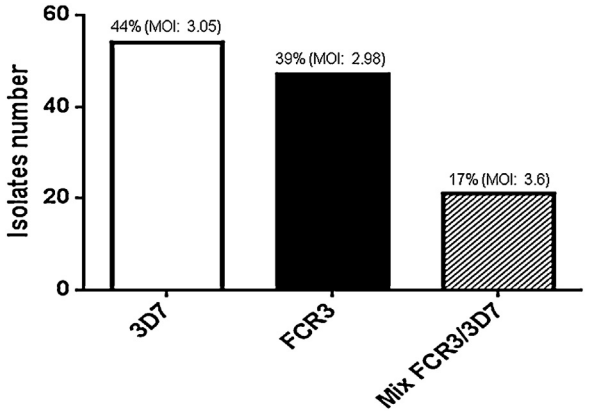

Fig. 1. Distribution of the dimorphic sequence signatures of VAR2CSA DBL2X among parasites infecting pregnant women. The dimorphic sequence motif (DSM) in DBL2X that discriminates the *var2csa* alleles into two subgroups (the 3D7 and FCR3 types) was genotyped in parasites collected from pregnant women in Benin. Shown are proportions of 3D7 type (white histogram), FRC3 type (black histogram) and the mixture of both genotypes (hashed histogram). The multiplicity of infection (MOI) was indicated for each category.

5.2. Specific IgG induced against the NTS-DBL2X of FCR3 and 3D7 types recognized isolates from pregnant women equally well

The labeling of VAR2CSA expressing IE was measured by flow cytometry on 47 freshly isolated, *in vitro* matured *P. falciparum*-IE. Among these isolates, 16 transcribed the 3D7 DSM, 21 the FCR3 DSM and both genotypes were detected in 10 isolates. Both anti-NTS-DBL2X IgG against the 3D7 and FCR3 variants, and IgG raised against the full-length VAR2CSA were used in this assay. IgG purified from non-immunized rabbit showed no significant reactivity with all tested isolates. The range of MFI values are shown in the supplementary Table 1. Higher levels of reactivity were observed with antibodies against the full-length VAR2CSA (median = 1.4) as compared to antibodies directed to the NTS-DBL2X fragments (medians, 1.13 and 1.17 for 3D7 and FCR3 respectively) ($P < 0.05$). No difference was found in the reactivity of the two types of anti-NTS-DBL2X IgG ($P = 0.59$). This reactivity was similar on isolates expressing either the 3D7 or FCR3 DSM-type, (Fig. 2, $P = 0.66$ for NTS-DBL2X/FCR3 and $P = 0.63$ for NTS-DBL2X/3D7).

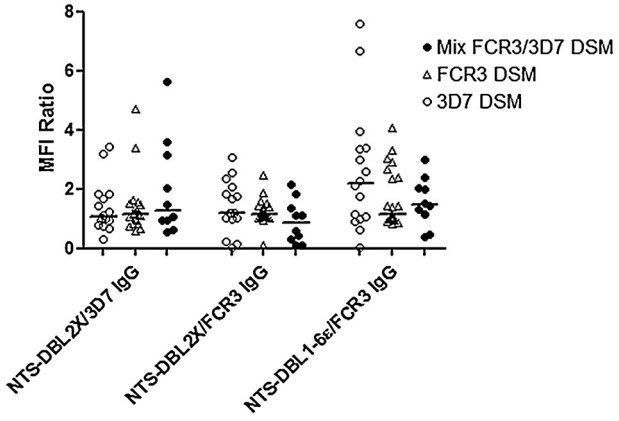

Fig. 2. Recognition of surface expressed VAR2CSA in field isolates by IgG raised against VAR2CSA constructs. By flow cytometry analysis, the expressed VAR2CSA recognition ability of IgG elicited to NTS-DBL2X constructs (NTS-DBL2X/3D7 and NTS-DBL2X/FCR3) and to the full-length extra-cellular part of VAR2CSA of the FCR3 strain (NTS-DBL1-6ε/FCR3) was assessed on field isolates. Parasites were segregated according to their DSM category (3D7, FCR3 or Mix FCR3/3D7 DSM). Bar indicates the median of the MFI ratio (MFI corresponding to the reactivity of IgG from hyper-immune animal/MFI corresponding to the reactivity of pre-bleed IgG).

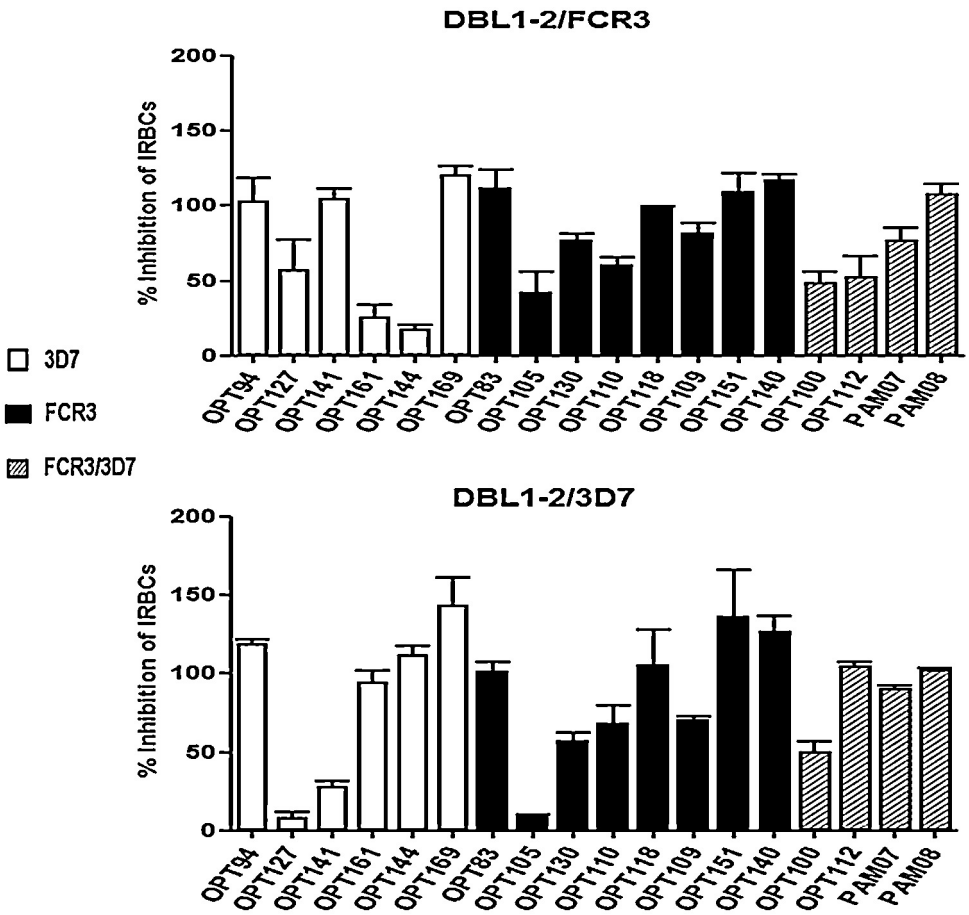

Fig. 3. Adhesion inhibitory activity of IgG raised against each of the two variants of NTS-DBL2X on field isolates. Binding inhibitory activity was evaluated on 18 freshly collected *P. falciparum* isolates from pregnant women. Data are shown as percent of inhibition of each antibody that were normalized by CSA inhibition value (used here as a reference of the maximal binding inhibition). An arbitrary threshold of two times the limit observed on a panel of pre-bleed rabbit sera was defined at 40% inhibition. Histograms were labeled according to DSM type detected in the isolate: (□) for 3D7 DSM type, (■) for FCR3 DSM type (▨) for mixture of both genotypes.

5.3. Adhesion inhibitory capacity of anti-NTS-DBL2X IgGs

The anti-adhesion capacity of the antibodies was further assessed on 18 isolates collected from pregnant women in Benin. Weakly binding isolates (<250 IE/mm^2) were excluded from this testing. All of the 18 highly binders tested in the IBA were significantly labeled by anti-VAR2CSA IgGs while excluded isolates were weakly or not recognized (all $P < 0.05$). No difference was observed between excluded and included isolates regarding the clinical characteristics of parasite donors or the DSM distribution. Six of the highly binding isolates expressed only the 3D7 DSM-type, 8 had only the FCR3 DSM-type, and a mixture of both types was found in 4. Significant adhesion inhibition was obtained with post-immunization IgG induced with both NTS-DBL2X constructs (Table 2). Data were further normalized against that of soluble CSA used as reference of the maximal binding inhibition as presented in Fig. 3. The interquartile range (IQR) of inhibitory activity of post-immunization antibodies on all isolates was [51–100%] for anti-NTS-DBL2X/FCR3 IgG and [55–100%] for anti-NTS-DBL2X/3D7. No inhibition was observed with pre-immunization IgG. Sixteen out of the 18 isolates tested were significantly inhibited (range, 43–100%) by anti-NTS-DBL2X/FCR3 IgG, whilst a high inhibitory activity of anti-NTSDBL2X/3D7 was observed on 15 out of 18 isolates tested (range, 50–100%). No significant difference was observed overall ($P = 0.84$) in the activity of both types of antibodies according to the DSM expressed by the isolates. However, significantly contrasting activity of both antibodies can be observed on the same parasite (Fig. 3). Of the 8 isolates expressing only the FCR3-like DSM, binding to CSPG for all was significantly blocked by anti-NTS-DBL2X/FCR3 (range, 43–100%) while binding of all but one isolate was inhibited by antibodies against the 3D7 type. Likewise, of the 6 isolates transcribing the 3D7 DSM-type, only 4 were actually inhibited by antibodies against each DSM-type, and 2 different isolates were not inhibited. Isolates OPT161 and OPT144 expressing the 3D7 DSM-type, were inhibited by IgG specific to the NTS-DBL2X/3D7 variant, but not by antibodies raised against the FCR3 variant. The opposite profile was observed with isolates OPT127 and OPT141 expressing the 3D7 DSM-type, which were inhibited by IgG specific to the FCR3 DSM-type and not by IgG against the homologous DSM.

The two IgG types were further tested individually or as a mixture on laboratory-adapted parasite lines selected on BeWo cells. The FCR3 and HB3 lines (which share the FCR3 DSM-type), and the NF54 (3D7 background) were used. High inhibitory activity was consistently obtained with antibodies toward IE belonging to homologous DSM. Anti-NTS-DBL2X/FCR3 IgGs completely inhibited CSA-binding of FCR3 and HB3 parasite lines (Fig. 4), but had no effect on binding of the NF54 parasite line. Anti-NTS-DBL2X/3D7 IgG highly inhibited (85%) the CSA-binding of NF54 parasite, and had only partial effect (20–40%) on the FCR3 and HB3 parasite lines. Interestingly, a mixture of anti-NTS-DBL2X IgG from both FCR3 and 3D7 strains conserved the ability to almost totally abrogate (>82%) the binding of all 3 parasite lines to CSPG.

Table 2
Adhesion inhibitory capacity of antibodies induced against two variants of the NTS-DBL2X constructs on *P. falciparum* infected erythrocytes collected from pregnant women.

Isolates	Gestational age of women	DSM-type	MFI Ratio for NTS-DBL2X/3D7	MFI Ratio for NTS-DBL2X/FCR3	MFI Ratio for NTS-DBL6ε/FCR3	Bound IE/mm2 on BSA	Bound IE/mm2 on CSPG	NTS-DBL2X/3D7 inhibition (%)		NTS-DBL2X/FCR3 inhibition (%)		CSA inhibition (%)
								Prebleed	Immune	Prebleed	Immune	
OPT094	12	3D7	1.1	1.2	3.0	3	417	23.00	91.59	15.27	73.05	76.96
OPT127	31	3D7	1.0	1.7	3.4	1	446	22.45	7.74	5.78	49.06	82.14
OPT141	24	3D7	1.0	2.1	3.7	1	402	4.28	22.88	20.00	86.25	82.00
OPT161	18	3D7	1.2	0.9	1	1	401	5.38	73.98	8.57	20.49	77.47
OPT144	28	3D7	1.7	1.1	1.7	1	414	6.15	89.74	0.00	14.96	80.05
OPT169	16	3D7	1.8	3.1	6.7	2	263	0.00	89.15	1.38	75.80	62.45
OPT083	22	FCR3	1.5	1.4	1.2	1	274	0.00	88.08	0.00	97.07	87.21
OPT105	38	FCR3	1.4	1.3	2.7	3	255	8.15	6.98	12.81	31.24	79.19
OPT109	27	FCR3	1.1	2.5	2.3	2	275	10.00	63.70	13.42	73.23	89.63
OPT110	37	FCR3	1.1	1.4	1.4	2	261	17.00	50.45	17.32	44.61	75.50
OPT118	29	FCR3	1.3	1.5	3.6	3	250	0.00	73.19	10.00	70.16	70.81
OPT130	24	FCR3	1.2	1.9	4.0	2	746	9.45	48.12	15.72	65.04	84.31
OPT140	33	FCR3	1.3	1.3	1.6	3	253	16.72	96.36	15.40	89.56	76.18
OPT151	28	FCR3	4.7	1.6	3.0	2	715	0.00	91.10	0.00	73.98	69.02
OPT100	32	Mix	1.0	1.1	1.3	3	552	11.54	42.77	0.00	47.86	90.05
OPT112	20	Mix	1.1	1.3	3.0	4	244	0.00	94.36	1.14	47.75	90.00
PAM007	37	Mix	3.1	1.8	3.1	1	1256	0.42	72.45	21.00	61.32	80.00
PAM008	39	Mix	3.6	2.2	2.9	0	964	10.51	93.62	0.00	98.60	91.38

Inhibition of binding was calculated as percent relative to blank (binding without competitor).

6. Discussion

Several lines of evidence identify VAR2CSA as the key target for the development of a vaccine to prevent PM [4,7,8,32]. However, constraints that include the high molecular weight of VAR2CSA and inter-clonal polymorphisms are the major obstacles in the process of vaccine development. An effective vaccine should elicit broadly reactive antibodies that inhibit adhesion of IE to placenta regardless of primary sequence variations between isolates. An optimal region within VAR2CSA that retains functional and strain-transcending epitopes was previously identified in the NTS-DBL2X fragment [20,21]. As some isolates remained weakly or not inhibited by antibodies raised against a single variant of NTS-DBL2X construct from the FCR3 parasite strain, this invited further analysis to understand the contribution of antigenic polymorphism to the adhesion inhibitory capacity of anti-VAR2CSA antibodies. In this study we were interested in the DBL2X where a site with dimorphic polymorphism was previously described. Although this VAR2CSA domain taken alone does not bind CSPG, it was essential for optimal binding ability of a bigger construct and induction of anti-adhesion antibodies [21,24]. The DSM defined in DBL2X can discriminate two major variants (FCR3 and 3D7) and the existence of this clear-cut dichotomy motivated the exploration of the ability of antigens bearing these signatures of inducing antibodies with similar properties. We first investigated the distribution of the two DSM variants among *P. falciparum* parasites isolated from pregnant women in Benin, and assessed the capacity of antibodies generated against two DSM-specific NTS-DBL2X constructs to alter IE adhesion to CSPG. The antibodies raised against NTS-DBL2X/FCR3 or NTS-DBL2X/3D7 variants were able to label the surface of several isolates at a similar magnitude, irrespective of the DSM type expressed. Higher level of labeling, however, was noted with antibodies raised against the full-length construct of VAR2CSA. This might be explained by the fact that surface recognition involves several epitopes on several domains, some of which might be immuno-dominant and will increase the labeling ability of antibodies to a larger protein than truncated ones. Interestingly, antibodies raised against each NTS-DBL2X construct could block the binding to CSPG of several field isolates, suggesting that these antibodies target strain-transcendent epitopes supporting IE adhesion. The evaluation of the antibodies on the phenotypically well-characterized laboratory parasite strains clearly highlighted distinct inhibitory properties on the homologous and heterologous strains as defined on the genetic background relative to the DSM. This differential activity is also visible on field isolates, where one can easily note distinct patterns of inhibition by the two kinds of antibodies on 5 of the 18 isolates analyzed. Although the DBL2X dimorphic antigenic polymorphism does not seem to totally explain the variation in the inhibitory capacity of the antibodies on various field isolates, as there is no significant difference in our data set to suggest that binding inhibitory antibodies are DSM specific, it is still striking to note that the adhesion of those 5 field isolates which was not inhibited by antibodies with specificity for one construct was indeed inhibited by antibodies with specificity for the other construct. The combination of the overlapping inhibitory patterns of both types of antibodies analyzed here and showing differential inhibitory properties on some isolates actually resulted in significant inhibition of all isolates studied. We assume that the few critical and functional variations between isolates are covered by the two different antigenic constructs. This observation is of major importance in the ongoing effort to create an optimal VAR2CSA-based vaccine that will likely be essential for the induction of a sufficiently protective immunity. The data from this study, although generated on a small number of samples, clearly suggest that a higher proportion of parasite isolates would be targeted by a multivalent vaccine approach comprising of two genotypes or

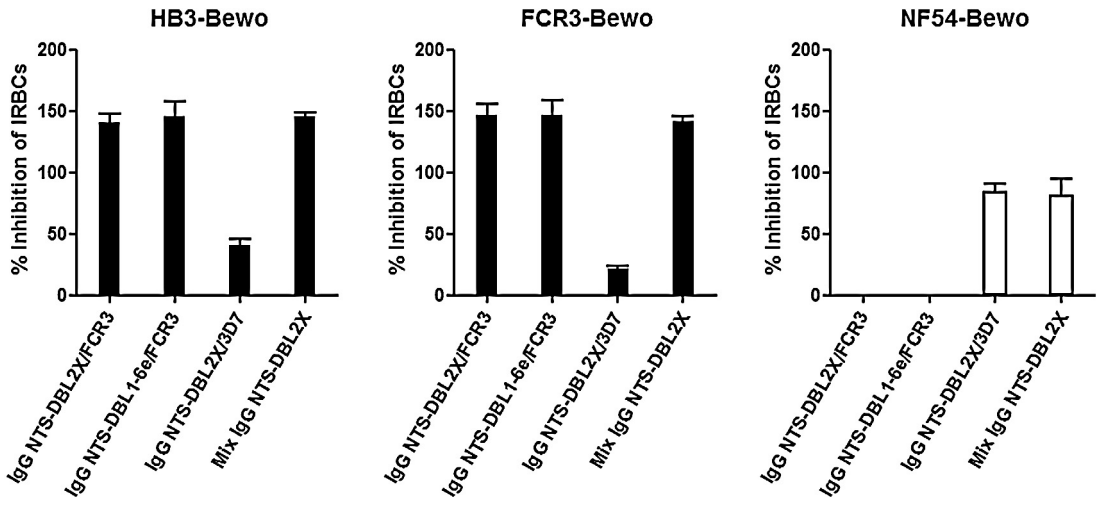

Fig. 4. Inhibition profile of antibodies on selected and laboratory-adapted CSA-binding strains. FCR3, HB3 and NF54 strains selected on Bewo-cells (FCR3-Bewo, HB3-Bewo and NF54-Bewo) were used to assess the ability of the induced antibodies to inhibit binding of 3 laboratory-adapted strains to CSPG. The *in vitro* functionality of the mixture of both antibodies was also evaluated. Values are normalized with CSA inhibition value.

more. In addition, the mixing of the two antibody preparations did not affect their individual activity. Analysis of isolates in which we observed strong contrasts in the properties of both types of antibodies clearly suggests that the DSM region is not the main target for adhesion inhibitory antibodies. A possible explanation might be that the sequences of these isolates significantly differ from their counterparts at other places. Thorough sequence and functional analysis of these isolates is underway.

In conclusion, this study has demonstrated that the NTS-DBL2X domain of both the FCR3 and 3D7 parasite strains is able to induce strain-transcendent adhesion-inhibitory antibodies. The combination of individual antibodies properties suggests that optimal inhibitory spectrum on field isolates might be achieved with antibodies targeting two variants of the N-terminal VAR2CSA. The results thus provide evidence on the relevance and the need for molecular optimization of the VAR2CSA-based vaccines currently in development. Conducting such studies on larger numbers of samples is needed to further refine these observations.

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Conflict of interests

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.07.072.

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