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Immunogenicity of a recombinant fusion construct composed of intrinsically unstructured, low polymorphic segments derived from merozoite surface protein 2 and trophozoite exported protein 1

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ABSTRACT

To overcome the extensive polymorphism found in human Plasmodium antigens and to avoid the lengthy characterization of their 3 dimensional structure and subsequent production of the native proteins we have been concentrated in large unstructured, non-or low-polymorphic fragments present in the blood stage of *P. falciparum*. Three fragments derived from the 2 family-specific and constant regions of merozoite surface protein (MSP2) and PFF0165c protein were previously selected for evaluation as potential single vaccine candidates. In order to increase and optimize their potential efficacy against *P. falciparum* infection the 3 antigens were combined in a single DNA recombinant product (FusN) and compared its antigenicity with that of single antigens in sera of volunteers living in endemic countries. Immunogenicity of the FusN was then compared with that of the mixture of 3 antigens in 3 strains of mice. Antigen specific, affinity purified human antibodies were then tested in antibody dependent cellular inhibition and merozoite opsonization assays. In addition, the antigen specific antibody response and its association with protection from malaria infection were determined. The data collected indicate that the recombinant product is an equal or better antigen /immunogen than fragments used either alone or as a mixture for vaccination in combination with adjuvant. In addition, antibody response to FusN shows a stronger association with protection than single fragments. The use of a single construct as vaccine would drastically reduce the cost of manufacturing and development of the GMP product.

1. Introduction

To overcome the extensive polymorphism found in human Plasmodium antigens and to avoid the lengthy characterization of their 3 dimensional structure and subsequent production of the native proteins we have focused on relatively conserved and unstructured fragments present in the blood stage *P. falciparum* proteins. One of these is the merozoite surface protein 2 (MSP2) targeted by protective antibodies (Abs) and by functional Abs with anti-parasitic activity *in vitro* [1–4]. MSP2 is a GPI-anchored protein present on the merozoite surface consisting of about 200–250 amino acids. MSP2 contains conserved N- and C-terminal (C) regions flanking a highly polymorphic central repetitive region [5]. In addition, a non-repetitive, dimorphic (D) region defines the two allelic families of MSP2, 3D7 and FC27 of low sequence polymorphism [6]. Although its function is not known, MSP2 appears to be essential for viability and completion of the *Plasmodium* life cycle in humans [3,4]. D and C regions families display low structural complexity due to the high percentage of hydrophilic residues, and are predicted and shown to represent "intrinsically unstructured regions" [7–9]. It has been shown that specific semi-immune Ab against MSP2 protein was predominantly cytophilic IgG3, as in other blood

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stage proteins [1,2,8,10]. These cytophilic (IgG1 and IgG3) Abs are thought to play important roles in antibody-mediated mechanisms of parasite clearance [11,12]. Otherwise, a full-length recombinant MSP2 protein was tested in clinical trials as one of the constituents of a three-component malaria vaccine, Combination B [13–16], containing ring-infected erythrocyte surface antigen (RESA), MSP1 and MSP2 (3D7 variant). The unstructured fragment P27A presenting a single limited polymorphic position (E293G) and derived from the trophozoite exported protein 1 (Tex1, PFF0165c; aa 223–326), whose function is not known, was also selected as a potential vaccine candidate based on its immunological and biological properties [17,18]. Phase 1A, 1B clinical trials confirmed the immunogenicity of P27A and the concomitant parasite inhibitory biological activity associated with anti P27A antibodies present in the trial volunteers [19]. Thus, in order to optimize malaria vaccine formulations for higher immunogenicity and protective properties against malaria infection a construct, called FusN, made of intrinsically unstructured fragments covering the MSP2 D family specific, C-terminal and P27A fragments was produced by DNA recombinant technology. The MSP2 fragments were delineated by two recent studies in humans and mice [20,21]. FusN should represent a pan-malaria candidate vaccine.

In the present study, the prevalence of FusN recognition was determined in 2 different malaria endemic areas, its immunogenicity was compared to the mixture of the 3 fragments in mice and affinity purified human antibodies were purified and tested in the antibody dependent cellular inhibition (ADCI) and the merozoite opsonization assay (OP). Finally, antibody response in Senegalese volunteers and its association with protection against malaria infection were determined.

2. Materials and Methods

2.1. Antigens

For FusN expression, the cells were grown in LB medium containing 50 μg/mL kanamycin at 37 °C, 120 rpm, overnight. Five hundred mL Difco Select APS (Alternative Protein Source) medium (50 μg/mL kanamycin) were inoculated with an initial OD600 of 0,2 and incubated at 18 °C, 120 rpm for 48 h. Cells were harvested by centrifugation at 5856g for 15 min at 4 °C and suspended in 20 mM Tris, 100 mM NaCl, pH 8.0. The cells were homogenized using a Miccra D-8 homogenizer with a DS20/PF MIR dispersing tool (ART Prozess- & Labortechnik GmbH) at 23,500 min-1 for 1 min followed by sonification for 3 × 1 min using a Bandelin GM70 sonifier with SH70G/TT13 tip at output level 80%. After another centrifugation at 27,000g for 30 min at 4 °C the supernatant obtained was subjected to an affinity chromatography on Ni-NTA Superflow resin (Qiagen AG, 40724 Hilden, Germany). FusN was eluted in a linear imidazole gradient from 5 to 250 mM imidazole using 20 mM Tris, 100 mM NaCl, 500 mM imidazole, pH 8.0 as elution buffer. FusN containing fractions were pooled and underwent a final buffer exchange on a Sephadex G25 column (GE Healthcare, Berlin, Germany) into 10 mM NH4HCO3, pH 8.0. The protein containing high molecular weight fraction of this gel filtration step was collected. The endotoxin content (Limulus Amebocyte Lysate assay) is 2,07 I.U./mg. Aliquots were freeze-dried and stored below −20 °C.

Synthetic fragments were synthesized by solid-phase Fmoc chemistry using Applied Biosystems 431A and 433A synthesizers (Foster City, CA) as previously described (Fig. 1). Peptide purity was assessed by analytic C18 HPLC and mass spectrometry (MALDI-TOF, Applied Biosystem, CA) and was higher than 80%. All the reagents used were purchased from Fluka (Buchs, Switzerland) and Novabiochem (Laufelfingen, Switzerland).

2.2. Plasma collection

Human plasma were collected during the malaria transmission season from adult donors living in endemic areas: Burkina Faso (BF) and Tanzania (TZ). Sera from Swiss naive donors who had no malaria history were pooled and used as a negative control. Blood was taken by venipuncture into tubes containing EDTA according to the ethical clearances from the different countries.

2.3. Mice and immunization

CB6F1, C3H and outbred ICR mice (5 animals/group) were immunized with 20 μg of FusN and 20 μg of peptide mixture containing 1/3 of each fragment 3 times at 3 week intervals. Formulations containing alum (500 μl) were injected ip while those containing 20 μg GLA in stable emulsion (GLA-SE) were given sc at the base of the tail [22]. Seven-10 days after the third immunization mice were bled and sera were stored at −20 °C.

2.4. ELISA

Antibody (Ab) titers were determined in the enzyme-linked immunosorbent assay (ELISA) as previously described (1). Briefly, antigens were diluted in PBS and used to coat ELISA plates at 0.5 μg/mL (50 μl) in duplicates. Secondary Ab, Goat anti-human or sheep anti-mouse polyvalent immunoglobulins (G, A, M)-AP (Sigma®) (50 μl/well), was used at the recommended dilution in PBS-T plus 2.5% milk. Titers were determined as the last dilution of sera with mean OD higher than the mean OD + 3 SD of negative controls (naïve human, NHS, or mouse sera, NMS). Samples were considered positive if the ratio of OD of test samples divided by mean OD of negative controls was > 3 fold higher and Ab titer of test samples was higher than 1000. Titers are expressed as geometric mean titer (GMT).

2.5. Affinity purified human antibodies

Purification of human antibodies was performed according to Olugbile et al. [17] with minor modifications. Briefly, isolation of specific antibodies were obtained from a single donor from Burkina Faso who was tested positive for the 3 antigens (Donor 9060005) and sera were diluted 5 times with PBS (1×) containing 0.5 M sodium chloride and mixed with the antigen-sepharose conjugate. This mixture was then stirred gently on a wheel overnight at 4 °C. After centrifugation the supernatant was collected and stored at −20 °C for further use. The elution of bound antibody was achieved with glycine (0.1 M, pH 2.5). The fractions obtained were instantly neutralized with PBS (1 M, pH 7.2) and the antibody concentration was determined by the absorbance of the solution at 280 nm.

2.6. Parasite culture and merozoites isolation

P. falciparum strain NF54 and FC27 were cultured and the merozoites isolated as described elsewhere [23,24]. Briefly, parasites were maintained in culture using 2.5% hematocrit of human blood group O in parasite growth medium (PGM) consisting of RPMI 1640 (Lonza, USA) supplemented with 0.5% Albumax, 25 mM HEPES, 2 mM L-glutamine, 24 mM NaHCO3, 25 μM gentamicin and 10% (v/v) and heat-inactivated human blood group AB serum. Culture was maintained at 37 °C in 25-cm^2 flasks after gassing with a gas mixture containing 5% O2, 5% CO2 and 90% N2. Parasite culture was double synchronized with 5% D-sorbitol. Late stage of parasite cultures are mac purified, treated with 10 μM E64 for 6–10 hr and filtrated through a 1.2 μm/32 mm syringe filter. The filtrate is mac purified to remove the hemozoin and the merozoites

FusN

MGSSHHHHH HSSGLVPRGS HMESSSSGNA PNKTDGKGEE SEKQNELNES
TEEGPKAPQE PQTAENENPA APENKGTGQH GHMHGSRNNH PQNTSDSQKE
CTDGNKENCG GGGGSGGSGS GGGAEASTST SSENPNHKNA ETNPKGKGEV
QEPNQANKET QNNSNVQQDS QTKSNVPPTQ DADTKSPTAQ PEQAENSAPT
AEQTESPELQ SAPENKGTGG GAGGAGGAGG AGGAHNNNEK NISYDKNLVK
QENDNKDEAR GNDNMCGNYD IHNERGEMLD KGKSYSGDEK INTSDNAKSC
SGDEKVITSD NGKSYDYVKN ESEEQEEKEN MLNNKKRS

in black, His 6x plus thrombin cleavage site and linkers, in blue family-specific MSP2 FC-27 aa 143-190, in green constant region aa 191-230, in magenta family-specific MSP2 3D7 aa 111-198 plus 8aa from constant region (aa 199-206), and in red TEX1 aa 223-326 (P27A)

Synthetic peptides (37)

LR 246, P27A-P27, TEX1 223-326-spacer-845-871 (KKRNVEEELH SLRKNYNIIN EEIEEIT)

LR55, MSP3 186-281

RKTKEYAEKA KNAYEKAKNA YQKANQAVLK AKEASSYDYI LGWEFGGGVP
EHKKEENMLS HLYVSSKDKE NISKENDDVL DEKEEEAEET EEEELE

LR236, MSP2 3D7 family specific region 111-198

AEASTSTSSE NPNHKNAETN PKGKGEVQEP NQANKETQNN SNVQQDSQTK
SNVPPTQDAD TKSPTAQPEQ AENSAPTAEQ TESPELQS

M-3, MSP2 FC-27 family specific region

ESSSSGNAPN KTDGKGEESE KQNELNESTE EGPKAPQEPQ TAENENPA

MR140, MSP2 constant region 189-229 (3D7 numbering)

APENKGTGQH GHMHGSRNNH PQNTSDSQKE CTDGNKENCG

Fig 1. Sequence of antigens used.

are collected in the run through. Following staining with 10 μg/ml of ethidium bromide (EtBr), the merozoites are counted.

2.7. Antibody dependent cellular inhibition assay

The ADCI assays were performed as previously described [25–27], with slight modification. Briefly, about 2×10^5 monocytes/well were selected by adherence to a 96-well flat-bottom culture plates (Nunc, Roskilde, Denmark) after a 2 h incubation of 2 million PBMCs/well of healthy Danish blood donors never exposed to malaria at 37 °C and 5% CO2 in monocyte medium (RPMI medium containing 5% NHS, 1% glutamine and 1% penicillin-streptomycin). Highly synchronized NF54 parasite culture, at late schizont stage of 0.5% parasitaemia and 2.5% hematocrit were added, 100 μl/well. The negative and positive control IgG used in the ADCI assays are respectively pooled IgG from malaria-naïve Danish individuals (PNIG) and pooled IgG from hyper-immune African adults (PHIG). Controls and test antibodies were added to respective wells at 1 mg/ml and 5 μg/ml respectively and the total volume adjusted to 200 μl with PGM for wells containing uninfected RBCs (uRBCs) and blank controls. Control wells included: (i) parasite culture without monocytes, (ii) culture with monocytes but without IgG, (iii) culture with pool of naïve Danish IgG (PNIG), (iv) culture with monocytes and PNIG, (v) culture with pool of hyper immune African adult IgG (PHIG), (vi) culture with monocytes and PHIG. At 48 h and 72 h an additional 50 μl of PGM was added to each well and after 96 h the assay was stopped. Final parasitaemia was determined as described (6) and specific growth inhibitory index (SGI) calculated: SGI = 100 X (1 − (% parasitaemia with MN and test antibodies/% parasitaemia test antibodies)/(% parasitaemia with MN and PNIG/% parasitaemia PNIG)).

2.8. Opsonic phagocytosis assay

The OP assay of merozoite was performed as described elsewhere with slight modifications [28]. In brief, three different

dilutions (three fold dilution) starting at 15 μg/ml of the affinity purified IgG from the FusN construct, its 4 components alone (and control IgG (50 μg/ml) was used to opsonize freshly isolated EtBr-stained merozoites (NF54 and FC27 strain) for 1 Hr. After three washes, the pellet is resuspended in 50 μl and coincubated with THP-1 cells (1×10^5 cells/150 μl/well) in fetal calf serum–coated, 96-well, U-bottom plates for 25 min at 37 °C in a 5% carbon dioxide humidified incubator. The phagocytosis is arrested by centrifugation of the plates for 5 min at 500g in a pre-chilled centrifuge, followed by 2 washes in ice-cold fluorescence-activated cell sorting (FACS) buffer (PBS + 0.5% BSA + 2 mM ethylenediaminetetraacetic acid). The pellet is re-suspended in 200 μl of FACS buffer and analyzed in a Beckman Coulter cytometer. The level of phagocytosis is calculated by gating the percentage of THP-1 cells containing EtBr-stained merozoites.

2.9. Association of the immune response with protection from malaria

This study is based on the samples collected in the context of the Dielmo and Ndiop projects [29,30]. The follow-up protocol was explained to the assembled village population, and any individual could withdraw from the study at any time. Written informed consent to participate to the project was obtained and regularly renewed either from adults enrolled in the project or from parents or guardians of children who provided blood samples. The study received clearance from the National Ethics Committee for Health Research of Senegal and ad-hoc committees of the Ministry of Health, and the Pasteur Institutes (Dakar and Paris).

Plasma samples were obtained by venous puncture from each of the 94 inhabitants selected in Ndiop village a malaria mesoendemic area of Senegal with an estimated annual entomological inoculation rate of 79 infected bites/person/year in 2000 [29]. A parasite density of 30 parasites / 100 leucocytes with hyperthermia i.e., a temperature greater than or equal to 38 °C was considered as a characteristic of a clinical malarial attack and rapidly treated once confirmed. A dispensary remained opened day and night so that any patient reporting an episode of illness was rapidly examined by a medical doctor and clinical information was recorded on standardized cards. Active clinical survey was characterized by a close and intense monitoring of each villager including three systematic weekly visits and a daily visit of each compound. Since free medical care was available 24 h/d, self-treatment was most uncommon, as ascertained by systematic detection of anti-malarial drugs.

The volunteers enrolled in the present study were all born in the village of Ndiop where they remained most of the time as most of the other inhabitants who live mainly from agriculture (peanuts, millet, livestock). The selected individuals were present in the village for 83.6 ± 26% of the time during the first year and 70.0 ± 30% of the time during the 3 years of daily and individual monitoring following the initial blood sampling used for the analyses. 90.8% of the infections occurring in the village were due to *P. falciparum* and only infections by this parasite were included in the study. The cohort included 47 males of 16.0 ± 15.1 years of age and 47 females with a mean age ± 1SD of 17.5 ± 12.3 years, and no pregnant woman was enrolled or became pregnant during the study. Biological tests, including hemoglobin electrophoresis and tests for glucose-6-phosphate dehydrogenase (G6PD) deficiency, were available for most subjects participating in the study. 18 out of the 94 individuals selected had a G6PD deficit (G6PD < 6.9 μl with a mean of 2.44 ± 1.93 μl), hemoglobin AA was present in 81 subjects whereas 9 were AS and 4 were AC carriers. These parameters were considered as potential confounders and controlled for in the statistical analyses. Overall, this Ndiop cohort corresponded to a representative subgroup of the inhabitants of this rural village where the individuals less than 30 years of age accounted for the dominant fraction of the population.

The parasite-specific antibody plasma content from the initial blood samplings was evaluated by ELISA, using 4 *P. falciparum* antigens namely: FusN, LR246, p27A and LR55. IgG1 and IgG3 subclass antibodies specific for these antigens were detected and quantified essentially as previously described [31,32]. Briefly, high-binding 96-well microplates (Nunc Maxisorp®, Denmark) were coated with 5 μg of antigen per well and incubated overnight at 4 °C. The optimal plasma dilution was determined to be 1:200 and optimal concentrations were 1:2000 for anti-IgG1 (clone NL16), and anti-IgG3 (clone ZG4), two monoclonal antibodies from Skybio, UK.

Antibody responses were expressed as net Optical Densities (OD) values corresponding to the test OD values obtained for a given test sample minus a background level defined as the mean plus 3 SD of the OD values obtained using a subsample of negative control sera originating from malaria-naïve blood donors.

2.10. Statistical analysis of data

ANOVA tests (GraphPad Prism, version 7.04) were used to determine p values between 2 corresponding mouse groups immunized with FusN or mixture of the 3 antigens in antibody level (Tables 2a and 2b). Field data were analysed using the JMP® and Statview® 5 softwares, both from SAS Institute (Cary, North Carolina, United States). Univariate and logistic multivariate analyses were carried out to provide information between subgroups or on the strength of the relationship between antibody responses and the occurrence of clinical malaria attacks. Age, the precise amount of time spent by each individual in Ndiop village during the periods of daily active monitoring and Log-transformed net OD values were included in our analyses. The (Log + 1)-transformed total incidence of clinical episodes observed during 1 or 3 years of continuous active follow-up after the blood sampling tested was used as a continuous variable or transformed as dichotomous variables as in previous studies [33,34].

When the pattern of *P. falciparum* specific humoral responses was determined as a function of the occurrence or non-occurrence of clinical malaria attacks identified during 1 or 3 years of follow-up, odds ratio (OR) values were determined and indicated as summary scores of the antibody effects. *P* values less than 0.05 were considered as significant but when several univariate tests were carried out a lower threshold p value was determined by Bonferroni correction and indicated in the Results section.

3. Results

3.1. Production and characterization of the MSP2-Tex1 construct

A synthetic gene was constructed coding for the three fusion fragments with N-terminal 6xHis and a thrombin cleavage site as shown with MW 35.43 KD (Fig. 1). The recombinant plasmid pLEXWO315-10 was transformed in BL21 (DE3) and the recombinant construct called FusN was purified from the strain following Ni-NTA affinity chromatography. Western blotting confirmed the identity of the recombinant purified product with an apparent MW of about 70 KD (Fig. 2) due to its unstructured nature. FusN exhibited positive reaction with all 3 specific antibodies directed against the 3 fragments and His-tag (data not shown). FusN was more than 98% pure and cross-reaction with anti-*E. coli* serum was not observed.

3.2. Prevalence of the antibody response in human populations

To determine the prevalence of the antibody response toward the FusN construct, sera/plasma obtained from donors living in different endemic areas were used at 1: 200 dilution in ELISA and

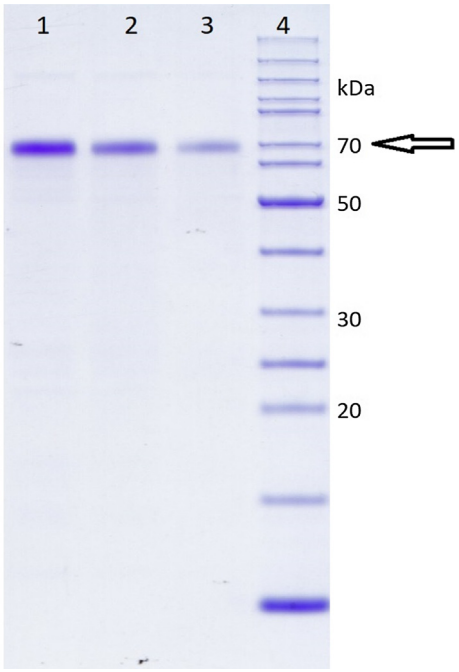

Fig. 2. SDA-PAGE of FusN. SDA-PAGE after purification of FusN at 3, 2 and 1 μg, respectively.

compared with the single constructs. FusN is recognized 100% in the 3 regions tested compared to 97–100, 93–100 and 85–96% to 3D7, FC27 family specific and constant regions and P27A, respectively (Table 1). Average OD was also higher (>2) but it is difficult to compare given that all antigens were used at 1 μg/ml concentration. Thus, concentration of each individual antigen present in FusN is about 1/3 of FusN concentration.

3.3. Immunogenicity in mice

Sera of B6F1, C3H and outbred ICR mice immunized with FusN or the mixture of the 3 antigens present in the recombinant product were tested after the 3rd immunization in ELISA. As observed in Table 2 immunization with FusN gives rise, in general, to superior titers compared to that of peptide mixture. In particular, this difference is more striking in CB6F1 and ICR mice. In addition, the number of seroconversion for groups of mice and adjuvants used is greater for FusN than the antigen mixture (Table S1). Pooled sera from different mouse groups were also tested in immunofluoresence assay, and results are shown in Table 2.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2019.07.048.

3.4. ADCI

In a first experiment, affinity purified IgG were tested in three fold serial dilutions ranging from 15 to 1.7 μg per assay. Some of the samples showed unspecific inhibition of parasite growth at the highest concentrations. It was therefore decided to test all samples at the same low concentration of 5 μg/ml per assay in two independent experiments (Table 3). Mean and standard deviation of the specific growth inhibition (SGI) and direct inhibition (DI) are expressed in %. Percentages with a negative value represent enhancement of parasitemia. All the affinity purified IgG against the FusN construct and its 4 components alone showed an inhibitory effect on the parasite growth in the presence of monocytes ranging from 15.8 to 45.7%. In contrast, none of the DI observed was significant.

3.5. Merozoite opsonisation

The antiparasitic potency of affinity purified IgG specific to the FusN construct and its 4 components alone was compared in opsonic phagocytosis assays over a range of 3-fold Ab concentrations ranging from 15 to 1.7 μg/ml using merozoites from NF54 and FC27 strain. There was a clear dose-dependent effect, and the OP index induced by FusN construct IgG was consistently greater than the inhibition induced by its 4 components alone (Fig. 3). Moreover the activity of FusN IgG were not strain specific. In general, at the lowest concentration of FusN IgG (1.7 μg/ml), the OP index was higher against NF54 than FC27 strain.

3.6. Antibody response and association with protection against malaria infection

During the first year, 139 malaria attacks were identified in 57 patients whereas during the 3-years of follow-up, 350 episodes of malaria attacks were observed and treated in 70 patients. 24 volunteers of the cohort (25.5%) did not have sign of *P. falciparum* infection at any time, 19 had 1 single episode of clinical malaria whereas 51 patients presented from 2 to 13 attacks (mean ± SD = 6.467 ± 3.415 episodes) during the 3 years of investigations. 71.3% of the Ndiop cohort had received anti-malaria treatments at least once prior to the present study.

Relationships between antibody response levels and occurrence of clinical malaria attacks were assessed during the daily and active monitoring of the Ndiop cohort. Besides FusN and P27A, an already studied vaccine candidate as control, MSP3 186–281 (LR55; 34) and a new potential one (P27A extended with an α coiled-coil segment (P27A-P27; LR 246; see Fig. 1) were included in the antibody response analysis and compared. MSP-2 fragments were already analysed and found or loosely associated with protection or not at all [8]. For the other antigens analyzed, IgG median values and 95% Confidence Intervals (CI) are shown in Table 4a. The highest levels of IgG1 and IgG3 responses were those specific

Table 1
Prevalence of response in endemic countries.

	Burkina Faso (N = 37)			Tanzania (N 42)		
	Mean OD + 3SD (%)	OD Ratio (%)	Mean OD	Mean OD + 3SD (%)	OD Ratio (%)	Mean OD
FusN	100	100	2.19	100	100	2.06
P27A	91	83	0.71	86	78	0.66
MSP2-3D7	100	100	2.18	nd	nd	nd
MSP2-FC27	100	100	1.60	nd	nd	nd

Prevalence: % of donors whose serum gave an OD value in ELISA higher than the mean OD + 3 standard deviation of negative controls (naïve European donors) or OD ratio ≥ 2. Sera dilution = 1:200. Mean OD comprises the mean of all OD.

Table 2a
Immunogenicity of FusN in mice.

Immunisation with FusN (Post 3)							
Mouse strain	Adjuvant	Antigenic molecule	GMT	SD	Responders	p-value	IFAT
CB6F1	GLA-SE	FusN	4.48	0.40	5/5	<0.0001	Pos
		P27A	4.39	0.34	5/5	0.00	
		MSP2-3D7	3.72	0.26	5/5	<0.0001	
		MSP2-FC27	3.91	0.34	5/5	<0.0001	
C3H	GLA-SE	FusN	5.24	0.40	5/5	0.85	Pos
		P27A	5.44	0.21	5/5	0.86	
		MSP2-3D7	4.48	0.71	5/5	<0.0001	
		MSP2-FC27	4.48	0.52	5/5	0.00	
ICR	GLA-SE	FusN	4.86	0.26	8/8	0.04	Pos
		P27A	4.86	0.51	8/8	0.22	
		MSP2-3D7	3.97	0.47	8/8	<0.0001	
		MSP2-FC27	4.86	0.62	8/8	<0.0001	
CB6F1	Alum	FusN	3.43	0.67	3/5	0.01	nd
		P27A	3.43	0.83	4/5	0.01	
		MSP2-3D7	2.76	0.54	1/5	0.03	
		MSP2-FC27	3.15	0.54	2/5	0.02	
C3H	Alum	FusN	5.63	0.26	5/5	0.45	Pos
		P27A	5.53	0.26	5/5	0.49	
		MSP2-3D7	4.77	0.52	5/5	<0.0001	
		MSP2-FC27	5.63	0.26	5/5	0.53	
ICR	Alum	FusN	4.09	0.88	7/8	0.11	nd
		P27A	3.91	0.88	8/8	0.14	
		MSP2-3D7	3.13	0.99	3/8	<0.0001	
		MSP2-FC27	3.73	1.19	5/8	0.00	

Table 2b
Immunogenicity of antigen mixture in mice.

Immunisation with Mix MSP2 + P27A (Post 3)						
Mouse strain	Adjuvant	Antigenic molecule	GMT	SD	Responders	IFAT
CB6F1	GLA-SE	FusN	2.29	0.26	0/5	nd
		P27A	2.48	0.58	1/5	
		MSP2-3D7	2.19	0.26	0/5	
		MSP2-FC27	2.00	0.00	0/5	
C3H	GLA-SE	FusN	5.34	0.34	5/5	Pos
		P27A	5.53	0.26	5/5	
		MSP2-3D7	2.57	0.52	1/5	
		MSP2-FC27	2.86	0.71	1/5	
ICR	GLA-SE	FusN	4.03	1.27	6/8	Pos
		P27A	4.33	1.15	6/8	
		MSP2-3D7	2.12	0.34	0/8	
		MSP2-FC27	2.30	0.57	0/8	
CB6F1	Alum	FusN	2.00	0.00	0/5	nd
		P27A	2.00	0.00	0/5	
		MSP2-3D7	2.00	0.00	0/5	
		MSP2-FC27	2.00	0.00	0/5	
C3H	Alum	FusN	5.24	0.40	5/5	Pos
		P27A	5.15	0.80	5/5	
		MSP2-3D7	3.15	0.80	2/5	
		MSP2-FC27	5.34	0.34	5/5	
ICR	Alum	FusN	3.43	1.53	4/8	nd
		P27A	3.25	1.73	3/8	
		MSP2-3D7	2.00	0.00	0/8	
		MSP2-FC27	2.54	1.15	1/8	

Mice were immunized with FusN or a mixture of the 3 components as described in Materials and Methods. Titers are expressed as GMT. P values are determined between the corresponding groups of these tables.
Nd: not determined.

for FusN and there existed a trend for less clinical episodes of malaria to be observed in subjects with high plasma levels of anti-FusN IgG3. The relationship between the number of past malaria attacks (i.e. clinical episodes observed before the blood sampling) and the level of anti-FusN IgG3 was moderate (Adjusted $R^2 = 0.077$; $p = 0.0039$). It was marked during the first year of prospective study (Adjusted $R^2 = 0.121$; $p = 0.0003$) and stronger during the 3 years of investigations (Adjusted $R^2 = 0.183$; $p < 0.0001$). Of note, no similar indication was observed between the number of malaria and the level of anti-FusN IgG1. As expected the strongest relationships between antibody responses was found between anti-p27A and anti-LR246 antibody responses (Adjusted $R^2 = 0.589$; $p < 0.0001$ for IgG1 and Adjusted $R^2 = 0.439$; $p < 0.0001$ for IgG3).

The mean ± SD incidence of malaria attack in the cohort was 1.63 ± 2.07 attack/person/year (during the first year of study) and

Table 3
Functional activity of affinity purified antibodies in ADCI assay.

	Experiment 1		Experiment 2	
	SGI%	DI%	SGI%	DI%
α-FusN F1	20.00	9.00	17.00	4.00
α-FusN F2	28.00	−4.00	29.00	11.00
α-P27A F2	19.60	−12.00	17.50	4.00
α-LR236	50.00	2.00	40.90	4.00
α-M3	34.00	0.00	32.00	−5.00
α-MR 140	13.00	−3.00	19.00	11.00
*Positive control	43.00	−2.90	40.00	−3.10

SGI and DI of two independent experiments of affinity purified IgG (5 μg/ml) against FusN, its 3 components and MSP2 constant region alone. F1 and F2 are 2 fractions of the same purification experiment.
* Pool of hyper immune IgG (1 mg/ml).

1.65 ± 1.56 attacks per person per year (during the 3 years of continuous and active follow-up).

The median OD values obtained in the absence or in the presence of clinical malaria attack(s) observed after 1 year or after 3 years of intensive monitoring are indicated in Tables 4b and 4c (p values were determined by median test).

Because 6 univariate tests were carried out in each of these Tables, it might be wise to consider that, according to Bonferroni correction, p values < 0.008 (i.e. 0.05:6) correspond to a more appropriate threshold of significance than $p < 0.05$. As a result, the most significant indications were those found for IgG3 anti-FusN and for both IgG1 and IgG3 anti-L246 responses during the first year and for IgG3-FusN and IgG1-L246 during the 3 years of investigations.

Results of multivariate logistic regressions carried out to analyse the associations between the binary outcome variable [clinical attack(s) or no clinical attack following 1- or 3-years of follow-up] and age, time spent in Ndiop by each member of the cohort and the various antibody responses tested as predictor variables are illustrated in Table 4d. IgG3-FusN response was found significant both after 1 and 3 years of study whereas IgG3-LR55 was found significant after 3 years of investigations only. This indicated that when holding age and time spent in the village at fixed values, one unit increase of either anti-FusN or anti-LR55 antibody responses contributed to reduce the odds of malaria attack after 1 and/or 3 years of active monitoring.

4. Discussion

The work presented here is the culmination of several investigations in which we concentrated our effort in the discovery and development of *P. falciparum* blood stage protein fragments as candidate malaria vaccine. Our original approach was dictated by the logical necessity to select and developed non-polymorphic, structurally defined antigens in order to optimize the effort, in terms of time and cost, of obtaining an efficacious blood stage vaccine.

The choice of two secondary structure motifs, easily identifiable by informatics and easily characterized, i.e. α-helical coiled coil and intrinsically unstructured protein segments, appeared in our view as the most coherent approach to the principle outlined above. Focusing on no- or low polymorphic unstructured segments is, in our opinion, a straightforward way to select and developed malaria vaccines. It was determined that 40–50% of *P. falciparum* proteome contains intrinsically unstructured segments over 50 amino acids long [35,36]. Some of the traditional antigens developed early on contained this structural motif (MSP2, MSP3, circumsporozoite protein). Thus, we concentrated our initial work on the two MSP2 family (3D7 and FC27) specific and constant domains, which present low or no polymorphism, respectively. In

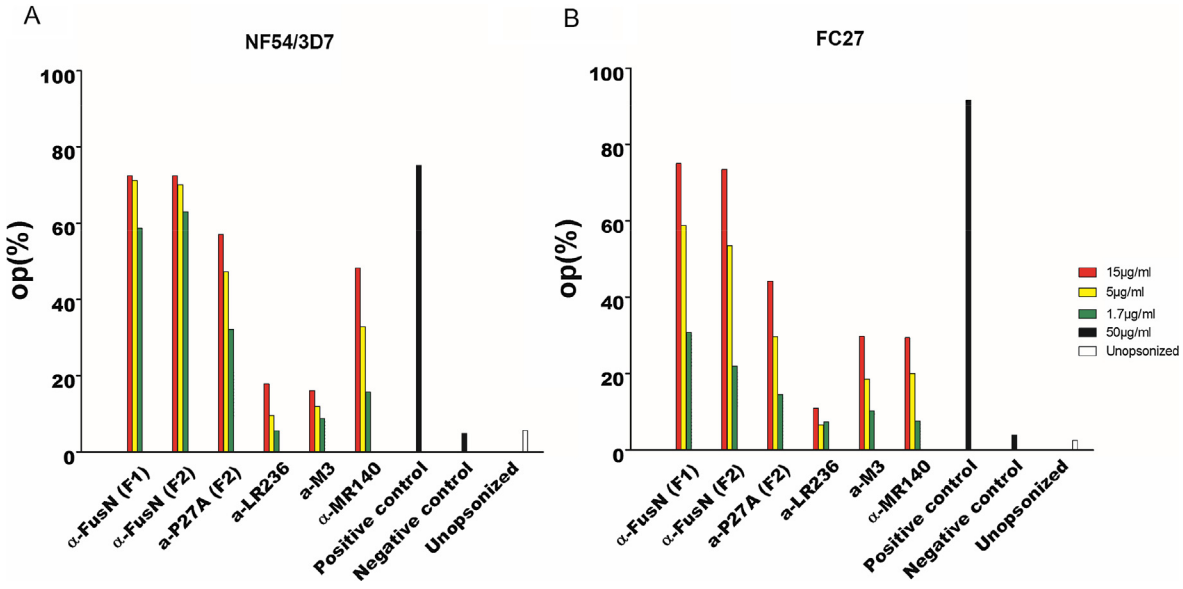

Fig. 3. Biological activity of affinity-purified IgG. Three fold serial diluted of FusN and its 4 components in opsonic phagocytosis assay with (A) NF54/3D7 and (B) FC27 *P. falciparum* parasites.

Table 4a
Antibody response and association of protection from parasite infection.

IgG1-FusN	**IgG3-FusN**	IgG1-LR246	**IgG3-LR246**	IgG1-LR55	**IgG3-LR55**	IgG1-p27A	**IgG3-p27A**
0.899	0.575	0.501	0.123	0.538	0.330	0.232	0.134
0.653–1.096	0.303–0858	0.258–0.716	0.046–0.251	0.265–1.22	0.122–0.719	0.119–0.402	0.057–0.541

Median values (and 95% Confidence Intervals) of IgG1 and IgG3 antibody responses found against FusN, LR246, p27A and LR55 antigens.

Table 4b
Antibody response and association of protection from parasite infection.

	IgG1/Fus	**IgG3/Fus**	IgG1/246	IgG3/246	**IgG1/LR55**	**IgG3/LR55**
No attack:	0.983	0.823	0.678	0.205	1.049	0.477
(N = 24)	0.778–1.175	0.527–1.024	0.486–0.838	0.081–0.505	0.350–1.418	0.195–0.815
Yes attack	0.791	0.449	0.410	0.079	0.440	0.239
(N = 70)	0.602–1.044	0.170–0.685	0.188–0.544	0.032–0.204	0.203–0.992	0.100–0.632
	***P* = 0.0088**	***P* < 0.0001**	***P* = 0.0001**	***P* = 0.0021**	*P* = 0.0108	*P* = 0.0535

IgG median OD values (and 95% CI) found in the groups of individuals without or with occurrence of clinical malaria attack(s) in Ndiop village during the first year of follow-up after the blood sampling (p values were determined by median test). Results of P values in bold indicates that even after Bonferroni correction, it might be appropriate to consider differences significant because they are less that 0.05/6 = 0.008.

Table 4c
Antibody response and association of protection from parasite infection.

	IgG1/FusN	**IgG3/FuN**	IgG1/246	IgG3/246	**IgG1/LR55**	**IgG3/LR55**
No attack:	0.987	0.877	0.671	0.218	1.237	0.680
(N = 24)	0.770–1.189	0.552–1.130	0.484–0.840	0.097–0.557	0.334–1.804	0.235–0.942
Yes attack	0.820	0.510	0.424	0.085	0.477	0.260
(N = 70)	0.624–1.065	0.193–0.778	0.217–0.613	0.036–0.222	0.245–1.028	0.111–0.595
	P = 0.0686	***P* = 0.0001**	***P* = 0.0079**	***P* < 0.0098**	***P* = 0.0095**	*P* = 0.0264

IgG median OD values (and 95% CI) found in the groups of individuals without or with occurrence of clinical malaria attack(s) in Ndiop village during 3 years of active follow-up after the blood sampling (p values were determined by median test). Results of P values in bold indicates that after Bonferroni correction, it might be appropriate to consider differences significant because they are less that 0.05/6 = 0.008.

Table 4d
Antibody response and association of protection from parasite infection.

	1 year study OR	P values	**3 year study OR**	P values
LogIgG3-**Fus**	0.120 [0.014–0.634]	P < 0.0001	0.032 [0.002–0.308]	P = 0.001
LogIgG3-LR246	0.352 [0.111–1.037]	P = 0.0583	No OR value	
LogIgG3-**p27A**	0.301 [0.095–0.879]	P = 0.0278	0.333 [0.097–1.045]	P = 0.0597
LogIgG3-LR55	No OR value		0.028 [0.001–0.319]	P = 0.0007

Reports the odds ratio (OR) values and [95% confidence interval of the OR] calculated during the first year of monitoring and after the 3 year-study period. NA = not available.

addition, we searched in the protein PFF0165c, also called TEX1 and identified in our laboratory, low polymorphic unstructured regions. One of these (P27A) seemed quite interesting for its length (104 aa) and for a single amino acid residue substitution (E/G; 60/40%; 17). The 3 segments were first studied as single antigens, and were shown to be widely recognized by sera from volunteers from different malaria endemic areas, targets of parasite inhibitory properties, immunogenic in animal models and the antigen specific antibody response was associated with protection. P27A was further tested in phase 1a and 1b with good safety profile, immunogenicity and *in vitro* parasite inhibitory biological activity [19].

At this point to increase the probability of developing an efficacious malaria vaccine with antigens operating at different stages of the parasite cycle (merozoite, trophozoite), the combination of the 3 antigens as a single product was thought to be very attractive and cost-effective.

As observed from the data obtained, the fusion product is equal or superior as antigen with respect to the single components as determined in ELISA using sera from volunteers living in malaria endemic areas. In addition, higher parasite inhibitory biological activity is associated with the antibody response to FusN than that of its components in the merozoite opsonization assay.

In terms of immunogenicity in mice, immunization of FusN elicits a stronger response to the 3 components than that observed for their physical mixture (Tables 2 and S1). This is most likely due to the fact that helper epitopes presents in the fusion product can, in principle, help antigen specific activation of any B cell response present in the fusion construct.

With regard to the analysis of antibody response and association with protection from parasite infection emphasis was put on IgG1 and IgG3 isotype responses because both IgG1 and IgG3 are cytophilic antibodies are the most abundant and found effective *in vitro* against P. falciparum multiplication in ADCI [32,33]. In addition, similar to antibodies directed against LR55 [37], those specific to P27A were previously found effective in ADCI [17]. In the present survey, IgG1 and IgG3 anti-FusN response levels were higher than antibody reponses against the other antigens tested. Of note, anti-FusN IgG3 and anti-LR246 IgG1 responses were higher in the plasma samples of Ndiop inhabitants with no attack than in those with malaria attacks both after 1 and 3 years of active monitoring. Finally, in a logistic regression analysis, anti-FusN IgG3 responses were found significantly linked with a lower risk of malaria attack both after 1 and 3 years of follow-up. In contrast, the other antibody responses did not reach significance except IgG3 responses against LR55 when taking into account the occurrence or absence of malaria attacks over a 3-year period. Therefore, it is remarkable to note that raised anti-FusN IgG3 responses are markedly associated with a reduced risk of clinical episode in this malaria endemic area of Senegal.

In conclusion, the FusN construct seems to display all of the biological properties of an efficacious malaria pan-vaccine candidate than the antigen mixture, is cost-effective, and should be further developed for testing in human clinical trials.

Declaration of Competing Interest

All the authors declare that they do not have any conflict of interest.

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