ANNEXES

1) Article soumis à Vaccine

Antibody dependent oxidative burst and phagocytosis of *Plasmodium falciparum* merozoites by activated neutrophils: a functional antibody assay for malaria vaccine candidates

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ABSTRACT

To prioritize merozoite surface protein (MSP) malaria vaccine candidates for clinical testing, functional assays are needed to evaluate natural and vaccination-induced antibody responses. A new functional antibody assay is described based on antibody dependent oxidative bursts and phagocytosis of merozoites (ADPm) by activated polymorphonuclear neutrophils (PMN), detected by chemiluminescence monitored with luminol. In meso and holoendemic areas, ADPm was positively correlated with cytophilic IgG1 and IgG3 titres, age related natural immunity, and reduced risk of clinical malaria episodes. The ADPm assay appears to be a relevant, reproducible functional correlate of antibody based immune protection for recombinant MSP vaccine candidates.

Key words: malaria vaccine; merozoite surface proteins; chemiluminescence antibody assay

Short Title: Chemiluminescence functional *Pf* merozoite antibody assay

1. Introduction

There are around 300-500 million clinical malaria cases annually, and 1-3 million deaths of primarily young African children [1]. *Plasmodium falciparum* causes the most morbidity and mortality, and widespread multi-drug resistance has accentuated the need for an effective vaccine. Merozoite surface proteins (MSPs) are relevant vaccine targets, because they mediate erythrocyte invasion, and are accessible to antibodies and complement. Antibodies to MSPs have been statistically associated with natural immunity acquired by endemic populations [2-5], and in vitro inhibition of erythrocyte invasion and parasite growth, indicate they are implicated in protective immune responses [6-8]. Antibody interactions with circulating polymorphonuclear neutrophils (PMN) and blood monocytes lead to phagocytosis of free merozoites [9,10] and cellular toxicity directed against intracellular blood stage parasites in vitro [11,12] and in vivo [13]. Reactive oxygen species (ROS) produced by neutrophils have been implicated in parasite clearance in Gabonese children [14]. Neutrophils internalize and destroy pathogens using ROS and granule hydrolytic proteins. Activated neutrophils are highly effective at generating ROS such as $O_2^{\cdot-}$ and H_2O_2 by a process known as the respiratory burst mediated by NADPH oxydase. ROS can be detected by a chemiluminescence dye (luminol), which emits quantifiable light as a measure of phagocytic activity [15,16], triggered by Fc receptor binding of antibody complexes [16-18]. Functional analysis of human antibodies by this technique has been limited due to technical difficulties, and inter-study comparisons have been hindered by methodological variability [9,11,19,20]. To address the dearth of functional assays currently available for evaluating antibody mediated immune responses to parasites [8,21-23], we have developed a standardized, reproducible high throughput protocol that permits inter-assay statistical analyses. We show that neutrophil release of ROS is (i) induced almost exclusively by cytophilic IgG1 and IgG3 antibodies specific for merozoites, (ii) positively correlated with age-related anti-merozoite antibody responses, and (iii) a functional antibody read-out which is significantly correlated with a reduced risk of clinical malaria.

2. Materials and methods

2.1. Study site

Blood samples were collected from Dielmo and Ndiop, two Senegalese villages with perennial and seasonal transmission respectively, after approval by the *ad hoc* Ethics Committee [24,25]. The project protocol and study design were explained to the assembled villagers, and informed consent was obtained individually from all participants or their parents or guardians. The protocol was approved by the Conseil de Perfectionnement of the Institut Pasteur de Dakar directed by the Ministry of Health of Senegal. In July 2002, 114 healthy villagers from each site were enrolled in a longitudinal and cross-sectional study. The mean age of the Ndiop and Dielmo cohorts was 24.0 (3.4 – 76.9) and 28.6 (3.4 – 80.5) years respectively. After venous puncture, RBCs were removed by centrifugation and plasma was stored at –20°C.

2.2. Antibodies

Sera were de-complemented by heating 20 min at 56°C. Hyper-immune sera from 30 adult residents of Dielmo were pooled for a positive control (HIS). A pool of non-immune sera for a negative control (NIS) was obtained commercially (Valbiotech, France). Total IgG was purified using

Ultralink® immobilized Protein-G (Pierce) according to the manufacturer's instructions. Briefly, sera (0.4mL) were diluted 1:5 in binding buffer, passed 3 times over 2mL of packed resin and washed with 15 column volumes of binding buffer. IgG was eluted with 10mL of elution buffer, neutralized with 1M Tris pH 8.0, dialysed against PBS, and concentrated to 0.4mL (Amicon Ultra, 5,000 MWCO; Millipore). IgG-depleted sera were obtained by concentrating flow-through fractions to the initial 0.4mL volume.

2.3. Parasite culture and merozoite preparation

Palo Alto (FCR3) *P. falciparum* cultures were maintained continuously on O+ erythrocytes in RPMI containing 0.5% Albumax and 1µg/mL gentamycin, in candle jars [26]. Merozoite extracts were prepared by three different methods. (1) Cultures with >5% parasitemia were centrifuged 5 min at 400xg. RBCs (pellet) were replaced in culture, and merozoites in supernatants were recuperated by centrifugation 20 min at 1500xg. (2) As above, except culture supernatants were first centrifuged 30 min at 50xg to remove hemozoin and cell debris, and then centrifuged as above to obtain hemozoin-free merozoite extracts. (3) After synchronization using D-sorbitol, cultures containing mature schizonts were centrifuged 10 min at 700xg at RT on a 75% isotonic percoll cushion. Merozoites in the RPMI-percoll interface were washed once in PBS, while infected erythrocytes in the pellet were washed in RPMI and replaced in culture. Merozoite viability was tested by culturing 40µL of fresh merozoite extract for 1 day at 6% or 1 week at 3% hematocrit. Merozoites were stored at -20 °C, and minimally three aliquots were pooled for each experiment.

2.4. ELISA

Merozoites resuspended in PBS were lysed with 3 freeze-thaw cycles, dosed by BCA (Pierce), diluted to 10µg/mL in PBS and coated on MaxiSorp plates (Nunc). The ELISA protocol was described previously [4,22]. The HIS positive control was titered on each plate in 2-fold dilutions starting from 1:200 for IgG1 and IgG3, or from 1:5,000 for total IgG. Samples were analysed at 3 dilutions, either 1:200, 1:400 and 1:800 for IgG1 and IgG3, or 1:5,000, 1:10,000 and 1:20,000 for total IgG. Arbitrary titers was extrapolated from the HIS regression curve using a four-parameter logistic fit.

2.5. SDS-PAGE and immunoblotting

Parasite extracts were lysed as above, re-suspended in 25mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100 [27], and 10µg was migrated on NuPAGE™ 4-12% Bis-Tris gels (Invitrogen) under non-reduced conditions, and stained with silver nitrate or transferred to nitrocellulose membranes overnight at 12V using the Invitrogen transfer system. Primary antibodies were diluted 1:1000 in TBS-milk 1%, 0.1% Tween-20 and alkaline phosphatase conjugated secondary antibodies were diluted 1:5000 (Promega).

2.6. PMN preparation

Blood samples from healthy individuals presenting for routine blood tests at the Pasteur Institute, Dakar were obtained with informed consent. Blood was collected into EDTA-K3 tubes (better than lithium heparin (LH) or citric acid-dextrose treated (ACD) tubes; 6-7mL from each of 6-7 donors), layered onto Ficoll-Hystopaque (density 1077, Sigma) and centrifuged at RT for 30 min at 400xg. RBC and PMN were harvested at the interface, and residual RBCs were lysed by incubation in 8.32g/L NH_4Cl,

0.8g/L sodium bicarbonate, and 0.043g/L EDTA 8 min at 4°C. After centrifugation at 400xg for 5 min, purified PMN were washed twice with Hank's balanced salt solution (HBSS), enumerated using trypan blue, and resuspended in PBS at a concentration of 5.10^7 cells mL^{-1}. Alternatively, blood was layered onto double Ficoll-Hystopaque (density 1077 and 1119) and centrifuged 30 min at 700xg. PMN were recovered at the ficoll interface and washed twice with HBSS.

2.7. Chemiluminescence monitoring of ROS generation

Chemiluminescence was measured using opaque 96-well plates (Berthold), and a MicroLumat Plus 96 luminometer (Berthold), controlled with WinGlow software. Chemiluminescence was recorded as relative light units (rlu). A 2mg/mL stock solution of luminol (4-aminophthalhydrazide; SIGMA) prepared in DMSO, was stored in aliquots at –20 °C in the dark, and used at a final concentration of 0.02mg/mL in sterile PBS. To facilitate rapid handling, only 40-50 wells per plate were used, with the HIS internal control systematically deposed in the first and last wells. Merozoites (40µL) were incubated with 10µL of test or control sera or IgG for at least 30 min at 37 °C. PMN (100µL at $5x10^7$ cells mL^{-1}) and luminol (100µL) were loaded rapidly using an Eppendorf multipipette 4780. Plate reading started immediately, and continued for 1 h. Data are presented as a standardized activity index of Antibody Dependent Phagocytosis of merozoites calculated as: ADPm = (rlu maximum sample / rlu maximum HIS) x 1000 where rlu maximum HIS is an average of the first and last wells on the plate. An additional internal control with the same positive serum was included in each run.

2.8. Statistical analysis

Antibody levels and/or ADPm comparisons were analyzed for statistical significance using the Wilcoxon signed rank test and the Spearman rank correlation test for non-normally distributed data. The Mann Whitney test was used to compare unpaired data, and only *P* values <0.05 were considered significant.

A Poisson regression model was used to analyze the relationship between the ADPm index and the incidence of clinical episodes during the following transmission season in Ndiop. Malaria episodes were defined as fever and/or malaria-like symptoms, and ≥30 *P. falciparum* trophozoites per 100 leukocytes, determined by experienced microscopists. Time at risk was calculated as number of days spent in the village during the 5.5-month follow-up. Episodes were considered independent if separated by >15 days. Risk was reduced by 15 days following a diagnosis of malaria, or by 8–15 days following anti-malarial treatment (regardless of diagnostic criteria), depending on the drug administered (8, 10, and 15 days for quinine, chloroquine, and sulfadoxine-pyrimethamine, respectively). This analysis included 114 individuals, with a total of 121 clinical episodes during the follow-up period. The mean incidence of clinical malaria per 1000 person-days as a function of age bracket was (i) 2–14 years: 15, n=49 (ii) 15–29 years: 4, n=32 and (iii) ≥30 years: 1, n=33 ($P<0.001$). Age stratification was based on parasitological and clinical data obtained at this site during a 10-year longitudinal study [24,25,28]. First-level interactions between variables were checked and included in the model where significant. The ADPm index stratification was determined by use of Akaike's information criterion (AIC). Statistical analyses were performed with Egret (version 3.01; Cytel) and Statview (version 5.0; SAS Institute) software.

3. Results

3.1. Merozoite preparations

Since merozoites are the key target of PMN phagocytosis, reproducible measures of ADPm depended on the quality and quantity of target material. Percoll gradient extraction of merozoites from synchronized cultures of mature schizonts gave the cleanest, most concentrated merozoites, and best chemiluminescence read-out (Fig. 1(A)). However, this technique yielded limited material and damaged cultures, so it wasn't suitable for high throughput analyses. In contrast, large quantities of merozoites could be regularly harvested from post-invasion media of cultures maintained at 5-10% parasitemia, but they contained hemozoin and membrane debris. To determine if these contaminants could activate PMN, they were isolated by differential centrifugation, and tested alone in the ADPm assay. A strong signal was induced with NIS, decreasing with HIS (Fig. 1(B)). However, with merozoites added, HIS induces 3-fold higher chemiluminescence than NIS with and without hemozoin. Merozoite preparations were compared by SDS-PAGE, after silver nitrate staining or immunoblotting with HIS antibodies, or an anti-MSP1p19 monoclonal antibody (Fig. 1(C)). No major differences were observed on immunoblots. Chemiluminescence signals were comparable using pools of frozen or fresh merozoites (data not shown), considerably facilitating the assay.

3.2. Effector cell population

The activity of PMN and PBMC harvested from the same donors were compared in the ADPm assay using the HIS pool. Although PBMCs are easier to harvest and have longer half-lives, they consistently generated lower signals and had longer response times (data not shown). The best PMN preparations regarding both yield and activity, were obtained using a single step ficoll gradient separation followed by RBC lysis. Donor-dependant variability in PMN phagocytic activity was minimized by using pools from several donors, which also allowed more sera to be tested in parallel. PMN from non-infected European or Senegalese donors were equally reactive with immune sera from Dielmo and Ndiop (data not shown). The requirement for very fresh PMN is the *sine qua non* of this assay, and rapid harvest, purification and utilization is critical.

3.3. Chemiluminescence profiles and standardization

Fig. 2 shows chemiluminescence readout as a function of time from 3 experiments (3 different PMN pools), with and without anti-sera (Fig. 2(A-C)). NIS alone gives no significant background. Chemiluminescence peaks were instantaneous, but amplitudes varied depending on the experiment and/or PMN batch. However, using HIS rlu values as an internal standard, a reproducible arbitrary unit, the ADPm index, could be calculated. ADPm indexes for the same standard serum were 1183, 1072 and 1126 for the 3 experiments of Fig. 2, yielding a mean of 1127 +/- 5%. Intra-assay (1 PMN pool) reproducibility was >95 % (P=0.002), while inter-assay reproducibility (different PMN pools) was >80 % (P=0.004). Giemsa staining after 30 min, shows that merozoites and hemozoin are effectively internalized by PMN under the ADPm assay conditions (Fig. 2(D)).

3.4. Dependence of chemiluminescence activity on IgG or complement

To demonstrate the essential role of IgG for chemiluminescence activity, 7 sera with high ADPm values were individually depleted of IgG by passage over Protein-G,

and the flow-through was concentrated to the original volume (IgG depleted serum). Figure 3 shows that purified IgG gives the same chemiluminescence signal as whole (P=0.4) or decomplemented (P=0.6) sera, while depleted sera had negligible activity. These results demonstrate definitively that chemiluminescence activity is mediated by IgG alone, with no essential requirement for complement (P=0.5) using the Wilcoxon signed rank test.

3.5. Comparison of ADPm activity in meso or holo-endemic conditions

ADPm indexes were compared in sera from residents of Ndiop or Dielmo (114 each), corresponding to meso or holo-endemic settings respectively. Fig. 4(A) shows that the mean ADPm index for Dielmo was significantly higher than for Ndiop (308 and 271 respectively, P=0.0019). However, when the ADPm index was compared in individuals with (asymptomatic) or without circulating parasites at the time of sampling, a significant reduction was observed in the Dielmo cohort harboring 52% asymptomatic carriers (P<0.001, Fig. 4(B)). A similar trend was observed in Ndiop, but with only 22% asymptomatic carriers, the data didn't reach statistical significance. These results suggest that anti-merozoite antibodies are being consumed to control parasitemia in asymptomatic carriers.

3.6. ADPm is mediated by cytophilic IgG1 and IgG3 antibodies

To determine if ADPm is associated with particular IgG isotypes, sera from 88 aparasitemic Ndiop residents were monitored for merozoite antibodies using isotype specific reagents. While only 3 or 4 sera were above background for IgG2 and IgG4 respectively (dilution 1:100), all were positive for IgG1 and IgG3. Arbitrary total IgG, IgG1 and IgG3 titers were calculated for each serum using the HIS standard regression curve, and sera were stratified into 5 groups depending on ADPm index levels (Fig. 5(A)). Fig. 5(B) shows a highly significant correlation between total IgG titers and ADPm activity when comparing the low (+/-) and moderate (+) ADPm groups (P<0.0001), and a less significant correlation between the + and high (++) categories (P=0.04). Similar significant correlations are seen between IgG1 (Fig. 5(C)) or IgG3 (Fig. 5(D)) titers, and +/- or + ADPm index levels (P=0.0005 and 0.0002 respectively), and + or ++ ADPm index levels (P=0.048 and 0.0048 respectively). There were no significant correlations between IgG titers and ADPm index for negative (-) to +, and ++ to very high (+++) categories. To implicate preferentially IgG1 or IgG3 in ADPm activity, the Spearman test was used to compare IgG3:IgG1 ratios stratified in 3 groups: (i) < 0.5 (ii) ≥0.5<1 and (iii) ≥1 with unstratified ADPm indexes. A significant positive correlation was seen only for sera with an IgG3:IgG1 ratio of < 0.5 (P=0.0016), suggesting a predominate role for IgG1.

3.7. Association between ADPm and susceptibility to clinical episodes

Associations between ADPm index and number of cumulative clinical episodes experienced during the 2002 transmission season in Ndiop were analyzed. The best Poisson regression model according to AIC was obtained using a cut-off close to the mean ADPm value (250). A bivariate analysis showed that the variables significantly associated with the number of clinical attacks were (i) the continuous and stratified ADPm index (continuous rate ratio [RR]=1.004 per ADPm unit lost, P<0.001; dichotomized rate ratio, RR=3.67, P<0.001), (ii) age (15–29 vs. ≥30 years, RR^1=3.6; 2-14 vs ≥30 years, RR^2=15.9; P=0.01 and

P<0.001, respectively), and (iii) hemoglobin phenotype (normal vs AS, RR=3.31; *P*=0.001). Conversely, positive parasitemia at enrollment was not associated with the number of clinical attacks. Table 1 shows two alternative models, using continuous ADPm index, age and hemoglobin phenotype (model 1), or stratified ADPm index, age and hemoglobin phenotype (model 2). Hemoglobin phenotype was no longer significant in either model. ADPm index and age were still significantly associated with immune status when using the mean ADPm cut-off as shown in Table 1.

4. Discussion

This study describes a functional antibody assay measuring ADPm and generation of ROS by activated PMN, detected by chemiluminescence. While other *P. falciparum* blood stages may also be implicated [20], merozoites are by far the best stimulators of oxidative burst [29] and phagocytosis [9]. Frozen merozoites prepared from supernatants of continuous *P. falciparum* in vitro cultures, provide good reproducible readouts in this assay.

The primary limitation of the ADPm assay is its requirement for fresh PMNs, which have a short half-life and donor dependent activity, and no PMN cell line can substitute. Nevertheless, the use of PMNs, rather than PBMCs, is important for several reasons. First, PMNs have consistently shown the most intense oxidative respiratory burst when stimulated by *P. falciparum* asexual blood stage parasites, particularly merozoites [12,19,29]. Secondly, PMN growth inhibition of *P. falciparum* in vitro is substantially dependent on antibody opsonins in immune serum [30], while monocytes or differentiated macrophages inhibited equally well with normal or immune serum in a radiometric assay [31], and monocytes from malaria patients efficiently phagocytosed merozoites, in the absence of immune serum [9]. ADPm activity totally depended on IgG in immune sera, particularly cytophilic IgG1 and IgG3 isotypes, but was independent of complement, as observed by Kumaratilake et al. [11]. IgG1 and IgG3 isotypes have been associated with clinical protection against malaria [32,33], as opposed to IgG2 and IgG4, which may be antagonistic [34], and associated with increased susceptibility [35].

A major asset of the ADPm assay is its standardized protocol using an internal positive control to assign arbitrary ADPm values for each serum. This permits inter-experimental comparison and increases the statistical power to correlate functional activity of antibodies induced by natural exposure or vaccination, with clinical protection. Extensive epidemiological data from a Senegalese village (Ndiop) was analyzed in an age-adjusted Poisson regression model, to correlate sera having ADPm indices >250, with fewer clinical episodes. This corresponds to a 44% decrease in the risk of clinical malaria, and is the first statistical correlation between a functional antibody readout and clinical outcome [8,36,37]. The same analysis showed no correlation between anti-merozoite IgG levels (ELISA) and morbidity (*P*=0.91), indicating that ADPm is a more relevant parameter for predicting clinical protection than overall antibody titers.

ADPm induced chemiluminescence appears to have in vivo relevance, since antibodies mediating these effects are present in variable amounts depending both on the state of acquired immunity, and on their use to control parasitemia. Sera from asymptomatic carriers had significantly lower ADPm indexes than sera from aparasitemic individuals, indicating that antibodies measured by ADPm are actively consumed to control parasitemia. Parasite killing by PMN or monocytes-macrophages is mediated by both phagocytosis and ROS.

Merozoite phagocytosis by neutrophils has been observed both in vivo [13,38] and in vitro [30], and ROS release has been implicated in parasite destruction [14,39]. Inhibition of *P. falciparum* cultures by activated PMN, was partially reversed by histidine and tryptophan, but not by superoxide dismutase or catalase, suggesting that singlet oxygen is the main ROS inhibiting parasite growth [39].

TNFα, TNFβ, and INFγ increase both the initial peak and duration of chemiluminescence signals induced in PMN activated by opsonized merozoites [30], suggesting that ADPm levels could be augmented using these cytokines. However, since PMNs from different donors may respond differently to cytokine stimulation [40], standardized ADPm indexes, and their correlation with clinical protection should be analyzed under these conditions.

The ADPm assay and antibody dependent cellular inhibition (ADCI) described previously [12,41], may measure similar phenomena associated with phagocytosis of opsonized *P. falciparum* merozoites, and ROS release by activated PMN and monocytes/macrophages. PMN have a short in vitro half-life and are unlikely to be functional and/or viable during most of the long (48-96-h) ADCI culture times (even with daily renewal). Indeed, microscopic examination of 48-h ADCI cultures indicated that an unspecified percentage of effector cells had been destroyed and was greater for PMN than for monocytes [41]. Since PMNs must be freshly prepared using rapid techniques, and used immediately in relatively short assays (60 min here), their in vitro or in vivo contributions would not be properly evaluated using the ADCI protocol. The unidentified soluble factor released by monocytes mediating ADCI may also be ROS, since intra-erythrocytic parasites are highly sensitive, and activated monocytes also produce ROS. Singlet oxygen preferentially mediates *Plasmodium* growth inhibition by ROS, explaining the inability of superoxide dismutase and catalase to block ADCI activity [12,39]. Finally TNFα is a mediator of ADCI [12], but also increased ROS release by PMNs activated with opsonized merozoites [11], so it may function similarly in both assays.

In conclusion, ADCI and ADPm appear to measure similar phenomena of *Plasmodium* destruction triggered by IgG specific for MSPs. However, the ADCI protocol is complex, involving several parameters not easily controlled, including variability and viability of donor monocytes>macrophages, co-cultured for relatively long periods with finicky parasites, and requiring tedious, less precise microscopic readouts, compared to automated chemiluminescence. The ADPm technique has further validated the baculovirus PfMSP1p19 vaccine candidate, using transgenic parasites [7,42], and another recombinant MSP candidate (manuscript in preparation). If this assay is reproducible in other laboratories, it could help to reliably prioritize MSP vaccine candidates for clinical testing.

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Table 1. Poisson regression analysis of ADPm index values and clinical outcome. Two alternative models of Poisson regression were built to analyze the relation between ADPm and acute malaria morbidity. Both models include age but the first one includes the continuous (decreased) ADPm index and the second, the stratified ADPm index, categorized in two classes using the mean ADPm value cut point. The latter model is the best (AIC=120.4), indicating that the risk of malaria attack is 1.79-fold higher for individuals with ADPm <250 than for those with ADPm >250.

		Model 1			**Model 2**		
		RR	95% CI	*P*	RR	95% CI	*P*
ADPm							
	Continuous	1.002	1.001-1.003	0.049			
	Dichotomized						
	≥ 250				1		
	< 250				1.79	1.038-3.077	0.036
Age (years)				<0.001			<0.001
	≥ 30	1			1		
	15 - 29	3.298	1.220-8.915	0.019	3.203	1.182-8.681	0.022
	< 15	12.239	4.868-30.773	<0.001	12.627	5.036-31.660	<0.001
Deviance			118.6			118.4	
AIC			120.6			120.4	

Figure Legends

Fig 1. Comparison of merozoite preparations for ADPm activity. (A) CL tested in parallel with merozoites derived from mature schizonts and percoll fractionation, or centrifuged culture supernatants of cultures. (B) CL observed with or without NIS or HIS, using merozoites prepared with or without hemozoin/cell debris, or the latter alone without merozoites. The figure represents a typical profile using different PMN batches. (C) SDS-PAGE and immunoblots of 3 merozoite preparations: lane 1, percoll purified; lane 2, culture supernatants with hemozoin/cell debris removed; lane 3, culture supernatants. Gels were loaded with 10 g of protein from each preparation, and stained with silver nitrate or transferred to nitrocellulose membranes and probed with the HIS or the G17.12 mAb specific for MSP1p19 [43].

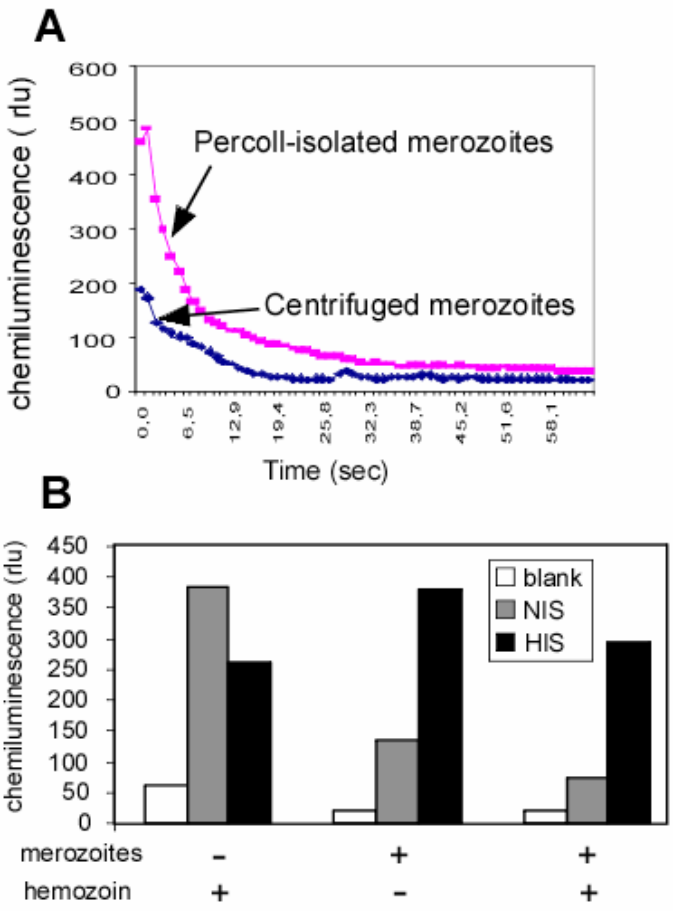
Fig. 2. Chemiluminescence activity profiles and standardization of ADPm (A-C) Chemiluminescence profiles from 3 different ADPm experiments (3 PMN pools) tested without serum (blank), or in the presence of NIS, HIS and a sample serum. The ADPm index for the sample serum, calculated as described in Materials and Methods using HIS as a positive standard, is shown for each experiment. (D) Giemsa stained smear of an ADPm reaction after 30 min in the absence of luminol; arrows indicate internalized merozoites and pigment.

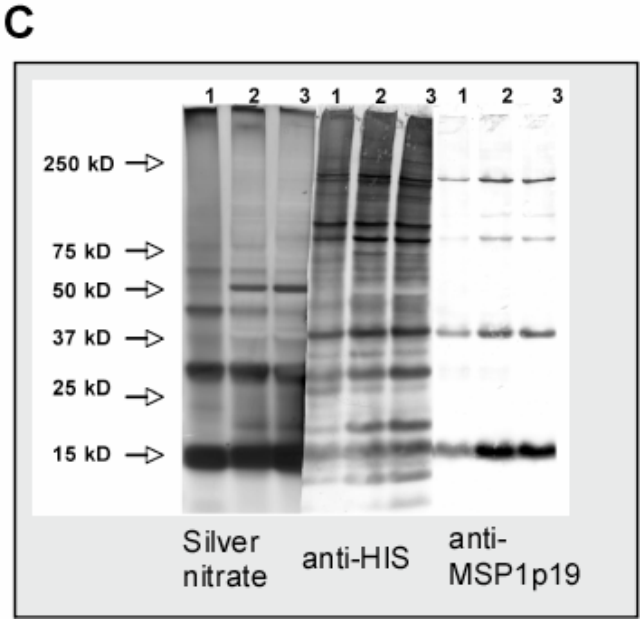
Fig. 3. Role of serum IgG and complement in the ADPm assay**.** ADPm indexes were determined using whole (lane 1), decomplemented (lane 2), and IgG depleted fractions (lane 3) of 7 different sera, as well as purified IgG (lane 4). Purified IgG gives the same signal as whole (P=0.4) or decomplemented (P=0.6) sera, showing that chemiluminescence activity is mediated by IgG alone, with no requirement for complement (P=0.5), using the Wilcoxon signed rank test. Distribution and median values are indicated by vertical and horizontal lines respectively.

Fig. 4. Comparison of ADPm index profiles for Dielmo and Ndiop**.** *(*A) The average ADPm index value detected for 114 age-matched individuals from Dielmo and Ndiop. (B) ADPm index values for the two villages as a function of parasite carriage at the time of blood sampling. Statistical significance was determined using the Wilcoxon signed rank test and significant *P* values are noted.

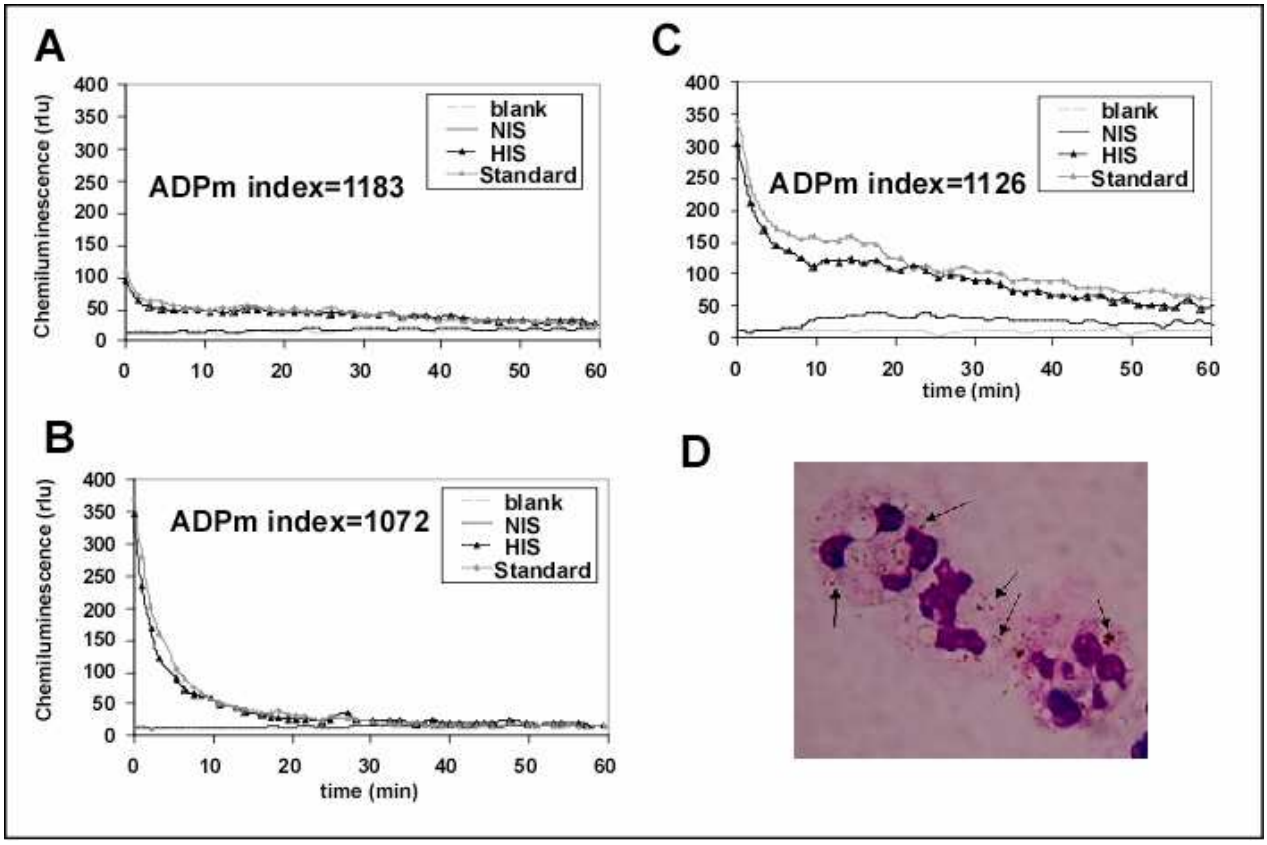
Fig. 5. Chemiluminescence dependence on IgG1 and IgG3 isotype titers. (A) ADPm indexes of sera from 88 aparasitemic Ndiop residents were stratified into 5 groups as indicated in the figure inset: (i) background <100; (ii) low=100-200 (+/-); (iii) moderately positive=200-400 (+); (iv) high positive=400-600 (++); and (v) very high positive >600 (+++). Stratified ADPm indexes are shown as a function of (B) total IgG titer, (C) IgG1 titer, and (D) IgG3 titer. Statistically significant differences, as detected with the Mann-Whitney test, are shown.

Vaccine, Joos et al. , Figure 1

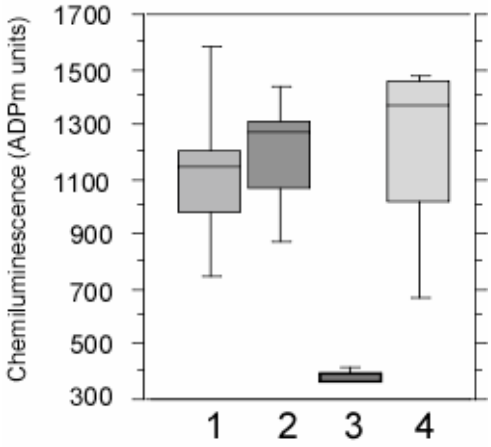




Vaccine, Joos et al. , Figure2



Vaccine, Joos et al. , Figure 3



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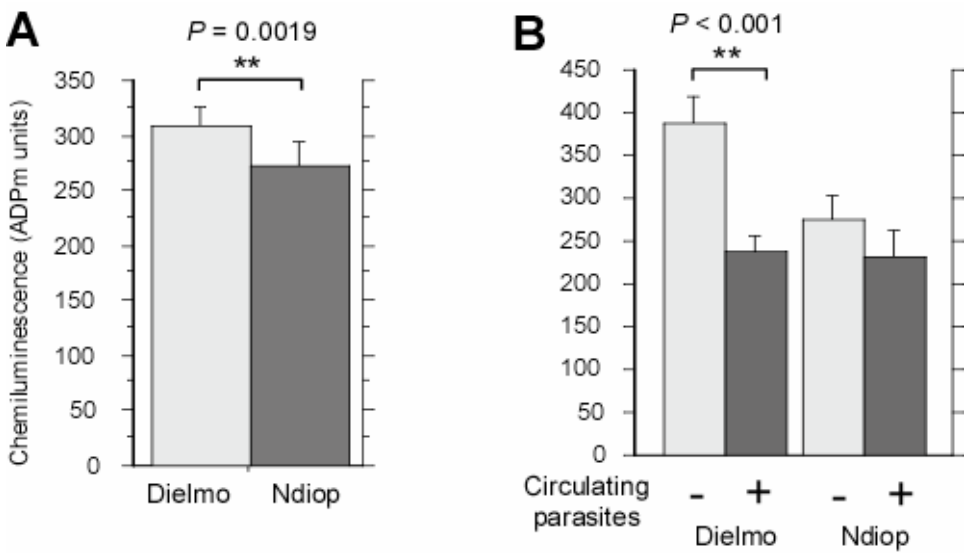
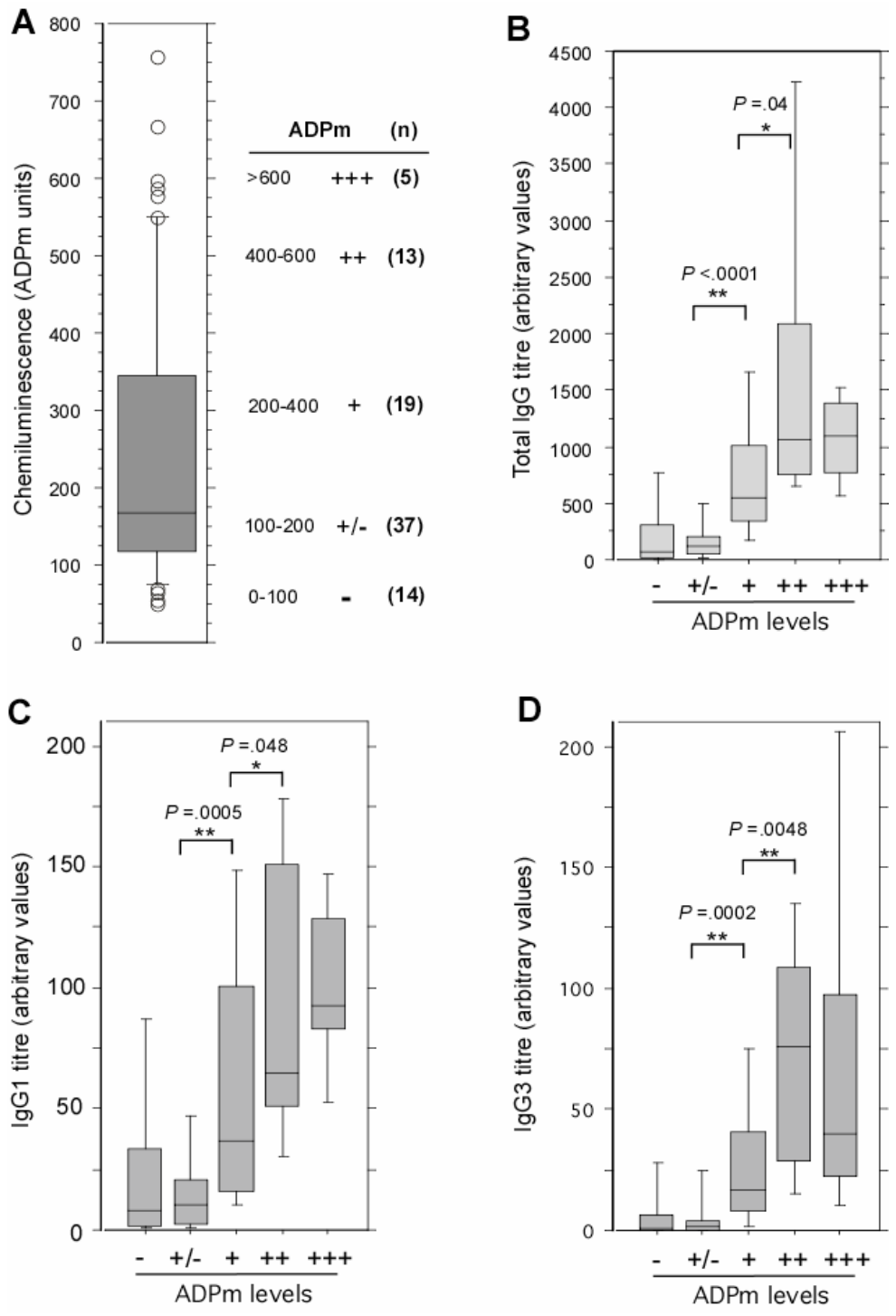


Vaccine, Joos et al. , Figure 5



2) Article en préparation - avant soumission

Antibodies specific for two leading malaria vaccine candidates are major components of antibody dependent neutrophil activation by merozoites.

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The project protocol and study design were explained to the assembled villagers, and informed consent was obtained individually from all participants or their parents or guardians. The protocol was approved by the Ethics Committee of the Ministry of Health of Senegal.
The authors do not have any commercial or other association that might pose a potential conflict of interest[1]

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Abstract
Merozoite surface proteins (MSPs) are malaria vaccine candidates because they are accessible to antibodies and mediate erythrocyte invasion. Functional antibody assays could help prioritize MSP candidates for expensive clinical testing. A new recently described functional antibody assay is based on antibody dependent phagocytosis of *P. falciparum* merozoites (ADPm), and activated neutrophil oxidative bursts, detected by chemiluminescence. Previous results are confirmed and extended by showing that ADPm statistically correlates with holoendemic clinical protection. This is the first time that *in vitro* functional activity of antibodies to parasite antigens in endemic sera have been statistically correlated with clinical protection from disease. Highly significant correlations were also found between the ADPm index and IgG specific for baculovirus recombinant MSP1p19, MSP4p20 and MSP5. An important role for PfMSP1p19 antibodies in ADPm activity was demonstrated using transgenic merozoites expressing *P. chabaudi* MSP1p19. Depletion of antibodies specific for PfMSP1p19 and/or PfMSP4p20 in endemic sera significantly reduces ADPm activity, strongly supporting the vaccine candidacy of these antigens.
Key words: MSP vaccines, functional antibody assay, neutrophil chemiluminescence

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1. Introduction

Plasmodium malaria parasites are responsible for enormous morbidity and mortality in tropical and sub-tropical developing countries. *P falciparum* causes the most severe, life-threatening forms of malaria, and is the target of multi-drug resistance, accentuating the need for an effective vaccine. Asexual extracellular merozoites invade erythrocytes, and merozoite surface protein (MSPs) are promising vaccine candidates because they are accessible to antibodies and complement, and they mediate a critical process for parasite survival [1]. Indeed, high antibody titres to some MSPs in endemic immune antisera have been statistically associated with protection against clinical episodes of malaria [2-6], and many current blood stage vaccine candidates are recombinant MSP homologues [7-8].

Baculovirus recombinant homologues of C-terminal MSP1 from *P. vivax*, *P. cynomolgi* and *P. falciparum* have been proposed as blood stage malaria vaccine candidates [9-11]. Most have been evaluated in primate vaccination-challenge trials, where both MSP1p42 and/or MSP1p19 showed substantial, but adjuvant dependent efficacy [12-14] (S. Longacre, C. Amaratunga, S. Handunnetti, unpublished data; J. Barnwell, S. Longacre, unpublished data; H. Contamin, J-C Michel, S. Longacre, unpublished data). In addition, high antibody titres to baculovirus PfMSP1p19 in endemic sera, were statistically associated with protection against clinical episodes in an age adjusted, multivariate analysis [2].

P. falciparum MSP4 and MSP5 have more recently joined the panoply of potential MSP vaccine candidates. Both proteins have a single C-terminal epidermal-growth-factor (EGF) like domain and are anchored to the merozoite surface by a glycosyl-phosphatidyl-inositol (GPI) moiety, similar to MSP1 [15]. PfMSP4, but not PfMSP5, is essential for parasite survival, although the latter may be required for optimal parasite growth *in vivo*. Analyses of PfMSP4 polymorphism in field isolates from several geographical sites [16], documented 18 non-synonymous polymorphic nucleotide sites for *Pfmsp4*, primarily near the N-terminus (residues 45-81), suggesting this region is subject to immune pressure. Strikingly, *Pfmsp5* is highly conserved, a rare feature for genes encoding MSPs, which usually harbour disproportionate numbers of non-synonymous polymorphisms [17-18].

We have produced baculovirus recombinant analogues of PfMSP4 and PfMSP5 as potential vaccine candidates, including a C-terminal protease resistant form of PfMSP4, which lacks most of the polymorphic region, PfMSP4p20 (Polson et al. article in preparation). While primate vaccination trials have often been used to validate vaccine candidates, this approach is unsuitable for evaluating the plethora of new candidates, because of animal cost and accessibility, reproducibility, inter-test comparability, and persistent doubts regarding the relevance of such studies. Recently, validation efforts have begun to focus on standardized *in vitro* functional antibody assays, which are less costly, avoid the logistical dilemmas of primate trials, and are more conducive to direct comparative testing (Long et al, submitted article; Arnot et al., submitted article). A new *in vitro* functional antibody test for MSP vaccine candidates was recently described, based on antibody dependent oxidative bursts and phagocytosis of merozoites (ADPm) by activated polymorphonuclear neutrophils (PMN), detected by chemiluminescence (Joos et al., submitted article). In a meso-endemic area, ADPm was positively correlated with cytophilic IgG1 and IgG3 titres, age related natural immunity, and reduced risk of clinical malaria episodes, an observation confirmed and extended in the present study in a holoendemic setting. Here, the ADPm chemiluminescence assay is used to evaluate the functionality of antibodies

specific for baculovirus PfMSP1p19, PfMSP4p20 and PfMSP5 vaccine candidates. A significant ADPm role for anti-PfMSP1p19 is demonstrated using transgenic *P. falciparum* merozoites expressing *P. chabaudi* MSP1p19 [19]. Endemic immune sera depleted of antibodies to PfMSP1p19 and/or PfMSP4p20, but not PfMSP5, had significantly reduced ADPm activity. Implications of these results for MSP based vaccine evaluation and development are discussed.

2. Materials and methods

2.1. Study site and sampling

Blood samples were collected in Dielmo and Ndiop, two Senegalese villages with perennial and moderate seasonal transmission respectively, after approval was obtained from the ad hoc Ethics Committee [20-22]. In July 2002, healthy villagers from Dielmo and Ndiop were enrolled in a cross-sectional prospective follow-up study. Cohorts of 116 sera from Dielmo, and 114 sera from Ndiop, were chosen to be representative of the population structure of each village. The mean ages of the Dielmo and Ndiop cohorts were 28.6 (3.4–80.5) yrs and 24.0 (3.4-76.9) yrs, respectively. After venous puncture, plasma and RBCs were separated by centrifugation and stored at –20°C.

2.2. Antigens

Purified baculovirus recombinant proteins corresponding to PfMSP1p19 [11], PfMSP4p20 (Polson et al. article in preparation) and PfMSP5 (Polson et al. article in preparation) were used as coating antigens for ELISA analysis and for sera depletions by immuno-affinity chromatography. The soluble, secreted proteins were produced in Sf9 or High Five (Invitrogen) insect cells infected with recombinant baculovirus, and were purified by metalloaffinity chromatography using a C-terminal hexa-histidine tag.

2.3. ELISA analysis.

Antigens were coated on Immulon-4 plates (Dynatech, Springfield, Va) at 0.5 µg.mL-1. The ELISA protocol has been described previously [2, 20]. For reliable inter assay comparisons, results were expressed as OD-ratio = OD-sample / OD-naïve, and used to measure the prevalence of specific antibodies to each antigen at a 1:200 dilution. To monitor specific antibody depletions quantitatively, samples were analysed at 1:200, 1:400 and 1:800 dilutions, and an arbitrary titre was extrapolated using a four-parameter logistic fit from a standardized positive control regression curve on each plate, determined using a pool of hyper-immune sera (HIS) from 30 adult residents of Dielmo and 2-fold dilutions starting at 1:200 (E. Remarque, unpublished technique). A non-immune sera negative control (NIS) was obtained commercially (Valbiotech, France).

2.4. Serum depletion

Sera from individuals with high ELISA responses for the different MSP test antigens were selected. Each test serum (100 µL) was diluted 1:3 in PBS and incubated in solution with 50 µg of MSP recombinant protein for 30 min at RT to allow efficient antigen-antibody binding. Packed TALON Metal Affinity Resin (Ozyme) pre-equilibrated with PBS (200 µL), was added and incubated with gentle mixing for 3 hr at RT, to allow antigen-antibody complexes to bind to the resin via the C-terminal hexa-histidine tag on the recombinant proteins. Depleted sera were recovered in the supernatant after centrifugation without further dilution, so that initial and depleted sera were directly comparable. Individual sera were depleted of IgG specific for PfMSP1p19, PfMSP4p20, or PfMSP5 alone, or of IgGs

specific for PfMSP1p19 and PfMSP4p20 (consecutive depletions).

2.5. Parasite culture and merozoite extraction

Palo Alto (FCR3) *P. falciparum* parasites were maintained in continuous culture on O+ erythrocytes in RPMI supplemented with 0.5 % Albumax and 1 μg/mL gentamycin, in candle jars [23]. After centrifugation of cultures with greater than 5% parasitemia for 5 min at 400 g, pelleted RBCs were replaced in culture, and merozoites were recuperated from culture supernatants following centrifugation for 20 min at 1500 g. The concentrated merozoite extracts were stored at –20°C until use.

2.6. PMN preparation

Whole blood samples were obtained from healthy individuals presenting for routine blood tests after informed consent. Freshly drawn peripheral blood was collected by veinipuncture using EDTA-K3 tubes (6-7 mL from 6-7 donors), layered onto Ficoll-Hystopaque (density 1077, Sigma) and centrifuged at RT for 30 min at 400 g. RBC and PMN were harvested at the interface, and residual RBCs were lysed by incubation in lysis buffer (8,32 g/L NH_4Cl, 0,8 g/L sodium bicarbonate, and 0,043 g/L EDTA) for 8 min at 4°C. After centrifugation at 400 g for 5 min, purified PMN were washed twice with Hank's balanced salt solution (HBSS), enumerated using trypan blue and resuspended in PBS at a concentration of 5.10^7 cells mL^{-1}.

2.7. Chemiluminescence monitoring of PMN ROS generation

Chemiluminescence was measured using opaque 96-well plates (Berthold), and a MicroLumat Plus 96 luminometer (Berthold), controlled with WinGlow software. Chemiluminescence was recorded as relative light units (rlu). A 2 mg/mL stock solution of luminol (4-aminophthalhydrazide; SIGMA) prepared in DMSO, was stored in aliquots at –20 °C in the dark and used at a final concentration of 0.02 mg/mL in sterile PBS. The HIS internal control is systematically deposed in the first and last wells and an additional common positive control was included. Merozoite preparations (40 μL) were incubated with 10 μL of test or control sera for a minimum of 30 min at 37 °C. PMN (100 μL of $5x10^7$ cells mL^{-1}), followed by 100 μL of luminol were added as quickly as possible using an Eppendorf multipipette 4780. Plate reading started immediately, and continued for 1 h. Data are represented as a standardized activity index of Antibody Dependent Phagocytosis of merozoites (ADPm) calculated as:

ADPm = (rlu maximum sample / rlu maximum HIS) x 1000

where rlu maximum HIS is an average of the first and last wells on the plate.

2.8. Statistical analysis of ADPm assay data

ELISA and/or ADPm data were analyzed for statistical significance using the Wilcoxon signed rank test and the Spearman rank correlation test for non normally distributed data. *P* values <0.05 were considered significant.

A Poisson regression model was used to analyze the relationship between the ADPm index and the incidence of clinical episodes during the following transmission season in Dielmo. A malaria episode was defined as the presence of fever and/or malaria-like symptoms, and ≥30 *P. falciparum* trophozoites/100 leukocytes as determined by an experienced microscopist. The time at risk for each villager, was calculated as the number of days actually spent in the village during the 5.5-month follow-up period. Episodes

were considered to be independent if they were separated by >15 days. Individual risk determinations were reduced by 15 days following a diagnosis of malaria, or by 8–15 days following anti-malarial treatment (regardless of diagnostic criteria), depending on the drug administered (8, 10, and 15 days for quinine, chloroquine, and sulfadoxine-pyrimethamine, respectively). This analysis included 116 individuals, with a total of 50 clinical episodes during the follow-up period. The mean incidence of clinical malaria per 1000 person-days as a function of age bracket was (i) 2–6 years: 18.8, n=18, (ii) 7-14 years: 1.8, n=31 and (iii) ≥15 years: 0.3, n=67 ($p<0.001$). The age stratification was based on parasitological and clinical data obtained at this site [24]. First-level interactions between variables were checked and included in the model where significant. Validities of models were determined by use of Aikake's information criterion (AIC). Statistical analyses were performed with Egret (version 3.01; Cytel) and Statview (version 5.0; SAS Institute) software.

3. Results

3.1. Confirmation of the correlation between ADPm and susceptibility to clinical episodes in a holoendemic setting

ADPm was previously shown to be positively correlated with age-related natural immunity and reduced risk of clinical malaria episodes in a mesoendemic Senegalese village (Ndiop) (Joos et al., submitted article). To determine if these relationships were valid in a holoendemic setting, the same parameters were analyzed using serum samples collected in 2002 in Dielmo, a holoendemic Senegalese village (Table 1). A bivariate analysis showed that the variables significantly associated with the number of clinical attacks were (i) the continuous and stratified ADPm index (continuous rate ratio RR=1.001 per unit lost; dichotomized rate ratio in 300 (mean value), RR=30.59; dichotomized rate ratio in 250 (Ndiop model value), RR=11.76; $P<0.001$ for all), and (ii) age (7-14 vs. ≥15 years, $RR^1=8.43$; 2-6 vs. ≥15 years, $RR^2=87.54$; $P<0.001$ for both). Conversely, hemoglobin phenotype and positive parasitemia at enrollment were not associated with the number of clinical attacks, although hemoglobin phenotype had a significant effect in multivariate models. Table 1 shows three alternative models, using continuous ADPm index, age and hemoglobin phenotype (model 1), or stratified ADPm index dichotomized to 300 units, age and hemoglobin phenotype (model 2), or stratified ADPm index dichotomized to 250 units, age and hemoglobin phenotype (model 3). ADPm index and age were still significantly associated with immune status for each model as shown in Table 1. The best Poisson regression model according to AIC was obtained with the same cut-off ADPm value used for the Ndiop cohorts (250, model 3; (Joos et al., submitted article)). For this model, hemoglobin phenotype is not significant but statistically important (score test Hb : $P = 0.035$).

3.2. Relationships between the amplitude of IgG responses to PfMSP1p19, PfMSP4p20 and PfMSP5 in holoendemic and mesoendemic antisera

Since the ADPm assay is a measure of functionality of antibodies specific for MSP antigens, it was of interest to determine the prevalence of IgG specific for three MSP vaccine candidates under study, as well as relationships among their IgG levels. The prevalence of IgG specific for the baculovirus recombinant PfMSP1p19, PfMSP4p20 or PfMSP5 vaccine candidates in endemic sera from Dielmo (holoendemic, 116 sera) or Ndiop (mesoendemic, 114 sera) was determined by ELISA analysis (OD ratio values above background at a 1:200 dilution). In Dielmo, 73% of sera carried antibodies

recognizing PfMSP1p19, 88% recognized PfMSP4p20, and 74% recognized PfMSP5. Corresponding values for Ndiop were 86% for PfMSP1p19, 78% for PfMSP4p20, and 66% for PfMSP5. The relationship between quantitative IgG responses to each pair of the three antigens in individual sera from both villages (OD ratio values at 1:200 dilution) was tested using the Spearman rank test. As shown in Table 2, there were highly significant positive correlations of IgG responses to PfMSP1p19, PfMSP4p20 and PfMSP5 (rho from 0.37 to 0.58, $P<0.0001$), indicating that individuals tended to respond with similar relative intensities to all three antigens. This linkage (varying around an average of 50%) prohibited using a Poisson regression to determine the impact due to IgG for any individual MSP under study.

3.3. Relationship between specific IgG to PfMSP1p19, PfMSP4p20 or PfMSP5 and ADPm activity in individual sera

To determine if IgGs specific for any of the three vaccine candidates might have a role in mediating ADPm activity, a statistical analysis was carried out using ADPm index and ELISA OD-ratio values determined for each serum (230 samples, Table 3). Highly significant correlations were found between the ADPm index and IgG responses for MSP1p19, MSP4p20 and MSP5 in both villages ($P \leq 0.0003$). Nevertheless, when the analyses were sub-divided according to age group, some correlations were no longer significant using the Bonferroni correction, as shown in Table 3, and this was different for the two villages.

3.4. A role for PfMSP1p19 specific antibodies in ADPm chemiluminescence readout

The role of PfMSP1p19 specific IgG in the ADPm assay was investigated using transgenic merozoites for 21 antisera having high anti-PfMSP1p19 ELISA OD-ratios. Chemiluminescence (rlu) was compared using either *P. falciparum* D10 (D10-PfM3') or transgenic D10 merozoites in which PfMSP1p19 has been replaced by the non-cross reactive *P. chabaudi* homologue (PcMEGF) train [19, 25]. Consequently, any differences in the observed chemiluminescence can be attributed to antibodies specific for PfMSP1p19. Fig. 1 shows that the transgenic merozoites elicited reduced chemiluminescence for all sera, varying from 7-54% inhibition, with a mean of 32%, which was highly significant ($P<0.0001$). The non-immune serum pool (NIS) mediated higher than usual non-specific chemiluminescence activity that was nevertheless unchanged using the transgenic merozoites. These results indicate that anti-PfMSP1p19 antibodies induced by natural infection have a substantial effect on neutrophil activation and phagocytosis of merozoites.

3.5. Efficacy of antigen specific IgG depletion

Since the transgenic merozoites were relevant only for PfMSP1p19, an alternative technique was developed, involving serum depletion of antibodies specific for MSP antigens. Individual sera were incubated first with hexa-histidine tagged recombinant antigens in solution, and subsequently with metallo-affinity resin. Depleted sera were recovered in the supernatant following centrifugation of affinity resin with bound antigen and antigen-antibody complexes. Quantitative estimations of depletion were obtained by analysing samples at 3 dilutions, permitting an arbitrary titre to be extrapolated using a four-parameter logistic fit from a standardized positive control regression curve on each plate. Fig. 2 shows an example of anti-PfMSP4p20 depletion from the HIS hyper-immune pool. HIS was titered for either PfMSP4p20 (Fig. 2(A)) or PfMSP1p19

(Fig. 2(B)) specific antibodies before and after depletion. The results show that the depletion protocol is specific and highly effective. Median titre units decreased from 511 to 1 after anti-PfMSP1p19 depletion, and from 816 to 8 after anti-MSP4p20 depletion, giving mean efficiencies of >99%. However, anti-PfMSP5 depletion was somewhat less efficient: 74% (from 351 to 90 units).

3.6. Impact of PfMSP1p19, Pf MSP4p20 and Pf MSP5 specific IgG depletion on ADPm activity

Fig. 3 shows the effects of specific IgG depletion on ADPm activity. Anti-PfMSP1p19 depletion in 20 individual sera resulted in a highly significant average 29% reduction of the ADPm index (P=0.0017), confirming the results obtained using the transgenic merozoites (Fig. 3(A)). Surprisingly, anti-MSP4p20 depletion in 23 sera gave a 46% average reduction of the chemiluminescence signal (P<0.0001), even greater than for MSP1p19. Double depletion of IgGs specific for PfMSP1p19 and PfMSP4p20 (10 sera), diminished the ADPm signal by 70% (P=0.0051). In contrast, sera depleted of anti-MSP5 IgG (8) showed no significant reduction of ADPm activity (P=0.78), although this could be due to less efficient depletion. Nevertheless, sera depleted of antibodies specific for all three MSPs no longer mediated an ADPm response.

4. Discussion

The high cost of clinical trials, requiring expensive GMP quality material, prohibits the direct evaluation of immunogenicity and efficacy in humans for all but a small fraction of available malaria vaccine candidates. Since blood stage candidates such as MSPs, are likely to protect by reducing, but not necessarily preventing parasitemia, Phase 2a efficacy evaluation would require allowing challenge infections to continue longer than is generally considered ethically acceptable. However, the primary protective mechanisms for blood stage infections appear to be mediated by antibodies, so *in vitro* functional antibody assays have been proposed as putative surrogate markers for clinical protection. The standardization of such assays would allow comparative testing to prioritize candidates for further GMP process development and clinical testing [26, 27].

A new previously described functional assay for antibodies to merozoite surface proteins (Joos et al., submitted) is based on antibody dependent oxidative bursts and phagocytosis of merozoites (ADPm) by activated polymorphonuclear neutrophils (PMN), detected by chemiluminescence in the presence of luminol (Joos, et al. submitted). The fact that the assay was developed as a high-throughput technique has permitted statistical validation of the results obtained. ADPm was positively correlated with cytophilic IgG1 and IgG3 titres, age related natural immunity, and reduced risk of clinical malaria episodes, in a mesoendemic Senegalese village (Ndiop). Results obtained in the present study, have confirmed and extended the latter observation, showing that ADPm is similarly correlated with clinical protection in a holoendemic setting (Dielmo). It is notable that this is the first time that *in vitro* functional activity of antibodies to parasite antigens in endemic sera have been statistically correlated with clinical protection from disease [27] (Joos et al. submitted article). Although *in vitro* growth inhibition assays (GIA) have been widely used to measure the capacity of antibodies to inhibit parasite growth and/or erythrocyte invasion, even in clinical trials [28], no clear correlation with morbidity in humans has been found (Long et al., submitted article).

IgGs are the principal effectors of ADPm activity, with or without complement [29] (Joos et al., submitted article), and it has been suggested that merozoite

phagocytosis may be better correlated than other functional antibody tests with the immune status of malaria-exposed individuals [30]. The data presented here and elsewhere, showing a highly significant age-independent correlation of ADPm with reduced clinical susceptibility to malaria in two different endemic settings, strongly supports this hypothesis, and indicates that the ADPm assay could be particularly relevant for the evaluation of antibodies induced by MSP based vaccine candidates in preclinical and phase I trials.

The ADPm test may be especially useful for comparing the functionality of antibodies elicited by the many different MSP1 derived vaccine candidates proposed, based on the expression of different fragments (e.g. complete MSP1, C-terminal p42 or p19, N-terminal block 2 etc.) in different expression systems (*E. coli*, *S. cerevisiae*, *P. pastoris*, baculovirus), or corresponding to different polymorphic variants. Here, the impact of antibodies specific for baculovirus PfMSP1p19 present in 20 individual high responder endemic human sera, on ADPm activity was demonstrated with two different approaches using (i) transgenic merozoites differing only in the MSP1p19 polypeptide expressed and (ii) antisera depleted for antibodies to PfMSP1p19. Both methods gave comparable, highly significant results leading respectively to an average of 32% ($P<0.0001$) or 29% ($P=0.0017$) reduction of ADPm activity in the 20 sera tested. These results were similar to the findings of O'Donnell et al. [19], where a 25% reduction of *in vitro* parasite growth was observed using the same MSP1p19 transgenic parasites and endemic sera from Papua-New Guinea. These results indicate that antibodies to baculovirus PfMSP1p19 mediate both PMN phagocytosis of merozoites accompanied by oxidative bursts, which are toxic to intracellular parasites, and the inhibition of merozoite reinvasion and parasite growth in the absence of effecter leucocytes. Together they provide a strong rationale for the observation that high levels of antibodies to baculovirus PfMSP1p19, are correlated with a 30% reduced risk of malaria clinical episodes in endemic areas [2], and further underscore the unique potency of this promising vaccine candidate.

The transgenic merozoite approach was valid for MSP1p19, because its function is apparently based entirely on its 2-EGF domain physical structure, relatively independent of the particular amino acid sequence used to arrive at that structure. This feature is very unusual and is unlikely to apply to other MSP antigens of interest. Nevertheless, the MSP1p19 results support the validity of the depletion approach for the comparison of other MSP vaccine candidates in the ADPm assay. Indeed, anti-MSP4p20 depletion of 23 individual high responder sera for MSP4p20, lead to an average 46% ($P<0.0001$) reduced capacity to mediate chemilumenscence in the ADPm assay. Equally striking, double depletion of IgGs in 10 sera that were high responders to both MSP1p19 and MSP4p20, led to an average 70% ($P=0.0051$) reduction of ADPm activity. These results give solid support for the relatively recent baculovirus MSP4p20 vaccine candidate, and provide a strong rationale for a vaccine containing both the baculovirus PfMSP1p19 and PfMSP4p20 antigens.

The fact that antibodies to MSP5 had no apparent role in ADPm, could be due to the fact that the depletion protocol used was not as efficient for MSP5 as it was for MSP1 and MSP4. Alternatively, MSP5 is known to be present in very small amounts on the merozoite surface, such that its density may not be sufficient to permit the antibody cross linking of FcR on PMNs that triggers phagocytosis and the oxidative burst. Nevertheless, antibodies to MSP5 may function to limit parasitemia by other mechanisms, since they are statistically associated with reduced clinical episodes (R. Perraut and C. Joos, unpublished data).

Although the depletion protocol used here gave promising results, it nevertheless depended on antigens having a hexa-histidine tag, allowing efficient binding of antigen-antibody complexes to the metallo-affinity resin following association in solution, which is much more efficient than if one reactant is immobilized on a solid support. Indeed, this feature was found to be critical for efficient depletion. However, bound antigen-specific antibodies could not be easily recuperated due to technical difficulties linked to the metallo-affinity technology. Nevertheless, the depletion protocol should be readily adaptable either to antigens carrying other affinity tags, or immobilized using suitable protocols. Alternatively, antibodies from endemic sera could be purified by affinity chromatography to vaccine candidates and shown to function in the ADPm assay.

A more direct application of the ADPm assay for validation of MSP vaccine candidates in preclinical and early phase clinical trials, would be to analyse the functionality of antibodies induced by them in experimental animals and humans. In this context, it is important to consider that antibody cytophilic isotypes (IgG1 and IgG3 in humans) and compatible Fc receptors on PMNs are critical parameters for ADPm activity. Thus, antibodies induced in animals (e.g. rabbits or mice) might not be expected to function with human PMNs, and indeed in our hands, the former does not. Consequently, for preclinical evaluations the assay may be most relevant using antisera from primates immunized with the vaccine candidates of interest. This assumes that anti-sera or purified IgG from primates (possibly depending on species) can function *in vitro* with human or primate PMNs, which remains to be demonstrated. For clinical trials, the assay could be applied in a straightforward way with PMNs from human donors and sera or IgG from immunized volunteers.

In conclusion, the ADPm chemiluminescence assay provides a new technique for evaluating immune responses to *P. falciparum* recombinant MSP vaccine candidates. It is anticipated that it can be validated in other laboratories, and subsequently integrated into a panel of standardized functional antibody assays for use in the comparative evaluation of competing malaria vaccine candidates.

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Table 1

Poisson regression analysis of ADPm index values and clinical outcome for Dielmo. Three alternative models of Poisson regression were built to analyze the relation between ADPm and acute malaria morbidity. All three models include age, but model 1 includes continuous decreasing ADPm index, and models 2 and 3 use a stratified mean ADPm index dichotomized to 300 or 250 ADPm units respectively. The latter model is the best (AIC=90.7), indicating that the risk of malaria attack is 17.51-fold higher for individuals with ADPm <250 than for those with ADPm> 250.

	Model 1			Model 2			Model 3		
	RR	**95%CI**	***P***	**RR**	**95%CI**	***P***	**RR**	**95%CI**	***P***
ADPm									
Continue	1.005	1.001-1.008	0.006						
Dichotomized									
≥300				1					
<300				12.15	1.653-89.302	0.014			
≥250							1		
<250							17.509	2.275-134.744	0.006
Age (years)			<0.001			<0.001			<0.001
≥15	1			1			1		
7-14	4.233	1.133-15.823	0.032	3.827	1.027-14.256	0.046	2.876	0.759-10.904	0.12
2-6	31.944	9.189-111.052	<0.001	41.13	12.590-134.371	<0.001	17.637	5.042-61.690	<0.001
Hb									
AA							1		
AS							0.79	0.291-2.141	0.643
scoretest Hb			NS			NS			0.035
Deviance	111.8			106.6			86.7		
AIC	113.8			108.6			90.7		

Table 2

Relationships between the amplitude of IgG responses to PfMSP1p19, PfMSP4p20 and PfMSP5 in holoendemic and mesoendemic sera

	MSP5		MSP4p20	
	Ndiop	**Dielmo**	**Ndiop**	**Dielmo**
MSP1p19				
rho	0,49	0,4	0,58	0,37
P	<0,0001	<0,0001	<0,0001	<0,0001
MSP4p20				
rho	0,5	0,44		
P	<0,0001	<0,0001		

Table 3.

Relationship between IgG specific for PfMSP1p19, PfMSP4p20 or PfMSP5 and ADPm activity in individual sera

	NDIOP - mesoendemic area				DIELMO - holoendemic area			
	ALL	2-14 years	15-29 years	≥ 30 years	ALL	2-6 years	7-14 years	≥ 15 years
MSP1p19 vs ADPm	rho = 0.68 *P* < 0.0001	rho = 0.53 *P* = 0.0003	rho = 0.67 *P* = 0.0002	rho = 0.62 *P* = 0.0004	rho = 0.71 *P* < 0.0001	NS	rho = 0.57 *P* = 0.002	rho = 0.67 *P* < 0.0001
MSP4p20 vs ADPm	rho = 0.53 *P* < 0.0001	rho = 0.39 *P* = 0.007	rho = 0.73 *P* < 0.0001	NS	rho = 0.40 *P* < 0.0001	rho = 0.73 *P* = 0.002	NS	rho = 0.42 *P* = 0.0005
MSP5 vs ADPm	rho = 0.40 *P* < 0.0001	NS	NS	NS	rho = 0.33 *P* = 0.0003	NS	NS	rho = 0.34 *P* = 0.006

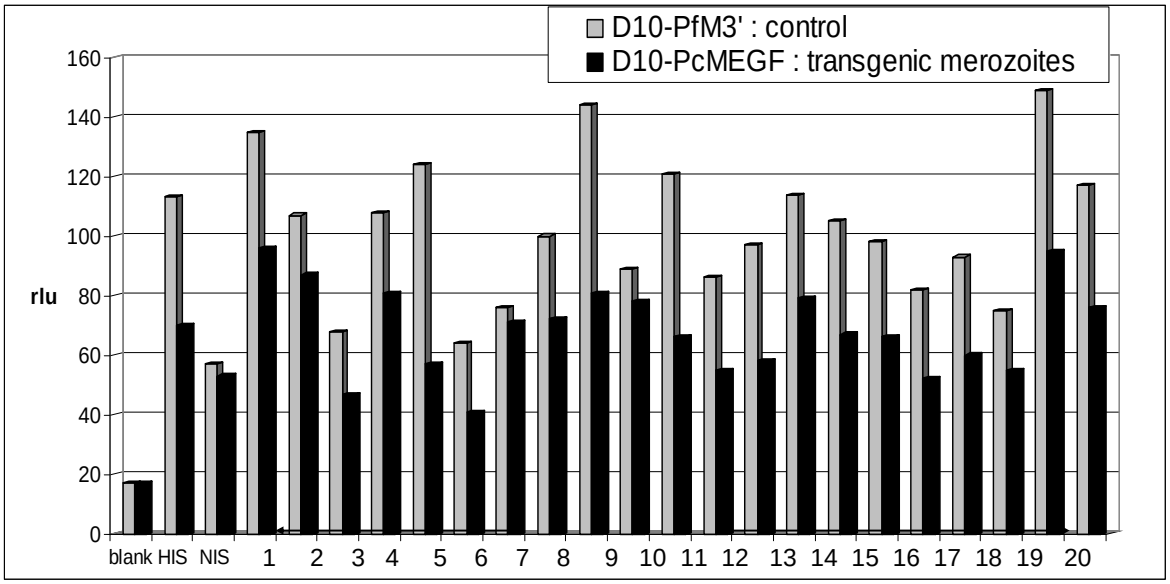
Figure Legends

Fig. 1. The impact of PfMSP1p19 specific antibodies on ADPm activity. ADPm chemiluminescence readouts (rlu) for 21 antisera having high anti-PfMSP1p19 OD-ratios, were compared using either *P. falciparum* D10 merozoites (D10-PfM3', black bars) or transgenic D10 merozoites with *P. falciparum* MSP1p19 replaced by the *P. chabaudi* homologue (PcMEGF, grey bars).

Fig. 2. Specificity of the antibody depletion protocol. The pool of 30 hyper-immune sera from Dielmo (HIS) were depleted of antibodies specific for PfMSP4p20 as described in Materials and Methods, and titered before (straight line) and after (dashed line) depletion, either against (A) PfMSP4p20 or (B) PfMSP1p19.

Fig. 3. Effect of anti-MSP1p19, anti-MSP4p20 and anti-MSP5 depletions on ADPm activity**.** Individual sera having high ELISA OD-ratios for each of the antigens under study were selected for the depletion experiments. The number of sera depleted individually of antibodies for each antigen is given in parenthesis. ADPm indices were determined before and after depletions. (A) anti-MSP1p19 depletion, (B) anti-MSP4p20 depletion, (C) anti-MSP1p19 and anti-MSP4p20 depletion, (D) anti-MSP5 depletion.

Vaccine, Joos et al., Figure 1



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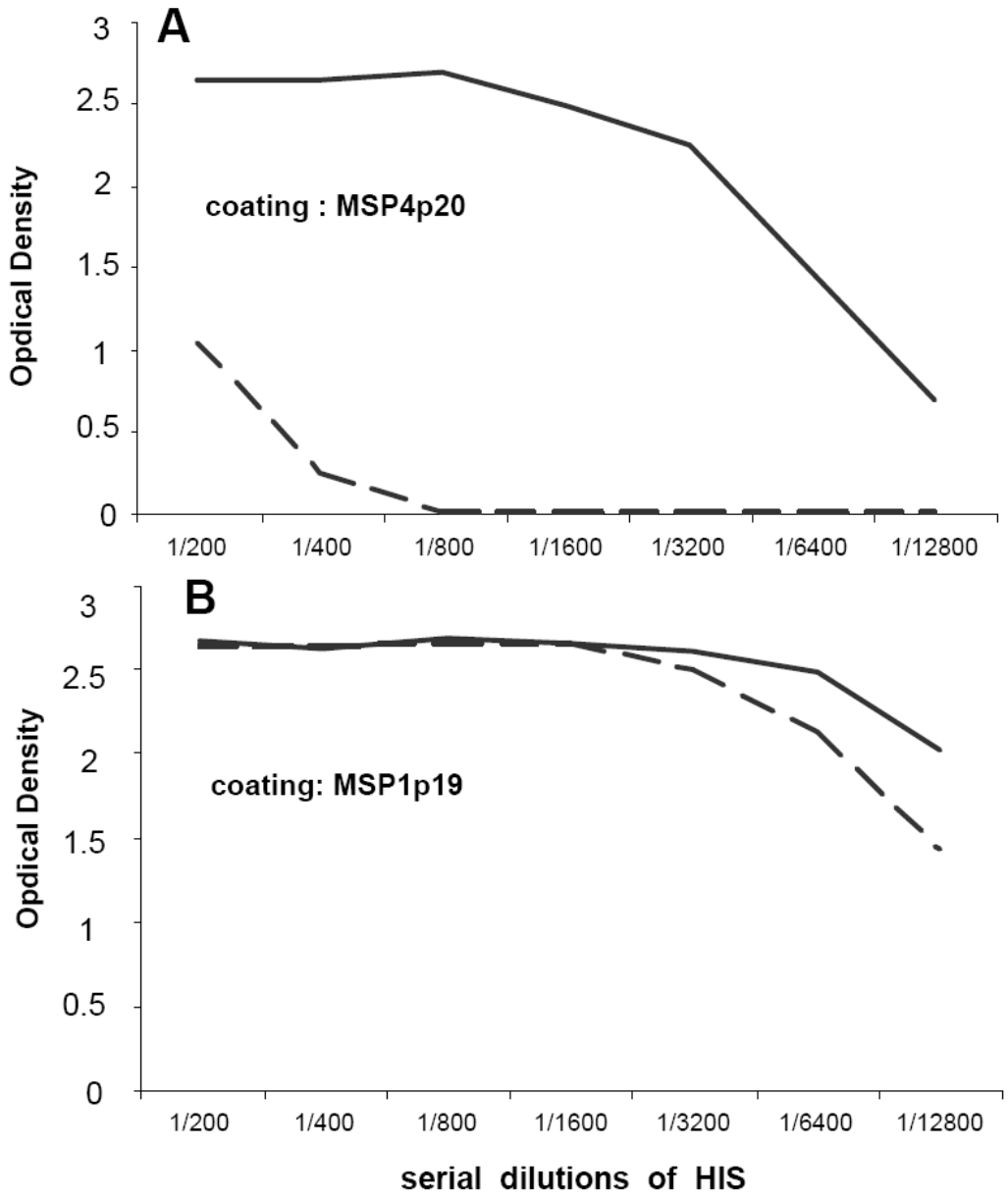

Vaccine, Joos et al., Figure 3