Comparative Testing of Six Antigen-Based Malaria Vaccine Candidates Directed Toward Merozoite-Stage *Plasmodium falciparum*[▽]

David E. Arnot,[1,2]* David R. Cavanagh,[2]† Edmond J. Remarque,[3]† Alison M. Creasey,[2]
Mercy P. K. Sowa,[1] William D. Morgan,[4] Anthony A. Holder,[4]
Shirley Longacre,[5] and Alan W. Thomas[3]

*Centre for Medical Parasitology, Institute for International Health, Immunology and Microbiology, University of Copenhagen,
Øster Farimagsgade 5, bygning 22 & 23, Postboks 2099, 1014 Copenhagen K, Denmark[1]; Institute of Immunology and
Infection Research, Division of Biological Sciences, Ashworth Laboratories, University of Edinburgh, King's Buildings, West Mains Road,
Edinburgh EH9 3JT, United Kingdom[2]; Biomedical Primate Research Centre, Department of Parasitology, Lange Kleiweg 1339,
2288 GJ Rijswijk, The Netherlands[3]; Division of Parasitology, MRC National Institute for Medical Research,
Mill Hill, London NW7 1AA, United Kingdom[4]; and Institut Pasteur, Rue du Dr. Roux, Paris, France[5]*

Immunogenicity testing of *Plasmodium falciparum* antigens being considered as malaria vaccine candidates was undertaken in rabbits. The antigens compared were recombinant baculovirus $MSP-1_{19}$ and five *Pichia pastoris* candidates, including two versions of $MSP-1_{19}$, AMA-1 (domains I and II), $AMA-1+MSP-1_{19}$, and fused $AMA-1/MSP-1_{19}$). Animals were immunized with equimolar amounts of each antigen, formulated in Montanide ISA720. The specificities and titers of antibodies were compared using immunofluorescence assays and enzyme-linked immunosorbent assay (ELISA). The antiparasite activity of immunoglobulin G (IgG) in in vitro cultures was determined by growth inhibition assay, flow cytometry, lactate dehydrogenase assay, and microscopy. Baculovirus $MSP-1_{19}$ immunizations produced the highest parasite-specific antibody titers in immunofluorescence assays. In ELISAs, baculovirus-produced $MSP-1_{19}$ induced more antibodies than any other single $MSP-1_{19}$ immunogen and three times more $MSP-1_{19}$ specific antibodies than the $AMA-1/MSP-1_{19}$ fusion. Antibodies induced by baculovirus $MSP-1_{19}$ gave the highest levels of growth inhibition in HB3 and 3D7 parasite cultures, followed by $AMA-1+MSP-1_{19}$ and the $AMA-1/MSP-1_{19}$ fusion. With the FCR3 isolate (homologous to the AMA-1 construct), antibodies to the three AMA-1-containing candidates gave the highest levels of growth inhibition at high IgG concentrations, but antibodies to baculovirus $MSP-1_{19}$ inhibited as well or better at lower IgG concentrations. The two *P. pastoris*-produced $MSP-1_{19}$-induced IgGs conferred the lowest growth inhibition. Comparative analysis of immunogenicity of vaccine antigens can be used to prioritize candidates before moving to expensive GMP production and clinical testing. The assays used have given discriminating readouts but it is not known whether any of them accurately reflect clinical protection.

After infection, humans make an antibody response to a large number of the 5,400 or so proteins encoded by the malaria parasite, *Plasmodium falciparum*. Tests of the efficacy of immunization have only been carried out with a few these proteins. Measurements of antibody or cellular responses to particular parasite antigens, following normal infection, have shown both positive and negative correlations with indirect measures of immune protection from malaria such as reduction in parasitemia, fever, or anemia (6, 7, 19). However, although immunoepidemiological data have influenced vaccine research, particularly in the selection of the variable erythrocyte surface adhesion antigens as vaccine targets, they have not provided sufficiently clear insights to support a scientific consensus on prioritizing antigens for vaccine development (21, 23). Malaria vaccine research has thus probably not yet achieved what has been termed the crucial "simplification of the complex" step in vaccine development (10).

Given the lack of evidence for a hierarchy of protective antigens, selection for vaccine development has frequently been based on the assumption that good vaccine candidates are likely to be antigens involved in biologically critical processes that may be susceptible to disruption by induced antibody binding. Prime targets include blocking invasion of liver hepatocytes by sporozoites, blocking red blood cell invasion by merozoites, and blocking infected erythrocyte cytoadhesion to host receptors on endothelium. Of around a hundred candidate malaria vaccines in various stages of development at present, around one-third contain sporozoite antigen sequences (circumsporozoite protein [CSP]), a further third of these prototypes contain merozoite surface protein 1 (MSP-1), and ca. 10% contain another merozoite protein, apical membrane antigen 1 (AMA-1) (14). These proteins were identified around a quarter of a century ago. Despite calls for new ideas in candidate selection, for blood-stage vaccines aimed at inhibition of replication of parasites in erythrocytes, antibody-mediated "invasion blockade" by targeting MSPs remains the paradigm that dominates the field.

* Corresponding author. Mailing address: Institute for International Health, Immunology and Microbiology, University of Copenhagen, Øester Farimagsgade 5, Bygning 22 & 23, Postboks 2099, 1014 Copenhagen K, Denmark. Phone: 45 35 32 78 65. Fax: 45 35 32 78 51. E-mail: d.e.arnot@cmp.dk.
† D.R.C. and E.J.R. contributed equally to this study.
▽ Published ahead of print on 11 June 2008.

Selection between competing candidates is a process common to drug and vaccine development programs. One constraint on the prioritization of antigens for blood-stage malaria vaccine development has been the lack of comparative data on antigenicity and immunogenicity and also of in vitro assays that have been proven to correlate with in vivo human immune responses (13, 20). Here we compare antibody titers and the in vitro functional immunoglobulin G (IgG) activity induced in rabbits by equimolar amounts of five merozoite surface vaccine candidate antigens and one combination of two of these antigens, all formulated in Montanide ISA720. The antigens included three MSP-1$_{19}$-based antigens, "near-wild-type" and modified versions produced in *Pichia*, and a baculovirus version with additional MSP-1 residues. *Pichia pastoris*-produced AMA-1 antigens containing domains I and II, either simply combined with, or genetically fused to, the modified *Pichia*-produced MSP-1$_{19}$ were also assayed. Standard protocols for enzyme-linked immunosorbent assays (ELISAs), immunofluorescence assays (IFAs), and parasite growth inhibition assays (GIAs) were carried out in two different laboratories, one operating with samples that had been blinded to the researchers. The significance of the results obtained and the usefulness of these assays for malaria vaccine candidate selection are discussed.

MATERIALS AND METHODS

Antigens. The antigens used in the immunizations were as follows.

First, the *P. falciparum* MSP-1$_{19}$ baculovirus antigen was produced in insect cells infected with a recombinant baculovirus containing a synthetic G+C-enriched (codon-optimized) *P. falciparum* MSP-1 gene fragment (Palo Alto allele) coding for the 43 N-terminal MSP-1 residues (the 19 residue MSP-1 signal sequence, which is removed by processing in baculovirus, plus 24 residues from the PfMSP-1 N-terminal block 1) and including the adjacent 16 amino acid residues upstream of the "classical" MSP-1$_{19}$ C-terminal sequence added to that sequence (3). The antigen was produced as an industrial scale (22L) batch by Serono SA (Geneva, Switzerland) under World Health Organization sponsorship 6 years previously and had been stored at 4°C in lyophilized form.

The second was a version of the MSP-1$_{19}$ protein fragment that has been genetically engineered to optimize codon usage from the original *P. falciparum* alle*e* (Wellcome clone) to that of the expression host and to remove potential N-glycosylation sites (by alanine replacement of the serine residue in the N-glycosylation motif) produced in *P. pastoris* (MSP-1$_{19}$ WT) (9; W. D. Morgan et al., unpublished data).

The third was a more structurally modified version of the wild-type MSP-1$_{19}$ recombinant antigen (MSP-1$_{19}$ mut) with Cys-2 and Cys-4 replaced (C12I/C28W), in the process removing the second disulfide bond in the wild-type protein (C. Uthaipibull et al., unpublished data). Codon optimization to the expression host usage was also carried out. Engineered modification of the structure of the recombinant *Pichia* expressed material was carried out to improve immunogenicity (9, 20).

For the fourth, domains I and II (residues 97 to 442) of AMA-1 (the allele present in the FVO clone) were expressed in *P. pastoris* (AMA-1 *Pichia*). The gene was engineered for production at the Biomedical Primate Research Centre (BPRC) (7) to conservatively replace potentially N-glycosylated sites by replacement of residues in the glycosylation motif, as previously described (13). The recombinant protein is reactive with the invasion-inhibitory, conformation-dependent monoclonal antibody 4G2 (13).

The fifth was a combined vaccine mixing separate preparations of the *P. falciparum* AMA-1 ectodomain construct described above with the *P. falciparum* MSP-1$_{19}$ C12I/C28W variant lacking one disulfide bond, both *P. pastoris* produced (AMA-1 +MSP-1$_{19}$ mix).

Finally, the sixth was a combined vaccine genetically fusing the *P. falciparum* AMA-1 ectodomain construct with the *P. falciparum* MSP-1$_{19}$ C12I/C28W variant described above, expressed in *P. pastoris* (AMA-1/MSP-1$_{19}$ fusion) (8).

Immunizations. The purified proteins were used to produce polyclonal antisera in New Zealand White rabbits. Antigen was emulsified in Braun Luer-type syringes without rubber pistons, through a 22-gauge needle, at a ratio of 3 parts antigen to 7 parts Montanide ISA720 adjuvant (vol/vol). This adjuvant is a squalene-based metabolizable oil containing a mannide mono-oleate emulsifier (Seppic S.A., France). Mixtures were used to immunize rabbits intramuscularly. Equimolar amounts of each antigen (20 μg for MSP-1$_{19}$, 80 μg for AMA-1, and 100 μg for the AMA-1/MSP-1$_{19}$ fusion) dissolved in 0.5 ml of phosphate-buffered saline (PBS) were used for each immunization. Doses were given at days 0, 28, and 56. Final bleeds were taken on day 70. Five rabbits were immunized with each of the six vaccine candidates. Two rabbits (one in the AMA-1 and one in the MSP-1$_{19}$ wild-type groups) died for reasons unrelated to the immunizations, resulting in 28 sets of pre- and postimmunization serum samples. The immunizations and serum collection were carried out by Eurogentec SA (Seraing, Belgium). The IFAs, ELISAs, and initial GIAs were performed "blind" at both testing centers after coding of the samples at the BPRC.

IFAs. Approximately 500 multispot slides were made from a single parasite culture batch of each of two different *P. falciparum* laboratory lines, the Wellcome and 3D7 clones. DNA from parasites used for IFA slides was preanalyzed by PCR and DNA sequencing to determine sequence, and then their AMA-1 and MSP-1$_{19}$ sequences were checked against the published sequence derived from these parasite clones. Both clones had an MSP-1$_{19}$ sequence identical to the published sequence for that clone (11). The AMA-1 (domain 1) sequence of 3D7 was identical to the published *P. falciparum* Genome Project-derived sequence. The Wellcome clone domain 1 AMA-1 sequence was, as expected, identical to the published FVO clone-derived sequence (13). 28 AMA-1 domain 1 amino acid residues differ between the proteins encoded by the 3D7 and Wellcome clones (8).

Mixed-stage schizont-rich cultures grown under standard conditions to 4 to 6% parasitemia were washed in PBS and resuspended to a 2% hematocrit. Then, 25 μl of this suspension was placed on each spot of 12-well multispot slides (Hendley-Essex, United Kingdom). The slides were dried overnight at room temperature and then stored with desiccant at −20°C. Immediately before use, the slides were thawed and fixed with acetone. Serial dilutions of the postimmunization sera plus control sera (1:50 to 51:51,200) were made in PBS containing 1% bovine serum albumin and 0.01% sodium azide. Next, 25-μl volumes of each dilution of antiserum were incubated on the multispots for 30 min at room temperature in a humidified chamber. The slides were washed three times (1, 5, and 5 min) in PBS and air dried. Each spot was then incubated as described above, with 15 μl of a 1:80 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Dako, United Kingdom) for 30 min at room temperature. After three further washes in PBS, the slides were immersed in a solution of 0.1% (wt/vol) Evans Blue and 0.001% (wt/vol) DAPI (4′,6′-diamidino-2-phenylindole; Sigma) in PBS for 5 min to counterstain erythrocytes and parasite nuclei, respectively. The slides were then washed twice in PBS (1 min each wash) and mounted under coverslips using the antifading agent Citifluor (City University, London). Parasites were visualized at 600× magnification by DNA-specific DAPI fluorescence with incident light at 450 to 495 nm. Antibody-reactive parasites were viewed by fluorescein isothiocyanate fluorescence with incident light of 390 to 440 nm. Reading of all slides was carried out "blind" by two experienced microscopists.

ELISAs. Antisera were tested by ELISA for their ability to recognize the recombinant antigens used as immunogens. Proteins were either redissolved if lyophilized in water or used directly if supplied as frozen aliquots. Protein concentrations were determined by BCA assay using bovine serum albumin (Pierce) as a standard. Each antigen was coated onto the wells of 96-well plates (Immulon-4 HBX; Thermo Dynex Inc.) at 0.5 μg ml^{-1} in 0.1 ml of coating buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$ [pH 9.3]) overnight at 4°C. This coating concentration was chosen after initial checkerboard titrations of antigens 1 to 4 with positive control sera. At this antigen concentration, it was possible to achieve maximum absorbance with positive control sera and minimum background reactivity with negative control sera, without loss of specificity. For reasons of sample availability, the antigens, as described above, were absorbed onto the test wells as follows: 1, MSP-1$_{19}$ baculovirus produced; 2, MSP-1$_{19}$ *E. coli* produced; 3, MSP-1$_{19}$ *P. pastoris* produced; 4, AMA-1 *P. pastoris* produced; and 5, AMA-1/MSP-1$_{19}$ *P. pastoris* produced fusion protein.

ELISA was performed with all rabbit antisera, against the above antigens, in duplicate on the same day with the same batch of ELISA plates. Each plate contained a positive control titration. The standard antiserum pool used for the standard curves on each plate was prepared by combining, in equal amounts, two other pools of high-titer rabbit antisera specific for AMA-1 and MSP-1$_{19}$, respectively. The standard pool was used at a maximum concentration of 1:8,000, titrated in doubling dilutions, in duplicate, to a final dilution of 1:512,000. Each test serum was titrated over eight titration points in duplicate from 1:1,000 to 1:128,000. Each antigen was coated onto separate plates, and the same pooled, positive standard dilutions were applied to control wells on each plate.

After antigen coating, the wells of each plate were washed three times in

washing buffer (0.05% Tween 20 in PBS). Unoccupied protein binding sites were blocked with 0.2 ml of blocking buffer (1% [wt/vol] skimmed milk powder in washing buffer) per well for 5 h at room temperature and washed again three times. Doubling dilutions (1:1,000 to 1:128,000) of rabbit sera in blocking buffer (0.1 ml per well) were added to duplicate antigen-coated wells, followed by incubation overnight at 4°C. After three washes, the wells were incubated for 3 h with 0.1 ml per well of horseradish peroxidase-conjugated polyclonal goat anti-rabbit IgG (at a 1:2,000 dilution) (Dako, Ltd., High Wycombe, United Kingdom) and then washed three times. Horseradish peroxidase substrate (0.4 mg of o-phenylenediamine Σ ml^{-1} and 0.012% H$_2$O$_2$) in development buffer (24.5 mM citric acid monohydrate and 52 mM Na$_2$HPO$_4$ [pH 5.0]) was added to each well (0.1 ml), followed by incubation for 15 min at room temperature. The reaction was stopped with 25 µl of 2 M H$_2$SO$_4$, and the absorbance was measured at 492 nm in a Multiskan Ascent plate reader (Thermo LabSystems, United Kingdom). The titer for each rabbit serum was determined by using the curve-fit algorithm within the Multiskan Ascent (version 2.4.1.) software for the ELISA reader. This algorithm plots the standard curve for the positive control serum pool for each plate, using four-parameter logistic regression, and then calculates the antibody titer as a factor of the positive control. Positive control serum at its lowest dilution (1:8,000) was given an arbitrary value of 1,000 U, from which titers were determined.

A slightly different ELISA protocol was used at the BPRC, where ELISA was performed in duplicate on serum samples in 96-well flat-bottom microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands). Wells were coated with 500 ng of purified AMA-1 or MSP-1$_{19}$ antigens ml^{-1} in PBS (pH 7.5) according to published methods (13). The sequence of reagents from the solid phase outwards was protein, antibody, alkaline phosphatase conjugate, and substrate. A standard curve with known amounts of antigen-specific IgG was used on each plate, and the concentrations of the unknowns were calculated by using a four-parameter fit.

IgG purification. IgG purification from all 28 experimental and control rabbit sera was carried out by affinity chromatography on HiTrap Protein G columns using an Åktaprime automated chromatography system (Amersham GE Healthcare). Serum samples diluted in PBS were passed through 0.2 µm filters (Minisart NML; Sartorius) before application to the column, thoroughly prewashed to remove unbound proteins before elution of the IgG with 0.1 M glycine-HCl (pH 2.7). Fractions eluting from the column were collected into glass tubes containing 1/10 volume of 1 M Tris (pH 9.0) to neutralize the sample and then concentrated and buffer exchanged into PBS using Vivaspin 6 (10-kDa cutoff; Sartorius) concentrators. The protein concentration of each sample was determined by using a Bradford assay (4) and adjusted with PBS to a concentration of 10 mg ml^{-1} for further use.

GIAs. Parasite invasion/GIAs were carried out with IgG purified from all sera, in both Edinburgh and at the BPRC. BPRC "blinded" the samples before dispatching them to Edinburgh on dry ice. Assays to test the ability of purified IgG from the immunized rabbits to inhibit invasion and growth of *P. falciparum* in cultures were carried out using Wellcome clone parasites in Edinburgh and using FCR3, 3D7, and HB3 clone parasites at the BPRC. Three methods were used to measure growth inhibition: (i) flow cytometric measurement of the parasitemia and the increase in nucleic acid content of the parasite-infected red blood cell, (ii) the parasite lactase dehydrogenase (pLDH) enzyme activity assay, and (iii) microscopic observation of the growth and morphology of the parasite in red blood cells.

Wellcome clone parasites were synchronized by three rounds of treatment with 5% sorbitol. After purification, each of the 28 rabbit IgG antibody samples (10 mg ml^{-1}) was serially diluted across six concentrations (neat, 1:1,1:2,1:4,1:8, and 1:16) using IgG from unimmunized normal rabbit serum at the appropriate concentrations to ensure that the same total concentration of IgG was present in each sample. Then, 50 µl of each IgG antibody dilution in PBS was dispensed in triplicate into the wells of a Costar microtiter plate (Corning, New York, NY). A portion (50 µl) of parasite culture at 0.3% parasitemia and 2% hematocrit, consisting mainly of mid-stage trophozoites, in twofold-concentrated normal culture medium, was placed in each well. At the initiation of the assay period therefore, parasites were present at 0.3% parasitemia in complete culture medium at 1% hematocrit. Test cultures had rabbit IgG present at a starting concentration of 5 mg ml^{-1}, serially double diluted to 0.15 mg ml^{-1}. Controls contained (i) 5 mg of normal rabbit IgG ml^{-1}, (ii) no rabbit IgG (PBS), (iii) culture in normal medium (10% human serum), or (iv) culture in normal medium with uninfected red blood cells. Plates were gassed with 1% oxygen, 3% carbon dioxide, and 96% nitrogen and then incubated at 37°C for 42 h.

After the 42-h incubation with the purified antibody, 50 µl of well-mixed parasites (Wellcome clone) from each assay well was dispensed into 0.25 ml of cold PBS in a fresh microtiter plate (Immulon-4 HBX). The plates were loaded into a Mistral 3000 centrifuge and centrifuged at 900 × g for 10 min at 4°C. A portion (0.24 ml) of supernatant was removed from each well, and the plates frozen at −80°C for 30 min to lyse the pelleted cells. After the plates were brought back to room temperature, 0.1 ml of complete pLDH substrate (100 mM Tris-HCl [pH 7.5], 40 mM sodium L-lactate, and 0.25% Triton X-100 containing 0.2 g of nitroblue tetrazolium ml^{-1}, 1 µl of 3-acetylpyridine adenine dinucleotide ml^{-1}, and 0.2 U of Diaphorase ml^{-1}) was added to each well. Plates were kept in the dark and read at 15, 30, and 60 min. The absorbance of wells in each plate was measured at 650 nm on a Multiskan Ascent plate reader (Thermo LabSystems), and the percent inhibition was calculated as $100 - (A_{650}$ of the test sample $- A_{650}$ of the RBC only)/(A_{650} of normal rabbit serum control $- A_{650}$ of RBC only) × 100 (12).

The BPRC used a slightly different protocol, where the effect of purified IgG antibodies on parasite invasion was evaluated in triplicate using 96-well flat-bottom plates (Greiner) with mature *P. falciparum* schizonts at a starting parasitemia of 0.2 to 0.4%, a hematocrit of 2%, and a final volume of 100 µl containing 10% normal human serum and 20 µg of gentamicin ml^{-1} in RPMI 1640. After 40 to 42 h, the cultures were mixed, and 50 µl was transferred to 200 µl of ice-cold PBS. The cultures were centrifuged, the supernatant was removed, and the plates were then frozen. The parasitemia was estimated by using a pLDH assay as described previously (12). Parasite growth inhibition, reported as a percentage, was calculated as $[100 - (A_{\text{experimental}} - A_{\text{background}})/(A_{\text{control}} - A_{\text{background}})] \times 100$, where A represent the absorbance value. Control IgG was isolated from rabbits that had been immunized with adjuvant alone.

Flow cytometry. Portions (15 µl) of well-mixed parasites (Wellcome isolate) from the assay wells were placed into 0.25 ml of 0.01 mM thiazole orange in FACSFlow buffer, followed by incubation for 15 min in the dark. The data from these samples were then acquired on a Becton Dickinson FACScan fluorescence-activated cell analyzer. The parasitemia in each sample was determined by using CellQuest software. Fluorescent staining of parasites was detected in both the FL2 and the FL3 channels, and the percent parasitemia was estimated for total parasites and mature trophozoite and ring-form parasites. Data from 50,000 cells were acquired for each sample.

Light microscopic assessment of stained parasites. In Edinburgh, after the triplicate samples had been removed for the flow cytometry and pLDH assays, the remaining sample material was pooled and concentrated by centrifugation. Supernatant was removed, and thin smears were made from the concentrated red blood cells. Slides were air dried, fixed with methanol, and then stained with 10% Giemsa reagent for 45 min before washing and viewing the samples using oil immersion light microscopy.

RESULTS

IFAs. The titers and staining patterns of the polyclonal antisera from the rabbits immunized with the recombinant proteins were compared by IFA. The results for each individual rabbit antiserum are shown in Table 1. In the majority of cases, the titers against the Wellcome clone and the 3D7 clone were the same. If antibody titer to the 3D7 clone was different to that observed against the Wellcome clone, that titer is given in parentheses alongside. The arithmetic mean titer to the Wellcome parasites has been calculated for each group of rabbits receiving the same antigen. All 28 postimmunization sera gave a reaction above the corresponding preimmunization sera, which were all negative at a 1:50 dilution (the highest concentration tested).

Two different patterns were observed in the sera that gave positive reactions. The first was that typical of a anti-MSP-1 antibody reaction in which the antisera reacted with all of the stages of the parasite within the infected erythrocyte. The second pattern was a punctate pattern observed on larger, late-stage mature schizonts only and typical of anti-AMA-1 reactivity. However, not all large schizonts in these samples showed the punctate fluorescence, possibly because the apparent size of schizonts is only an approximate measure of maturity. Since the frequency of positive reactions on an IFA slide can influence the microscopic assessment of the IFA titer, all

TABLE 1. Immunofluorescence titers of serum samples against
P. falciparum-infected red blood cells

Rabbit serum	Titer (3D7 titer)	Mean titer (Wellcome)	Pattern[a]	Immunogen
1	1:1,600 (1:400)	2,000	Punctate	AMA-1
8	1:1,600		Punctate	
15	1:3,200		Punctate	
20	1:1,600		Punctate	
2	1:1,600	960	All stages	MSP-1$_{19}$ mut
10	1:400 (1:800)		All stages	
13	1:1,600		All stages	
22	1:400		All stages	
28	1:800		All stages	
3	1:400 (1:800)	800	All stages	MSP-1$_{19}$ WT
11	1:400 (1:800)		All stages	
14	1:800		All stages	
21	1:1,600		All stages	
4	1:1,600	2,240	All stages	AMA-1 and
12	1:1,600		All stages	MSP-1$_{19}$ mut
16	1:3,200		All stages	
19	1:3,200 (1:400)		All stages	
26	1:1,600		All stages	
5	1:1,600	1,600	All stages	AMA-1-MSP-1$_{19}$
9	1:1,600		All stages	mut fusion
18	1:1,600		All stages	
24	1:1,600		All stages	
25	1:1,600		All stages	
6	1:1,600	3,520	All stages	MSP-1$_{19}$
7	1:3,200		All stages	baculovirus
17	1:3,200		All stages	
23	1:3,200		All stages	
27	1:6,400		All stages	
Preimmune control	1:50		No reactivity	
Positive control 1 (BPRC)	1:1,600		Punctate	
Positive control 2 (Pasteur)	1:1,600		All stages	

[a] The punctate pattern was typical of reactivity with AMA-1 in mature schizonts, whereas the reactivity with all parasite blood stages was typical of binding to MSP-1$_{19}$.

sera were retested on a second batch of slides from the Wellcome parasite clone, in which the overwhelming majority of parasites were mature schizonts. The IFA endpoint titer for each serum sample was recorded as the highest dilution at which clear, parasite-specific fluorescent reactivity was seen. Figure 1 shows a simple graphical representation of all of the endpoint titers of the IFA assays for each rabbit.

The results indicate that the baculovirus-produced MSP-1$_{19}$ immunizations produced fourfold-higher parasite-specific antibody titers than either of the two other single MSP-1$_{19}$ immunogens. This result was statistically significant in both cases (Kruskal-Wallis test, $P < 0.05$ in each case). The parasite-specific antibodies were of comparable levels in the AMA-1 and the two AMA-1/MSP-1$_{19}$ coimmunization groups, with a slight reduction in titer in the AMA-1/MSP-1$_{19}$ genetic hybrid (which gave 1:1,600 titers in each rabbit tested). None of the other between-group comparisons were statistically significantly different (Kruskal-Wallis test, $P > 0.05$ in all cases). Rabbits 1 and 19 immunized with the AMA-1 antigen (alone and combined, respectively) showed a substantially reduced IFA titer against the 3D7A clone (heterologous to the AMA-1 antigen used) compared to the Wellcome clone (homologous to the AMA-1 antigen). This suggests that the highly polymorphic AMA-1 antigen induces strain-specific antibodies, although much larger sample sizes would be required for statistical significance. In contrast, the three immunization groups

corresponding to the conserved MSP-1$_{19}$ alone showed the same or similar assay results with both lines. Overall, as measured by IFA, the baculovirus MSP-1$_{19}$ product induced the highest IFA titers, and the *Pichia*-derived MSP-1$_{19}$ products gave the lowest titers of parasite-specific antibodies.

ELISAs. The individual antibody titers, arithmetic mean antibody titers, and the standard deviations for each group of rabbits with each immunogen tested are given in Table 2. These data are shown graphically with the antibody titers for each immunogen in Fig. 2. In general, ELISA titers for each individual serum sample against the different MSP-1$_{19}$ antigens were not significantly different (analysis of variance, Tukey-Kraemer multiple-comparison test, $P > 0.6$ in all cases). However, rabbits immunized with MSP-1$_{19}$ generally had slightly higher titers to the homologous antigen than to MSP-1$_{19}$ recombinant protein derived from other expression systems. Baculovirus-produced MSP-1$_{19}$ showed this effect most strongly, with 17 to 34% higher titers to the homologous baculovirus-expressed MSP-1$_{19}$ than to either *Pichia*-expressed MSP-1$_{19}$ products (Table 2). Baculovirus MSP-1$_{19}$ induced between 5 and 12 times more antibodies than any other single MSP-1 immunogen and approximately three times more MSP-1 specific antibodies than the AMA-1–MSP-1 fusion. Titers to MSP-1$_{19}$ were significantly higher in the baculovirus-immunized group than in all other groups (Mann-Whitney U test, $P < 0.01$ for all except MSP-1$_{19}$ WT, $P < 0.05$). The ELISA results shown here are those generated in Edinburgh; results generated at the BPRC using the same serum samples, but with different positive control sera, were in very close accordance (R^2 for MSP-1$_{19}$-specific ELISA, 0.987; R^2 for AMA-1-specific ELISA, 0.932 [Spearman rank correlation coefficient]).

Interestingly, some competitive effects were noted between antigens when combined in a single immunization. Antibody titers induced by immunization with the modified, *Pichia*-produced MSP-1$_{19}$ alone were approximately twice that seen when the same antigen was mixed with AMA-1, although this effect was not statistically significant (Mann-Whitney U test, $P = 0.1508$). In contrast, no effect was seen on anti-AMA-1 titers after mixing
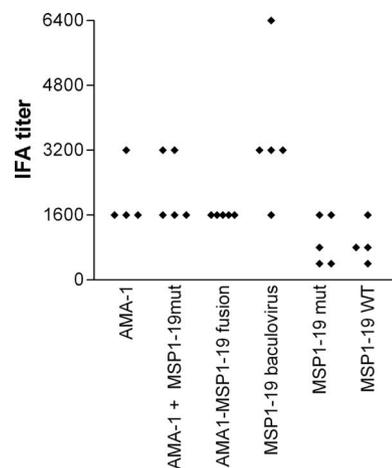

FIG. 1. Endpoint titers of groups of rabbit antisera as measured by IFA. Individual IFA titers against *P. falciparum*-infected erythrocytes for groups of sera from rabbits immunized with different recombinant antigens are presented.

TABLE 2. ELISA titers of serum samples against recombinant proteins produced in three different heterologous expression systems

Immunogen	Rabbit	ELISA titer against specified antigen[a]									
		$MSP\text{-}1_{19}$ baculovirus		$MSP\text{-}1_{19}$ E. coli		$MSP\text{-}1_{19}$ Pichia		AMA-1 Pichia		AMA-1/$MSP\text{-}1_{19}$ fusion (Pichia)	
		Individual	Mean (SD)	Individual	Mean (SD)	Individual	Mean (SD)	Individual	Mean (SD)	Individual	Mean (SD)
AMA-1 (Pichia)	1	0	0 (0)	0	0 (0)	0	21,731* (37,639*)	6,163,161	5,211,353 (2,214,190)	11,174,154	7,528,464 (3,264,936)
	8	0		0		86,924*		8,036,203		9,562,225	
	15	0		0		0		4,663,407		6,819,924	
	20	0		0		0		1,982,639		2,557,553	
AMA-1 and $MSP\text{-}1_{19}$ mut M(Pichia)	4	1,881,886	1,747,361 (1,249,585)	1,727,902	1,905,354 (1,504,295)	1,880,343	1,896,859 (1,283,201)	6,495,146	4,906,408 (1,882,904)	14,954,570	9,674,828 (5,196,097)
	12	3,942,091		4,704,904		4,056,689		7,529,256		16,578,072	
	16	1,830,801		1,891,172		2,328,646		4,514,991		8,006,075	
	19	706,647		750,935		790,794		3,657,063		5,256,569	
	26	375,380		451,857		427,822		2,335,582		3,578,853	
AMA-1/$MSP\text{-}1_{19}$ fusion (Pichia)	5	7,416,359	5,515,669 (1,761,481)	7,634,790	5,488,458 (1,879,153)	7,333,850	6,002,001 (1,968,330)	7,490,832	4,681,638 (2,285,393)	20,378,261	12,173,497 (5,034,747)
	9	5,868,007		5,021,620		6,752,750		3,795,046		10,721,569	
	18	6,440,689		6,011,829		6,709,397		2,596,616		9,097,366	
	24	2,213,221		2,136,340		2,092,871		2,205,802		5,802,182	
	25	5,640,067		6,637,713		7,121,134		7,319,895		14,868,104	
$MSP\text{-}1_{19}$ baculovirus	6	14,926,689	22,000,134 (14,134,561)	12,521,503	16,305,333 (9,595,486)	12,213,576	18,709,234 (12,338,749)	0	0 (0)	3,854,369	4,911,977 (2,654,392)
	7	38,288,771		24,041,240		29,029,435		0		6,147,857	
	17	9,306,937		6,874,438		8,427,230		0		1,699,378	
	23	7,670,374		7,165,041		6,350,238		0		3,457,721	
	27	39,807,896		30,924,444		37,525,692		0		9,400,561	
$MSP\text{-}1_{19}$ mut (Pichia)	2	4,475,443	3,129,648 (1,243,195)	4,663,469	3,649,773 (1,612,441)	5,411,953	4,183,259 (1,725,772)	354,278*	78,493* (138,235*)	18,331,361	5,811,042 (6,304,573)
	10	2,204,977		1,984,804		2,693,325		26,439*		2,129,327	
	13	3,113,266		4,266,222		4,388,542		0		3,620,664	
	22	1,349,413		1,567,243		1,853,468		11,747*		1,612,972	
	28	4,505,141		5,767,128		6,569,007				3,360,886	
$MSP\text{-}1_{19}$ WT (Pichia)	3	1,637,003	2,041,431 (1,434,600)	1,782,117	1,774,597 (739,213)	1,592,636	1,744,707 (826,550)	0	0 (0)	706,355	733,919 (243,088)
	11	922,446		1,120,200		1,146,232		0		487,766	
	14	1,121,437		1,218,199		1,102,684		0		608,523	
	21	4,484,837		2,977,872		3,137,275		0		1,133,032	

[a] *, possible reactivity with Pichia contamination (possible reactivity with baculovirus contamination could not be tested).
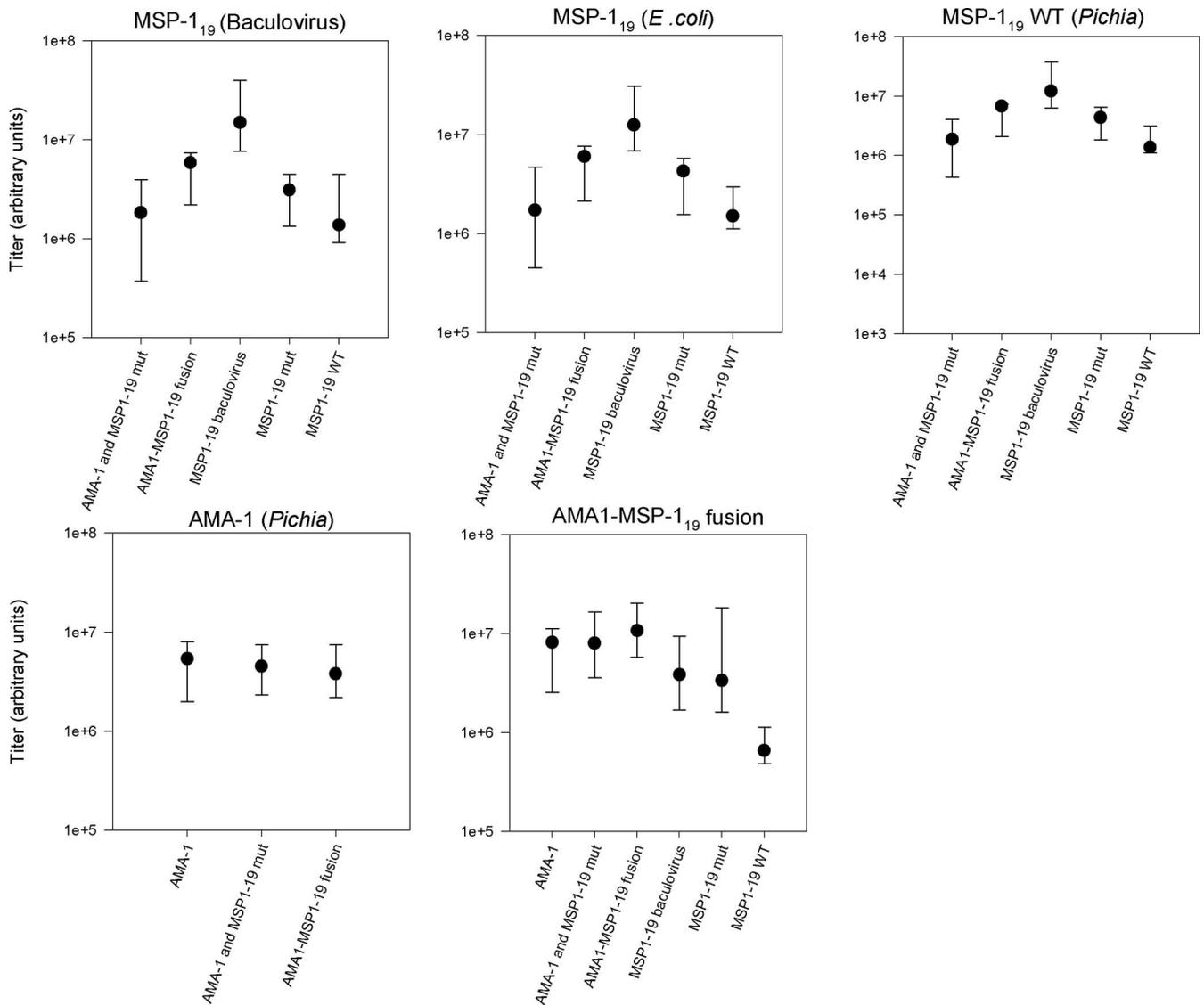
FIG. 2. ELISA titers of sera from groups of rabbits immunized with different immunogens, as measured against different coating antigens. Antibody titers for each group of rabbits are shown in separate panels, for each antigen on the ELISA plate. Sera from each immunization group are arranged along the x axis. Circular symbols represent the median antibody titer in each group of rabbits, with bars representing the highest and lowest titers in each group, expressed in ELISA units (see Materials and Methods).

with the mutated MSP-1$_{19}$ recombinant protein. Conversely, a significant increase in titer to MSP-1$_{19}$ was noted when the two immunogens AMA-1 and MSP-1$_{19}$ were fused genetically (Kruskal-Wallis test, $P < 0.01$ for all MSP-1$_{19}$ antigens). ELISA titers to the MSP-1$_{19}$ portion of the antigen were increased by 1.4- to 1.7-fold compared to titers of antibodies induced in the rabbits immunized with the modified MSP-1$_{19}$ alone and were ~3-fold higher than the titers of rabbits immunized with the mixture of AMA-1 and MSP-1$_{19}$. There was no adverse effect of the genetic fusion on anti-AMA-1 titers compared to AMA-1 immunization alone or to the mixture of AMA-1 and MSP-1$_{19}$. Rabbits immunized with AMA-1 showed no significant difference in levels of reactivity with both AMA-1 and the AMA-1-MSP-1$_{19}$ fusion protein (Kruskal-Wallis test, $P = 0.9463$).

Some rabbits (rabbits 2, 13, and 28, given the modified MSP-1$_{19}$ and rabbit 15 given AMA-1) showed detectable levels of, respec-

tively, AMA-1 or MSP-1$_{19}$ cross-reactive antibodies (10^4 to 10^5). It is possible that this is due to low levels of *P. pastoris* contaminants in the immunogen or test antigen preparations. It might also be due to rabbit serum antibodies cross-reacting with O-linked glycosylate additions to the protein made by the yeast, a phenomenon that has been noted with other recombinant proteins produced with *P. pastoris* (D. R. Cavanagh et al., unpublished data). No reagents were available to test for baculovirus cross-contamination and cross-reactivity effects, although antibodies induced with this antigen also showed the highest ELISA titers against *Pichia* and *E. coli* MSP-1$_{19}$ homologues. It seems unlikely that these antigens would have baculovirus or insect cell contaminants.

Invasion and GIAs. (i) Flow cytometric and pLDH measurement of growth inhibition. GIAs were carried out by adding purified IgG to synchronized Wellcome (Edinburgh) or FCR3
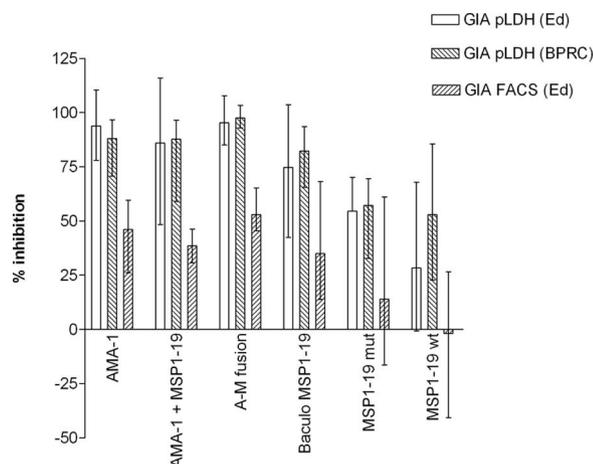
FIG. 3. Median percent parasite growth inhibition in the presence of purified IgG from rabbits immunized with malaria vaccine candidates. Each bar represents the median level of parasite growth inhibition for the IgG from each group of rabbits. For the Edinburgh data, the 5-mg ml^{-1} GIA results using the Wellcome parasite clone are plotted compared to the BPRC data (at 6 mg ml^{-1}) using the FCR3 parasite clone. The number of parasites in each sample was measured by using the pLDH levels and by flow cytometry after thiazole orange staining of parasites (for the Wellcome clone only). Error bars indicate the minimum and maximum inhibition levels within each group.

(BPRC) *P. falciparum* cultures. Control samples underwent an ~10-fold increase (from 0.5 to 5% parasitemia) in the assays shown. The percent growth inhibition was calculated as the experimental growth relative to growth in the positive control grown in the presence of unimmunized juvenile rabbit IgG. In vitro culture growth, as measured by the flow cytometric detection of thiazole orange-stained parasites, and the pLDH enzymatic assay is shown as the median parasite growth inhibition for the various immunization groups (Fig. 3). The data obtained at both testing sites are compared in this figure, and the results indicate that despite the minor differences the assay

conditions (described above), the pLDH assay gave similar results in both laboratories. IgG induced by all experimental vaccines showed a degree of inhibition at the antibody concentrations used (5 to 6 mg ml^{-1}). The median inhibition observed was >85% for the three AMA-1-containing candidates, somewhat less for baculovirus MSP-1$_{19}$, and lowest for the two *Pichia* MSP-1$_{19}$ antigens. It should be noted that the parasite lines used for this comparison (Wellcome and FCR3) both encode AMA-1 proteins homologous to the sequence of the immunization construct used. Because of sequence polymorphism, the results obtained with this antigen can be highly strain dependent. Due to the limited amounts of purified IgG available, the pLDH results of Fig. 3 were the only GIA data that were directly comparable between the Edinburgh and the BPRC testing centers. A more complete pLDH based data set was generated by the BPRC alone (see below).

Parasite growth inhibition measured by flow cytometry was carried out only in Edinburgh and was always less than that measured by the pLDH assay. This is due to the fact that flow cytometry measures changes in the nucleic acid content of parasite-infected erythrocytes. Parasite nuclei that have ceased metabolic activity will be scored by this technique. This will affect the extent of the increase in nucleic acid staining detected between growth-impaired cultures and growth-unimpaired cultures undergoing the rapid increase in nucleic acid content that occurs during intra-erythrocytic parasite development. The pLDH assay measures a parasite-specific enzyme activity generated by active parasite metabolism with no contribution to the measured signal from metabolically inert parasites.

(ii) pLDH measurement of growth inhibition at increasing IgG concentrations with three different *P. falciparum* clones. Figure 4 shows the results of pLDH assayed growth inhibition at a range of concentrations of IgG (0.75 to 6 mg ml^{-1}) from rabbits in each immunization group (shown as the average of the four or five animals in each group). These titrated assays were carried out at BPRC using cultures of the FCR3, HB3,
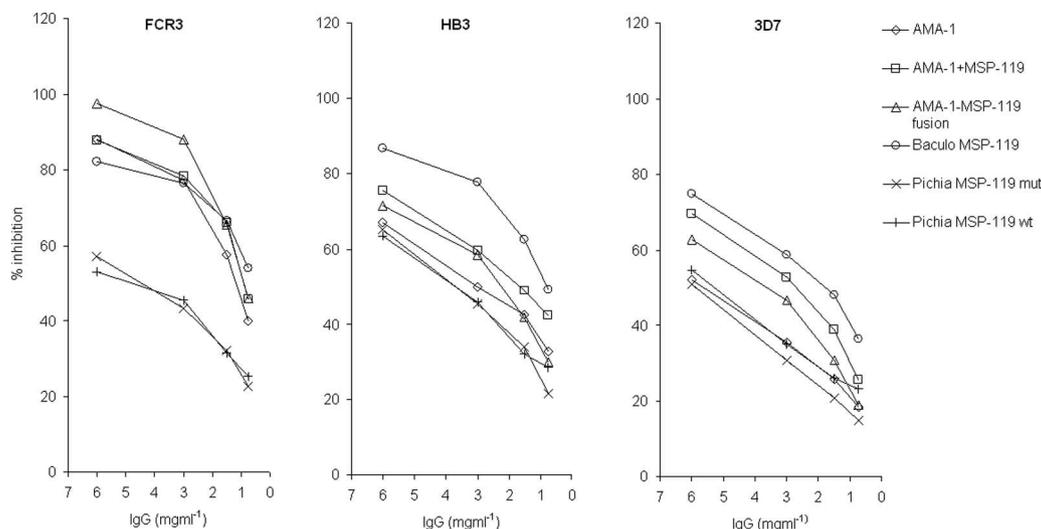

FIG. 4. Parasite GIA. The percent growth inhibition in cultures was measured by pLDH using three different clonal isolates of *P. falciparum* (FCR3, HB3, and 3D7). Each panel represents the growth inhibition data obtained from each isolate. The average parasite growth inhibition for each group of rabbits is plotted on the *y* axis against the IgG concentration (in mg ml^{-1}) on the *x* axis.
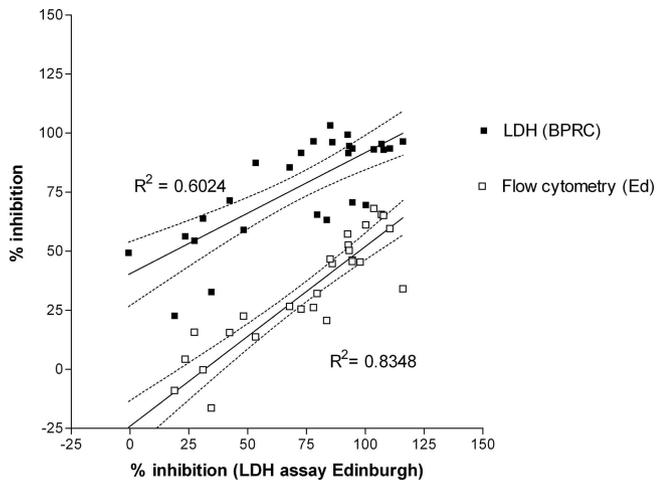
FIG. 5. Correlation between different methods for assaying in vitro parasite culture growth inhibition. The percent inhibition of all 28 rabbit IgG samples in the study measured as parasitemia by flow cytometry (in Edinburgh, open symbols) and pLDH assay (at BPRC, solid symbols) is plotted on the y axis against the inhibition measured using the same pLDH assay (x axis, Edinburgh pLDH assay). Solid lines indicate trend lines for each data set; dotted lines represent 95% confidence intervals for each trend line.
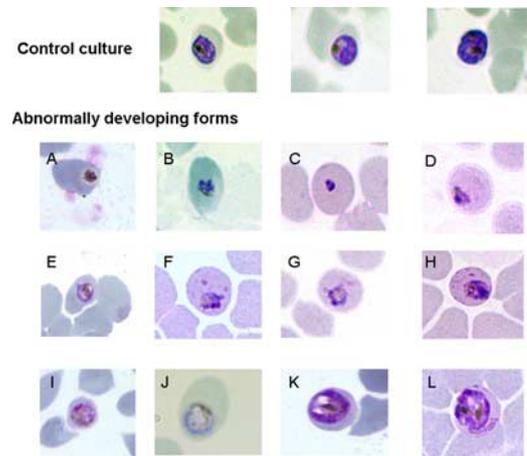

FIG. 6. Light microscopic comparison of normal *P. falciparum* trophozoites and schizonts from untreated cultures and a range of abnormally developing forms observed in cultures treated with anti-MSP-1_{19} antibodies. Plates A to D, misshapen and crumpled early ring forms; plates E and F, condensed and pycnotic early trophozoites; plates I to L, mature trophozoites that appear deformed, swollen, and less densely stained due to the appearance of vacuoles.

and 3D7 cloned isolates of *P. falciparum*. Growth inhibition as a percentage function of parasitemia compared to the control is shown plotted against the IgG concentration. A marked titration of the antibodies induced by immunization is observed with each immunogen.

Growth inhibition against the FCR3 isolate (homologous for the AMA-1 immunogen tested here) was strongest in animals immunized with the AMA-1/MSP-1_{19} fusion antigen, followed by the two other AMA-1-containing immunization groups, at the highest IgG concentration, although at lower concentrations anti-baculovirus MSP-1_{19} IgG inhibited as well as or better than the others. Anti-baculovirus MSP-1_{19} IgG showed the best growth inhibition at all IgG concentrations with the HB3 and 3D7 isolates (both nonhomologous to the AMA-1 immunogen used here), followed by the unfused and fused AMA-1–MSP-1_{19} combinations. Overall, the least growth inhibition was generally observed with antibodies to the two *Pichia* MSP-1_{19} recombinant products, a result also found in the experiments shown in Fig. 3.

(iii) Correlation between flow cytometric and pLDH-based measurements of parasite culture growth inhibition. To assess comparability of the two methods used for measuring in vitro culture growth inhibition in the presence of antibodies, the percent growth inhibition as measured by flow cytometry in Edinburgh and the pLDH assay at the BPRC was plotted against the growth inhibition measured (in the same culture) using the pLDH assay in Edinburgh (Fig. 5). Comparison of the medians of the GIAs (taken as a whole since individual groups are too small) shows that the pLDH GIA assays in Edinburgh and the BPRC did not give significantly different results (Friedman test of nonparametric repeated measures, $P >$ 0.05 [analysis of variance]). As noted above, the flow cytometric measurements give consistently lower growth inhibition percentages (Fig. 5) and therefore give significantly different results from either pLDH assay (Friedman test, $P < 0.001$).

This result is in agreement with the observations of Bergmann-Leitner et al. (2), who demonstrated differences in growth inhibition levels when using different nuclear staining dyes and pLDH assays, probably due to the different sensitivities with which these methods detect parasitemia and metabolic activity in cultured parasites.

(iv) Microscopic analysis of parasite growth in the antibody inhibition assays. In GIAs carried out in Edinburgh, Giemsa-stained blood smears were also prepared for microscopy. Microscopically determined estimates of culture parasitemia correlated well with the measurements recorded by automated flow cytometric analysis. Microscopy, however, permitted an analysis of parasite morphology in samples treated with antibody relative to the controls. Correlating morphological observation of intra-erythrocytic growth stage with precise estimation of the status of parasite nuclear division and the stage of the cell cycle in the parasite is difficult, and the cell cycle of *P. falciparum* is not completely understood (1). Without good biochemical markers for cell cycle stages, estimates of the "health" of *P. falciparum* cultures are necessarily subjective, although experienced parasite cultivators can recognize declining cultures with unsatisfactory growth rates. However, numerous parasites with abnormal morphology were observed in growth-impaired cultures during the GIAs in the presence of antibody. Examples of these abnormal intra-erythrocytic parasites, sometimes referred to as "crisis forms" (5), are shown in Fig. 6.

The antibodies produced by different immunizations showed certain consistent, but difficult to quantify, differences in their effects on the in vitro-cultured parasites. In cultures grown in the presence of anti-AMA-1 IgG, invasion was inhibited to various degrees, but development of the postinvasion intra-erythrocytic forms appeared normal. In contrast, in parasites grown in the presence of anti-MSP-1_{19} IgG, intra-erythrocytic development frequently appeared abnormal, containing misshapen rings and young trophozoites that appeared condensed

and pycnotic, while more mature trophozoites often appeared deformed, swollen, and less densely stained due to the appearance of vacuoles. The micrographs shown in Fig. 6 show several examples of the abnormal parasite morphologies seen in cultures grown in the presence of anti-MSP-1$_{19}$ IgG. Similar observations of the differential effects of antibodies on parasite growth have been obtained in another recently published study, also comparing functional assays of parasite growth inhibition (2).

DISCUSSION

Standardized immunogenicity measurements and in vitro efficacy assays have not featured prominently in malaria vaccine research, and a systematic comparison is presented here for the first time. Established laboratory procedures, logistical and funding difficulties, intellectual property concerns, and the competitive nature of the research process all inhibit objective comparative analyses of different vaccine candidates. However, there have been increasing calls to introduce industry-style "critical path decision-making" into malaria vaccine development, particularly since most competing vaccine development projects currently use sequences derived from the same small group of antigens. These antigens are being combined with a wide variety of delivery technologies and platforms, but few formulations have been compared for immunogenicity and antigenicity, let alone in any in vitro assay that arguably might have some correlation with the in vivo efficacy of a vaccine.

We compared the immunogenicity of six recombinant proteins that are being considered as immunogens in possible malaria vaccines. As measured by the concentrations of actively parasite binding antibody detected by IFA, rabbits immunized with the baculovirus produced MSP-1$_{19}$ had the highest parasite-specific IFA titer of the group, followed by: AMA-1+MSP-1$_{19}$ > AMA-1 > AMA-1-MSP-1$_{19}$ fusion > MSP-1$_{19}$ WT and MSP-1$_{19}$ mut (equal). Immunogenicity was also measured by ELISA, although the original immunogens used were not available as test antigens in all cases. Instead, three different versions of MSP-1$_{19}$ were tested, plus two antigens containing AMA-1. Again, the baculovirus-produced MSP-1$_{19}$ product was the most immunogenic antigen tested.

It was also possible to demonstrate a good correlation between flow cytometric analysis, microscopy, and the pLDH measurements of antibody-mediated growth inhibition. Some parasite growth inhibition was seen in the presence of antibodies raised by all of these immunogens. The inhibitory capacity of the antibodies was overall the highest in rabbits immunized with the baculovirus MSP-1$_{19}$ protein, using only 20-μg doses of a 6-year-old lyophilized preparation, attesting to its potency and stability. Importantly, the inhibition was strain independent. In contrast, the inhibitory capacity of antibodies induced by the two *Pichia* MSP-1$_{19}$ antigens was overall the lowest. Growth inhibition by antibodies to AMA-1 containing candidates was intermediate, except at high IgG concentrations using a homologous parasite test strain, where they gave the best inhibition. AMA-1 combination or fusion with MSP-1$_{19}$ generally elicited better inhibitory antibodies than did AMA-1 alone, although all tended to be strain dependent.

Taking all of the data together, the differences in antibody binding and functional activity that we have attempted to as-

sess result from either or both of two causes: (i) the particular protein being expressed induces more biologically relevant inhibitory or otherwise protective antibodies and/or (ii) the particular expression and purification systems used produced conformations of the antigen that were more immunogenic and/or more like the native parasite antigen and thus better able to induce inhibitory or otherwise protective antibodies.

Our data cannot differentiate between these possibilities. We have noted that different recombinant constructs based on the C terminus of MSP-1 are not in fact completely identical protein sequences. For example, the baculovirus MSP-1$_{19}$ product tested here has sequences from the N-terminal block 1 region of MSP-1, fused to a slightly larger portion of the C-terminal MSP-1 sequence than appears in the *Pichia*-derived MSP-1$_{19}$ products (3). The block 1 region of MSP-1 is known to contain at least one human T-cell epitope which might enhance the immunogenicity of this product in both animals and humans (17, 20).

Genetic fusion to create a recombinant hybrid protein of AMA-1 and MSP-1$_{19}$ enhanced the immunogenicity of MSP-1$_{19}$. However, simply mixing these individual components reduced the MSP-1$_{19}$-specific titers in ELISA and IFA. Neither fusion nor mixing appeared to affect the anti-AMA-1 titers. Thus, the mixture of the two individual vaccine candidate components may have resulted in some kind of immunological interference that led to some suppression of the anti-MSP-1$_{19}$ IgG response in these animals. This may be due to antigenic competition, a phenomenon known to immunology for over 100 years, and noted in recent immunizations with multiple recombinant malaria antigen plasmids (22). In some cases, a dominant antigen can prevent or reduce the antibody response to another antigen, if the two are administered simultaneously and in the same formulation. The degree of suppression of the immune response is determined by the relative quantities of the antigens in competition with each other (15). Encouragingly, as stated earlier, the fusion of the two antigens in one genetic construct ablated this effect and restored the anti-MSP-1$_{19}$ titers to the levels seen with single immunizations, or higher (Fig. 2).

GIAs have attracted the attention of the malaria vaccine field because they have been proposed to offer some correlation with the in vivo efficacy of a vaccine and could potentially be used as a prioritization tool. The correlation remains unproven, although it could be tested if significant amounts of human sera from an at least partially successful clinical trial were available. The assays are very sensitive to the in vitro culture growth conditions of the parasite, and suboptimal initial parasitemia and relatively low parasite replication rates in culture will translate into relatively small differences in the growth inhibition and uninformative assay results. The lack of "robustness" of the *P. falciparum* erythrocyte invasion-based in vitro culture system itself remains a concern in the interpretation of GIA results. However, the GIA data obtained at both testing sites were in very good agreement, following training of operators in the NIH assay, at that facility.

This indicates that results that are repeatable in different laboratories can be obtained, although this will be dependent on strict adherence to common protocols and the use of preferably identical, but at the very least agreed and equivalent reagents, antibodies, and parasite clones. Simpler assays of the immunogenicity of candidate antigens and more robust mea-

sures of the functional activity of antibodies elicited by immunization will continue to be developed. However, given the complexity of the malaria parasite's biology and the host's immune responses, interpretation of the biological relevance of in vitro assay of blood-stage malaria vaccine candidates requires a better understanding of the molecular mode of action of the various specificities of anti-parasite antibodies induced by both natural malaria infections and experimental malaria vaccines.

There is a possibility that different antibodies, by binding with different avidity and affinity to different parasite antigens, might disrupt parasite development in different ways, a conclusion that has been reached independently in another recent study (2). Anti-AMA-1 antibodies appeared to affect invasion only, whereas there were indications that anti-MSP-1 antibodies continued to affect postinvasion erythrocytic development and led to a degree of inhibition of normal parasite development. Clearly, these data are currently insufficient to draw any strong conclusions about this phenomenon, but this observed intra-erythrocytic inhibition of parasite growth is potentially important and deserves further investigation. A two-cycle GIA could increase the sensitivity of detection of growth-inhibitory antibodies (18). Previous reports on evasion of antibody-mediated growth inhibition by allelic variants of AMA-1 indicate that fundamental features of the assay, such as the choice of *P. falciparum* culture isolate, can affect comparative assay outcomes (2). Strain dependence is clearly an important parameter in these types of functional assays and requires further investigation because many *P. falciparum* antigens are very polymorphic in nature and a successful malaria vaccine must induce strain transcendent immunity.

These results clearly indicate that comparative preclinical immunogenicity testing of different *P. falciparum* blood-stage vaccine candidates can give discriminating results with regard to several different parameters. Indeed, the baculovirus MSP-1$_{19}$ candidate proved to be superior by most of the criteria used. Although the relevance of these criteria may be open to question for a variety of reasons, there do not appear to be many obvious alternatives at the present time for potential surrogate markers of protective immunity in humans. In reality, no candidate has yet been prioritized for clinical trials based on superior comparative immunogenicity and/or growth inhibitory activity, although the charitable foundations and funding bodies supporting malaria vaccine research are considering moving in this direction (16). Comparative immunogenicity testing will thus probably have a growing role in the vaccine optimization process and in dissecting immune responses following clinical trials. However, two major factors still confound in vitro assay-based prioritization. The first is the preparation and purity of the immunogens. Impure antigens can be significantly more immunogenic than highly purified, expensively produced GMP-grade products. The second is the more fundamental problem of the continuing lack of a proven correlation between any of our in vitro assays and in vivo protection from malaria.

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