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Immunogenicity and *in vitro* protective efficacy of a recombinant multistage *Plasmodium falciparum* candidate vaccine

(*Plasmodium*/vaccine/synthetic gene/B & T cell epitopes/efficacy)

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ABSTRACT **Compared with a single-stage antigen-based vaccine, a multistage and multivalent *Plasmodium falciparum* vaccine would be more efficacious by inducing "multiple layers" of immunity. We have constructed a synthetic gene that encodes for 12 B cell, 6 T cell proliferative, and 3 cytotoxic T lymphocyte epitopes derived from 9 stage-specific *P. falciparum* antigens corresponding to the sporozoite, liver, erythrocytic asexual, and sexual stages. The gene was expressed in the baculovirus system, and a 41-kDa antigen, termed CDC/NIIMALVAC-1, was purified. Immunization in rabbits with the purified protein in the presence of different adjuvants generated antibody responses that recognized vaccine antigen, linear peptides contained in the vaccine, and all stages of *P. falciparum*. *In vitro* assays of protection revealed that the vaccine-elicited antibodies strongly inhibited sporozoite invasion of hepatoma cells and growth of blood-stage parasites in the presence of monocytes. These observations demonstrate that a multicomponent, multistage malaria vaccine can induce immune responses that inhibit parasite development at multiple stages. The rationale and approach used in the development of a multicomponent *P. falciparum* vaccine will be useful in the development of a multispecies human malaria vaccine and vaccines against other infectious diseases.**

It is estimated that human malaria parasites cause 300–500 million illnesses and 1.5–3 million deaths throughout the world each year, with subSaharan Africa accounting for >90% of them. Most of the severe morbidity and mortality occurs in children and pregnant women and is caused by *Plasmodium falciparum* (1). Widespread and increasing resistance of the parasite to antimalarial drugs, development of resistance by *Anopheles* mosquito vectors to commonly used insecticides, population growth, and movement of nonimmune populations to malarious areas have worsened the malaria problem. It is envisioned that an efficacious vaccine together with other conventional control measures will provide a sustainable tool for control and prevention of malaria (2).

The complex life cycle of the malaria parasite provides a number of potential targets for vaccination. Over the last two decades, several *P. falciparum* vaccine candidate antigens have been identified. Three main types of malaria vaccines, based on different stages of the parasite life cycle, are currently under development. These are (*i*) pre-erythrocytic, including liver stage; (*ii*) asexual blood stage; and (*iii*) transmission-blocking vaccines. Although studies of immunogenicity and the results of *in vitro* protection experiments have been promising for many of the single stage-specific vaccine candidate antigens, the test of *in vivo* protection has not always been satisfactory. There is consensus, however, that a highly effective malaria vaccine would require a combination of key antigens and/or epitopes from different stages of the life cycle and that induction of both humoral and cellular immunity is required for optimal efficacy (3). Such a multicomponent malaria vaccine would also circumvent the problems associated with host genetic restriction and antigenic variability in the case of single antigen-based vaccines.

The first multicomponent *P. falciparum* vaccine, Spf66, was developed by Patarroyo *et al.* (4). This chemically synthesized vaccine contained a portion of each of the 35-kDa, 55-kDa, and 83-kDa blood-stage proteins linked by repeat sequence from the circumsporozoite protein of *P. falciparum*. Although Spf66 showed promising results in early trials (4), large-scale human trials revealed that it provides only limited protection (5). A vaccinia virus-based multistage *P. falciparum* vaccine (NYVAC Pf-7) that contains seven stage-specific antigens has been recently developed (6). In a phase I/IIa trial of this vaccine, cellular immune responses were detected in >90% of volunteers, while antibody responses were generally poor. Of the 35 volunteers challenged, only one was completely protected, although there was a significant delay until the onset of parasitemia (7). Another recent study has investigated the protective effects of a multivalent vaccine formulation against the exoerythrocytic stage of the parasite, in which 15 plasmodial cytotoxic T cell lymphocyte (CTL) epitopes and a B cell epitope were included. In a rodent model, immunization with this construct was shown to induce protective CTL responses (8).

Our strategy in the development of a multicomponent *P. falciparum* vaccine was to first delineate the characteristics of naturally acquired immunity and to then combine the promising protective epitopes identified through immunoepidemiologic studies and other *in vitro* and *in vivo* protection studies in model systems. We synthesized a gene that contains 12 B cell and 9 T cell epitopes derived from 9 stage-specific antigens as a candidate vaccine antigen termed CDC/NIIMALVAC-1 and have expressed the gene in the baculovirus expression system (unpublished data).

In this report, we describe (*i*) the antibody responses in rabbits immunized with a baculovirus-expressed multicomponent vaccine in various adjuvants; (*ii*) reactivities of the vaccine-elicited

Abbreviations: CDC/NII MAL VAC-1, baculovirus-expressed recombinant vaccine protein containing 21 immune epitopes derived from sporozoite-, liver-, sexual-, and asexual blood-stage antigens of *P. falciparum*; ADCI, antibody-dependent cellular inhibition; IFA, indirect immunofluorescence assay.

A Commentary on this article begins on page 1167.

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antibodies with various stages of *P. falciparum*; and (*iii*) *in vitro* antiparasite activity of the vaccine-elicited antibodies.

MATERIALS AND METHODS

Table 1 presents amino acid sequences of the 12 B cell and 9 T cell epitopes derived from 9 stage-specific vaccine candidate antigens of *P. falciparum* that were used in the development of CDC/NII MAL VAC-1 (refs. 9–21; unpublished data). One universal T cell epitope from tetanus toxoid (22) also was incorporated. Twelve overlapping single-stranded oligonucleotides spanning the entire CDC/NII MALVAC-1 gene were synthesized, and the vaccine antigen gene was assembled by using a strategy described elsewhere (unpublished data). The synthetic gene was cloned, and the recombinant protein was expressed in Sf9 insect cells. The baculovirus-expressed recombinant protein was purified from Sf9 cells at 72 hr postinfection by using Talon metal-affinity resin (CLONTECH) and its purity and specificity determined by using SDS/PAGE and Western blot analysis.

Four-month-old female New Zealand white rabbits (The Jackson Laboratory) were immunized intramuscularly with 100 μg per dose of purified CDC/NII MALVAC-1 protein in Freund's complete adjuvant (rabbit 787), in a potentially human-usable adjuvant, in nonionic block copolymer P1005 in water/oil emulsion (rabbit 789), and in the human-usable adjuvant aluminum hydroxide (rabbit 1015). A total of four immunizations were given at 3-week intervals (i.e., at weeks 0, 3, 6, and 9), and sera were collected after each immunization until weeks 33–51. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996.

Serum antibody titers against the vaccine antigen and individual peptide epitopes were determined by ELISA. Responses against sporozoites, asexual blood-stage parasites, and gametocytes were determined by using an indirect immunofluorescence assay (IFA). Sera collected from each rabbit during weeks 10–16 were pooled, and total IgGs were purified by using ammonium sulfate (Sigma) precipitation followed by DEAE (Pierce) batch purification according to methods described elsewhere (23). Following dialysis against PBS, the purified antibodies were used for immunoelectron microscopy, antibody-affinity testing, and an *in vitro* protection assay.

We employed a Biacore surface-plasmon resonance detector (Biacore, Piscataway, NJ) to evaluate the binding properties of antibodies to the vaccine antigen. Purified vaccine antigen was covalently immobilized in a "C1" (short-chain carboxymethyldextran) sensor cell by standard carbodiimide/*N*-hydroxysuccinimide methods. Mobile-phase analyte consisted of purified IgG preparations diluted to 50 μg of protein per ml in 10 mM Hepes buffer (pH 7.4) plus 150 mM NaCl. After establishing a stable baseline signal with buffer, the association reaction was initiated by switching to the analyte stream, and the resonance signal was followed in time (24). A flow rate of 5 μl/min was used throughout. Association-rate curves observed with different IgG preparations were adjusted to a common baseline and superimposed; initial velocities of association of the high-affinity components were estimated by measuring the initial linear slopes of the curves (e.g., in the interval of 430–500 sec on the abscissa of Fig. 1*B*)

To study ultrastructural localization of antibody reactivities with various stages of *P. falciparum*, sporozoite, exoerythrocytic-infected hepatocyte, gametocyte stage III-IV, and asexual blood-stage parasite were chosen for immunoelectron microscopy. Briefly, sections were incubated for 24 hr at 4°C with 1:800-diluted antibody for determination of reactivity with sporozoites and 1:200-diluted antibody for determination of reactivities with other stages of parasites. This was followed by a 1-hr incubation at 25°C with gold-labeled goat anti-rabbit IgG antibody. Method specificity was confirmed by incubating control sections with preimmune rabbit serum in place of the primary antibody, with the colloidal gold probe, or with colloidal gold alone. Reactivities of antibodies with parasites were examined in a Zeiss CEM902 electron microscope (25).

Table 1. Amino acid sequence and epitope-specific antibody responses of CDC/NII MAL VAC-1

Sequence	Antigen-epitope	Epitope-specific antibody responses in rabbits
KPKHKKLKQPGDGNP	CSP-B	<1:50
WSPCSVTCG	SSP-2-B	<1:50
KPKDELDYENDIEKKICKMEKCS	CSP-CTL	1:6,400–1:51,200
DIEKKICKMEKCSSVFNVVNS	CSP-T	1:400–1:3,200
NSGCFRHLDEREECKCLL	MSP-1-B	1:50–1:800
EDSGSNGKKITCECTKPDS	MSP-1-B	<1:50
KPIVQYDNF	LSA-1-CTL	1:400–1:6,400
3XNANP	CSP-B	1:6,400–1:102,400
DGNCEDIPHVNEFSAIDL	AMA-1-B	1:3,200–1:102,400
GNAEKYDKMDEPQHYGKS	AMA-1-B	1:800–1:1,600
LTPLEELY	RAP-1-B	1:200–1:1,600
KPNDKSLY	LSA-1-CTL	<1:50
QYIKANSKFIGITEL	P2-T	<1:50
SNTFINNA	MSP-2-B	<1:50
GQHGHMHG	MSP-2-B	<1:50
NEREDERTLTKEYEDIVLK	EBA-175-B	1:100–1:1,600
EFTYMINFGRGQNYWEHPYQKS	AMA-1-T	1:200–1:3,200
DQPKQYEQHLTDYEKIKEG	AMA-1-T	1:50–1:200
KPLDKFGNIYDYHYEH	Pfg27-B	1:50–1:1,600
SSPSSTKSSPSNVKSAS	RAP-1-T	1:100–1:3,200
LATRLMKKFKAEIRDFF	RAP-1-T	<1:50
GISYYEKVLAKYKDDLE	MSP-1-T	<1:50

For epitope-specific antibody responses post fourth immunization, endpoint titers were determined based on the highest dilution of the samples which generated an optical density (OD) greater than the cutoff value (mean plus 3 standard deviations of preimmunization sera). Since three rabbits were immunized with the vaccine antigen formulated in different adjuvants, epitope-specific antibody responses were presented by range of endpoint titers. Abbreviations: CSP, circumsporozoite protein; SSP2, sporozoite surface protein-2; LSA-1, liver-stage antigen-1; MSP-1, merozoite surface protein-1; MSP-2, merozoite surface protein-2; AMA-1, apical membrane antigen-1; EBA-175, erythrocyte-binding antigen-175; RAP-1, rhoptry associated protein-1; Pfg27, gametocyte 27-kDA antigen. B cell (B), T proliferative (T), and cytotoxic T cell epitopes (CTL) are shown.

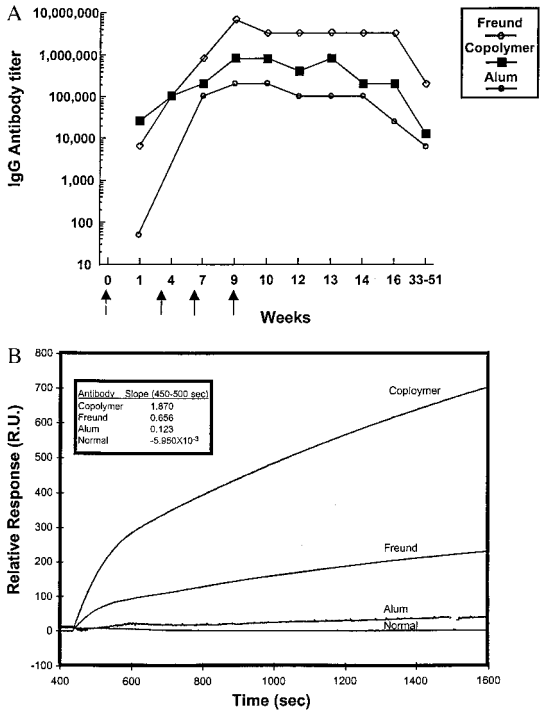

FIG. 1. Antibody responses in sera as measured by ELISA and binding of purified IgGs. (*A*) Rabbits were immunized with purified CDC/NII MAL VAC-1 by using different adjuvant formulations at weeks 0, 3, 6, and 9 as indicated by arrows. Antibody responses to CDC/NII MAL VAC-1 in the sera from rabbits receiving Freund's (◇), copolymer (■), and alum (○) were measured. Titers were determined based on the highest dilution of the samples that generated an OD greater than cutoff value (mean ± 3 SD of preimmunization sera). ODs lower than cutoff value at 1:50 dilution were considered negative responses. (*B*) Purified antibodies at concentration of 50 μg/ml were presented at 5 μl/min to a Biacore sensor cell loaded with CDC/NII MAL VAC-1 protein. *Inset* shows initial velocities of association (450–500 sec).

Inhibition of sporozoite invasion assays were conducted to determine the inhibitory effects of antibodies as described (26). Briefly, the purified antibodies were added at two different final concentrations (25 and 50 μg/ml) into the HepG2-A16 hepatoma cells, and 30,000 sporozoites were added. The cells were incubated at 37°C in 5% CO_2 for 3 hrs, rinsed twice with PBS, and fixed with methanol. Sporozoites that had entered hepatoma cells were visualized by immunohistochemical staining with a mAb to *P. falciparum* sporozoites (NSF1), peroxidase-conjugated goat anti-mouse Igs, and a substrate, 3,3-diaminobenzidine. All cultures were done in triplicate, and the number of invaded sporozoites was determined by light microscopy.

Antibody-dependent cellular-inhibition (ADCI) assays were carried out by methods described elsewhere (27, 28). Briefly, the purified antibodies were added at three different final concentrations (12.5, 25, and 50 μg/ml) into FC27 strain blood-stage parasite cultures (0.3% parasitemia with 60% schizonts and 1% hematocrit) along with 80,000 rhIFN-γ (100 ng/ml)-activated human monocytes. The cell cultures were incubated at 37°C in a mixed-gas environment containing 5% O_2/5% CO_2/90% N_2 for 72 hr, which required medium and antibody replacement every 24 hr. Parasites were stained with the vital dye hydroethidine, and parasitemias were determined by using a flow cytometry-based parasite-enumerating procedure using FACScan (Becton Dickinson). Transmission-blocking assays were performed by using membrane-feeding assays (29). In this assay, *P. falciparum* (3D7) gametocytes were used to infect *Anopheles stephensi* mosquitoes. Various IgG preparations were tested at the final concentration of 125–500 μg/ml.

RESULTS

Immunogenicity of the Multicomponent *P. falciparum* Vaccine. Antibody titers against the vaccine antigen and peptides complementary to 22 immune epitopes (Fig. 1; Table 1) were measured with ELISA after each immunization until week 51 for rabbit 787 (Freund's complete adjuvant), week 39 for rabbit 789 (copolymer as adjuvant), and week 33 for rabbit 1015 (alum as adjuvant). Fig. 1 shows that the vaccine antigen induced high level and long-lasting antibody responses against the vaccine in rabbits immunized with different adjuvants (1/3,276,800 titer for rabbit 787, 1/819,200 titer for rabbit 789, and 1/204,800 titer for rabbit 1015 after fourth immunization). Overall, the rabbit receiving Freund's as the adjuvant had higher antibody levels to the vaccine antigen as compared with those receiving copolymer and alum as adjuvants. The antibody responses reached maximal levels after the fourth immunization, remained at high levels until week 14–16, and decreased 10- to 15-fold from week 33 to 51.

Analysis of epitope-specific antibody responses by using ELISA showed that the vaccine-induced antibodies recognized both B cell (7 of 12) and T cell (6 of 10) epitopes in this vaccine construct. Among the epitope-specific antibody responses, antibody levels against B cell epitopes of CSP (P519, NANP repeat), AMA-1 (P600), and CTL epitope of CSP (P593) was significantly higher than antibody levels to other epitopes. Like antibody responses to the whole-vaccine antigen, the antibody titer against these epitopes also was higher in rabbits receiving Freund's and copolymer as adjuvants than those receiving alum-adjuvanted vaccine (data not shown). We observed medium-to-low antibody responses to other epitopes. Medium-level antibody responses were against the B cell epitopes of AMA-1 and RAP-1 (P601 and P545) and T cell epitopes of CSP and LSA-1 (P594 and P595). Low-level antibody responses were detected against B cell epitopes of EBA-175, MSP-1, and Pfg 27 (P546, P597, and P591) and T cell epitopes of AMA-1 and RAP-1 (P602, P603, and P604). Although the size of the epitopes did not appear to be a determining factor in inducing antibody responses, those epitopes to which medium-to-low responses were generated tended to cluster at the C terminus.

We next investigated the binding affinities of the vaccine-elicited antibodies in a Biacore assay. Antigen-antibody binding showed multiple kinetic components distinguishable into fast (450- to 500-sec) and slow (800- to 1,400-sec) components (Fig. 1*B*). Quantitatively, total IgG from the rabbit immunized in the presence of copolymer adjuvant contained much higher levels of high-affinity (i.e., rapidly binding) antibodies. This conclusion also was supported by the biological analysis described below.

Characterization of Immune Responses Against the Multicomponent Vaccine. Because the vaccine was designed as a multistage vaccine, the determination of antibody responses to various stages of the parasite life cycle is an important aspect of the evaluation of immunogenicity of this vaccine antigen. Serum samples collected at week 10 (first week after the fourth immunization) from all rabbits were first tested by IFA using sporozoites, infected erythrocytes, and gametocytes. All immunized rabbits had strong and comparable IFA titers against sporozoites (1:3,200). IFA titers against infected erythrocytes were of moderate levels (1:50–1:400), with the highest titer (1:400) observed in the rabbit receiving copolymer as adjuvant. Reactivity with gametocytes showed lower levels (1:25–1:100) of antibody responses in rabbits 787, 789, and 1015. These IFA reactivities were comparable to the natural antibody responses of clinically immune adults in western Kenya (data not shown). We also conducted ultrastructural localization of antibody reactivities with sporozoite, liver, blood, and sexual stages of *P. falciparum* by using immunoelectron microscopy. Numerous gold particles were observed on the surface and within the cytoplasm of sporozoites (Fig. 2*A*). Antibody recognized parasitophorous vacuole mem-

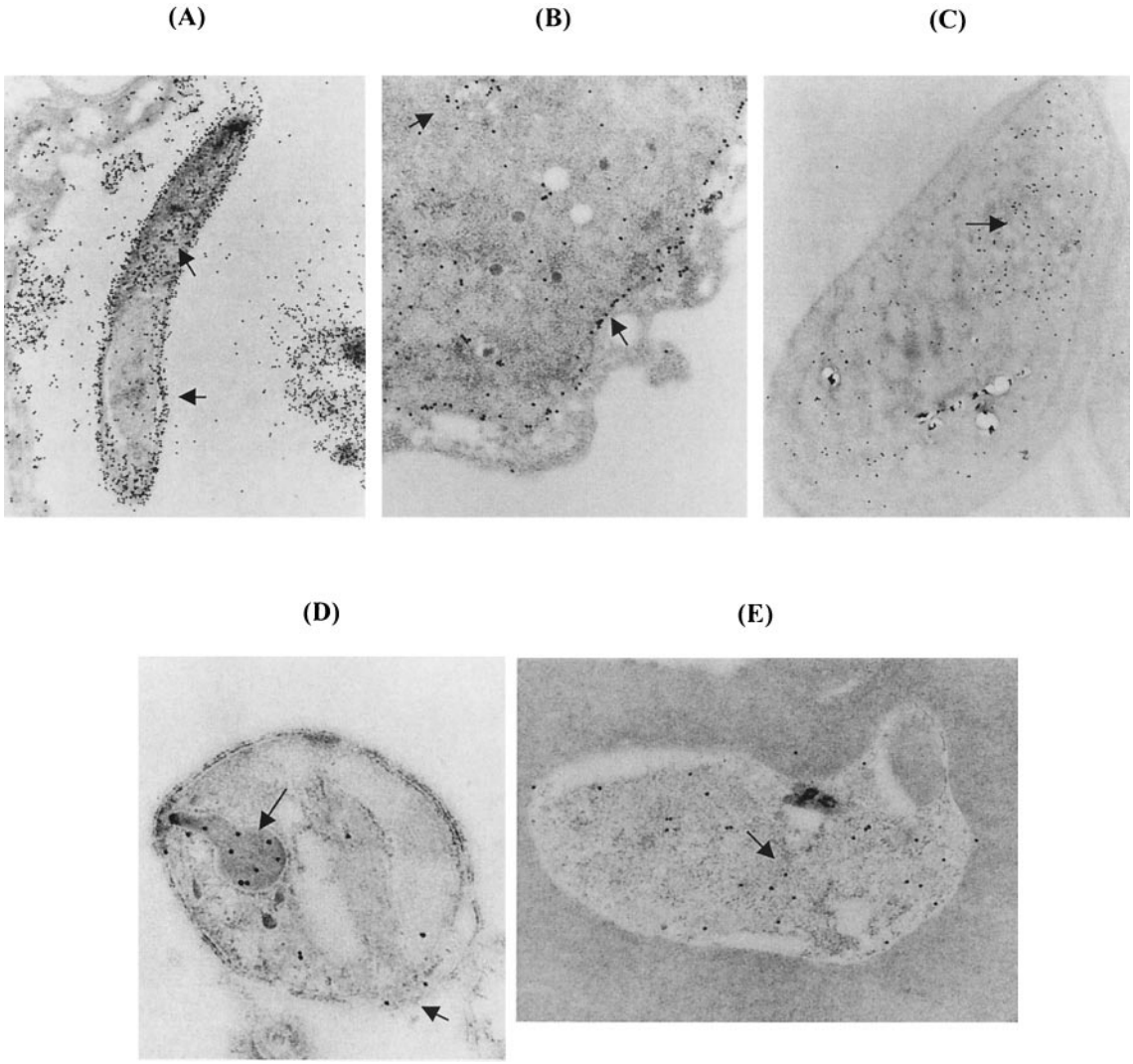

FIG. 2. Ultrastructural localization of antibody reactivities with various stages of *P. falciparum*. Reactivities of the purified antibody from rabbit 789 receiving copolymer as adjuvant are presented here. Gold particles were found on the surface and in the cytoplasm of the sporozoite (arrows in *A*), in the parasitophorous vacuole membrane and cytoplasm of exoerythrocytic stage (arrows in *B*), in the cytoplasm in gametocyte stage III-IV (arrow in *C*), in rhoptry and surface of merozoite of blood stage (arrows in *D*), and in the cytoplasm of trophozoite of blood stage (arrow in *E*).

brane and cytoplasm of exoerythrocytic-stage parasites (Fig. 2*B*). The cytoplasm of gametocytes contained randomly distributed gold particles (Fig. 2*C*). In infected erythrocytes, gold particles were found in rhoptries and on the surface of merozoites and in the cytoplasm of trophozoites (Fig. 2 *D* and *E*).

Determination of *in Vitro* Antiparasite Activity of Vaccine-Elicited Antibodies. Antibodies from rabbits immunized with the vaccine in different adjuvants strongly inhibited sporozoite invasion of HepG2-A16 cells (Table 2). The antibodies from rabbit that received the vaccine in the block-copolymer adjuvant almost completely inhibited sporozoite invasion (98% inhibition) at an antibody concentration of 50 μg/ml. This level of inhibition was comparable with the inhibition observed by using a positive mAb control. Antibodies elicited in the presence of alum and Freund's adjuvants also inhibited invasion of sporozoites, although at lower levels. At 25 μg/ml, antibody-mediated inhibition again was most prominent in the case of antibodies elicited with vaccination with block copolymer adjuvant.

ADCI experiments showed that the vaccine-elicited antibodies had significant effects on *in vitro* growth of blood-stage parasites in the presence of monocytes. No growth-inhibitory effects in the absence of monocytes were observed. The most striking ADCI activity was mediated by antibodies from the rabbit that received the vaccine-copolymer formulation (73% inhibition); lower activity was observed in those animals receiving vaccination in Freund's adjuvant (70%) and alum adjuvant (67%) (Table 3). We also observed an antibody concentration-dependent inhibition in the ADCI assay (Table 3). As compared with the ADCI activity with the rabbit antivaccine antibodies, we found that the purified IgGs from clinically immune Kenyan adults were much less effective (29% inhibition; Table 3). As compared with the sporozoite- and blood stage-inhibitory activities, the results of transmission-blocking assays did not reveal any significant inhibition (data not shown).

DISCUSSION

Based on the studies done over the past two decades that used model systems and vaccine trials in humans, it has become clear that multiple protective immune responses against multiple antigens from different stages will be needed to protect against malaria. Although a single-antigen and/or stage-specific vaccine could provide protection against infection, there are several reasons to advocate a multivalent, multistage malaria vaccine. A major concern with a single antigen-based vaccine is that an antigenic variant population of the parasite not recognized by the vaccine will cause infection (with heterologous parasites) and cause disease. In the case of single-stage pre-erythrocytic and/or transmission-blocking vaccines, a highly effective vaccine would

Table 2. Evaluation of *in vitro* antiparasitic activities of CDC/NIIMAL VAC-1-elicited antibodies (inhibition of sporozoite invasion)

	Antibody concentrations, ug/ml			
	50		25	
Antibodies	Invasion number	Inhibition, %	Invasion number	Inhibition, %
Preimmune	131 (10)	0	112 (7)	0
Freund	19 (3)	85	29 (3)	74
Copolymer	3 (2)	98	10 (3)	91
Alum	15 (2)	89	23 (4)	79
Positive control	ND	ND	2 (2)	98

Invasion number given as mean number of *P. falciparum* sporozoites that entered triplicate cell culture with SD given in parenthesis. Inhibition expressed as % relative to preimmune control culture (0%), which was calculated as follows: $100 \times [1 -$ (mean number of invaded sporozoites in test culture/mean number of invaded sporozoites in control culture)]. Preimmune, antibodies purified from preimmune rabbits used as negative control; Freund, antibodies purified from rabbit receiving Freund's as adjuvant; Copolymer, antibodies purified from rabbit receiving nonionic block copolymer P1005 as adjuvant; Alum, antibodies purified from rabbit receiving aluminum hydroxide as adjuvant. For positive control, mAb against sporozoites (NFS1) was used. ND, not done.

prevent the development and maintenance of immunity against the blood-stage forms of the parasite, and if the effects of the vaccine waned with time, the population would be at an increased risk of morbidity and mortality (30). In contrast to a single antigen-based vaccine, a multivalent, multistage human malaria vaccine that induces protective-antibody and cell-mediated immune responses against the different stages of the parasite may provide more efficacious and long-lasting protection against malaria-associated morbidity and mortality.

The objective of our multicomponent vaccine research effort is to develop an effective multistage vaccine eliciting long-lasting immunity against *P. falciparum* malaria by using conserved, immunogenic, and protective epitopes from the sporozoite, liver, asexual blood, and sexual stage-specific antigens of the parasite. Selection of the B cell- and T cell-proliferative and CTL epitopes used in the development of CDC/NII MALVAC-1 was based on (*i*) the results of our immunoepidemiologic studies in western Kenya that have investigated characteristics of naturally acquired immunity against infection and disease and (*ii*) *in vitro* and/or *in vivo* protection studies in model systems (refs. 9–21; unpublished data). By combining the epitopes that are naturally immunogenic with the epitopes that have been implicated in protection by *in vitro* studies, we envisioned the creation of a synthetic protein that contains multiple targets at different parasite stages for immune intervention.

One important test of vaccine efficacy is whether elicited antibodies can inhibit invasion, growth, and/or development of parasites. To answer this question, we immunized rabbits and used serum Igs in three *in vitro* assays of protection. Vaccine-elicited antibodies strongly inhibited parasite invasion in an inhibition of sporozoite-invasion assay, which tests the ability of antibodies to block the invasion of sporozoites into hepatoma cells. Likewise, these antibodies inhibited the growth of blood-stage parasites in the ADCI assay, which has been shown to correlate with *in vivo* protection against infection (27). These observations of the *in vitro* protective efficacy of vaccine-elicited antibodies coincided with our immunoelectron microscopic findings, which showed that the antibodies recognized different stages of parasites. Significantly, relatively higher levels of inhibition of sporozoite-invasion and ADCI activities were found in antibodies from rabbits receiving vaccine in the potentially human-usable copolymer formulation.

Although the vaccine-elicited antibodies recognized gametocytes in both IFA and immunoelectron microscopy, they did not inhibit parasite development at the mosquito stage. This failure could have been caused by the fact that only one epitope from Pfg27 was included in the vaccine and/or the antibody level against this epitope was lower (data not shown). Therefore, including additional immune epitopes from transmission-blocking vaccine candidates (especially the Pfs25 ookinete antigen) may increase the transmission-blocking efficacy of the multicomponent vaccine. Similarly, it will be possible to increase the parasite-inhibitory effects by incorporating additional B and T cell epitopes from the blood-stage and sporozoite antigens.

One of our concerns in the development of the multicomponent vaccine is that a tandem arrangement of the epitopes in this multivalent vaccine construct might result in a protein that fails to elicit epitope-specific immune responses caused by epitope competition and/or problems related with antigen processing and presentation. We chose to arrange the epitopes in the recombinant protein in an order that would maintain an overall hydrophilic nature, thus ensuring that the recombinant protein would be water-soluble and easily purified. Despite this rationale, the arrangement chosen might not have been optimal for induction of immune responses. Whereas the T cell proliferation and CTL data are not yet available, the antibody data show that immunization with the multicomponent vaccine elicited antibodies against 7 of 12 B cell epitopes and 6 of 10 T cell epitopes. For those epitopes that failed to elicit antibody responses, there did not appear to be an association with size, but they did tend to cluster at the C terminus of the construct. It is important to keep in mind that most of the epitopes included in CDC/NIIMALVAC-1 were discovered in human-based studies; thus, subtle differences in antigen processing, presentation, and T cell recognition between

Table 3. Evaluation of *in vitro* antiparasitic activities of CDC/NII MAL VAC-1-elicited antibodies [growth inhibition of the blood-stage parasite in the presence of monocytes (ADCI)]

	Antibody concentration, ug/ml								
	50			25			12.5		
	Parasitemia			Parasitemia			Parasitemia		
Antibodies	No monocyte	Monocyte	Inhibition, %	No monocyte	Monocyte	Inhibition, %	No monocyte	Monocyte	Inhibition, %
Preimmune	2.56 (0.07)	1.64 (0.1)	0	3.39 (0.09)	2.25 (0.065)	0	3.75 (0.13)	2.48 (0.105)	0
Freund	3.33 (0.07)	0.65 (0.025)	70	3.49 (0.04)	1.77 (0.125)	23	3.56 (0.025)	2.19 (0.095)	7
Copolymer	2.85 (0.09)	0.50 (0.025)	73	3.10 (0.12)	1.43 (0.11)	30	3.46 (0.01)	1.68 (0.06)	26
Alum	2.97 (0.14)	0.63 (0.11)	67	3.33 (0.05)	1.10 (0.05)	50	3.65 (0.125)	1.40 (0.17)	42
Positive control	3.17 (0.1)	1.17 (0.035)	29		ND			ND	

Parasitemia given as mean numbers of parasitemias in duplicate cell culture with SD given in parenthesis. Inhibition expressed as relative to preimmune control culture (0%) and taking into account the possible inhibition induced by monocyte and antibody alone, which was calculated as follows: $100 \times [(1 -$ [(mean parasitemia with test IgG and monocyte/mean parasitemia with test IgG and without monocyte)/(mean parasitemia with control IgG and monocyte/mean parasitemia with control IgG and without monocyte)])]. Preimmune, antibodies purified from preimmune rabbits used as negative control; Freund, antibodies purified from rabbit receiving Freund's as adjuvant; Copolymer, antibodies purified from rabbit receiving nonionic block copolymer P1005 as adjuvant; Alum, antibodies purified from rabbit receiving aluminum hydroxide as adjuvant. For positive control, mAb against sporozoites (NFS1) was used. ND, not done.

rabbits and humans could account for some loss of immunogenicity. Also, junction (flanking) sequences—those sequences formed by the end-to-end joining of epitopes in this vaccine—might have affected efficient processing of epitopes. Although in some cases flanking sequences have been shown to be important (31), there are examples of presentation of epitopes regardless of their context (32, 33). These issues could be resolved by increasing the size of the epitopes and altering their position in the construct, although continued assessment of the construct (particularly in monkey vaccination studies) will be necessary to fully evaluate the immunogenicity (of individual epitopes in CDC/NIIMAL-VAC-1) and protective efficacy. Notwithstanding these issues, the antibodies that were produced in immunized rabbits were clearly efficacious in mediating protection against the sporozoite and blood stages in *in vitro* assays.

The issue of antibody amount vs. affinity can be critical in protection, especially in the case of malaria parasites that are available for direct antibody-based intervention only for a short period of time. Although immunization with Freund's adjuvant produced higher levels of antibodies as compared with vaccination with block-copolymer adjuvant, Biacore biosensor assessment of IgG showed that the block-copolymer adjuvanted vaccine induced higher affinity antibodies. These data are in agreement with the IFA data, in which higher titers were seen with antibodies induced by vaccination with block-copolymer adjuvant. We have demonstrated that adjuvants can have a significant influence in the induction of high affinity antibodies in malaria vaccine research.

Although we have characterized the antibody responses and evaluated the *in vitro* protection provided by these antibodies after immunization with our construct, we have not yet evaluated cell-mediated immune responses and protection associated with CD8 + CTL responses. It is important to point out that the CTL epitopes included in the vaccine construct have been shown to induce protective immune responses in model systems and/or are associated with protection in epidemiologic studies (10, 11, 20). It is possible that the protective effects of this vaccine may also be directed against the liver stages, a possibility consistent with the observation with electron microscopy that antibodies react with the exoerythrocytic bodies. The cell-mediated responses and their association with protection will need to be addressed in subsequent studies including vaccine trials in nonhuman primate models.

The advantage of our multicomponent vaccine over vaccines based on single proteins is that multiple layers of protective immune responses cannot only prevent infection at multiple stages in the parasite's life cycle but can also help overcome problems associated with host genetic restriction of immune responses and genetic diversity in vaccine candidate antigens. The approach used in the development of a multicomponent vaccine can be easily manipulated to add or delete epitopes to enhance the immunogenicity and the protective efficacy of the vaccine antigen. The strategy also offers ways to design and develop multiple-species malaria vaccine constructs and vaccines against other infectious diseases.

In conclusion, we have developed a multistage and multicomponent recombinant malaria vaccine. The results of experimental immunization in rabbits with three different adjuvants revealed that this vaccine is highly immunogenic; the vaccine-induced antibodies recognize various stages of the parasite. More importantly, the vaccine-elicited antibodies produced significant anti-parasite activity as evidenced by *in vitro* assays against both sporozoite and blood stages of malaria. There is now a need to evaluate the protective efficacy of this malaria vaccine in nonhuman primates and then in humans. The results of this study are encouraging and provide a promising candidate for both travelers who are nonimmune and people living in malarious regions of the world.

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