Safety, Immunogenicity, and Efficacy of Prime-Boost Immunization with Recombinant Poxvirus FP9 and Modified Vaccinia Virus Ankara Encoding the Full-Length *Plasmodium falciparum* Circumsporozoite Protein

Michael Walther,^{1*} Fiona M. Thompson,¹ Susanna Dunachie,¹ Sheila Keating,¹ Stephen Todryk,¹ Tamara Berthoud,¹ Laura Andrews,² Rikke F. Andersen,² Anne Moore,² Sarah C. Gilbert,² Ian Poulton,¹ Filip Dubovsky,³ Eveline Tierney,³ Simon Correa,¹ Angela Huntcooke,¹ Geoffrey Butcher,⁴ Jack Williams,⁵ Robert E. Sinden,⁴ and Adrian V. S. Hill^{1,2}

Centre for Clinical Vaccinology & Tropical Medicine, Nuffield Department of Clinical Medicine, Oxford University, Churchill Hospital, Oxford OX3 7LJ, United Kingdom¹; The Wellcome Trust Centre for Human Genetics, Nuffield Department of Clinical Medicine, Oxford University, Roosevelt Drive, Oxford OX3 7BN, United Kingdom²; PATH Malaria Vaccine Initiative, Bethesda, Maryland 20814³; Department of Biological Sciences, Sir Alexander Fleming Building, Imperial College London, Imperial College Road, London SW7 2AZ, United Kingdom⁴; and Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, Maryland 20910⁵

Received 15 December 2005/Returned for modification 3 February 2006/Accepted 25 February 2006

Heterologous prime-boost immunization with DNA and various recombinant poxviruses encoding malaria antigens is capable of inducing strong cell-mediated immune responses and partial protection in human sporozoite challenges. Here we report a series of trials assessing recombinant fowlpox virus and modified vaccinia virus Ankara encoding the *Plasmodium falciparum* circumsporozoite protein in various prime-boost combinations, doses, and application routes. For the first time, these vaccines were administered intramuscularly and at doses of up to 5×10^8 PFU. Vaccines containing this antigen proved safe and induced modest immune responses but showed no evidence of efficacy in a sporozoite challenge.

Despite considerable efforts, *Plasmodium falciparum* malaria remains both a major health problem and a significant constraint to economic and social development in tropical countries (20). Drug-resistant parasites and increasing costs of effective treatment make the development of a vaccine imperative. The feasibility of a vaccine against the pre-erythrocytic stage of malaria is supported by the observation that immunization with irradiated sporozoites can elicit sterile immunity against a challenge with *Plasmodium* sp. sporozoites in mice and humans (9, 27). There is supporting evidence for a central role of gamma interferon (IFN- γ)-secreting CD4⁺ and CD8⁺ T cells in protection against the liver stages of malaria (11, 18, 34, 35, 38, 44). The observation that both an HLA class I antigen (HLA-B53) and an HLA class II haplotype (DRB1*1302-DQB1*0501) were independently associated with protection against severe malaria in children provides indirect evidence of a crucial role for T cells in humans too (16, 17).

Based on these findings, we hypothesized that a vaccination approach aiming to induce potent T-cell responses could be capable of eliminating infected liver cells. One such strategy, known as heterologous prime-boost, uses sequential immunization with different carriers such as DNA and viral vectors, delivering a common antigen. Priming with plasmid DNA encoding the pre-erythrocytic antigen circumsporozoite (CS) an-

tigen of *Plasmodium berghei* or thrombospondin-related adhesion protein (TRAP), followed by boosting with recombinant modified vaccinia virus Ankara (MVA or M) induced complete or almost complete protection in mice which correlated with CD8⁺ T-cell responses (13, 28, 37). With a recombinant fowlpox virus (FP9 or F) as the priming agent instead of DNA, immunogenicity and the level of protection could be increased even further (3).

Clinical phase I and IIa studies evaluating a variety of different carrier combinations proved them to be safe (24) and demonstrated unprecedented levels of T-cell responsiveness in humans. Priming with DNA plasmids encoding *P. falciparum* TRAP and a multiepitope (ME) string containing 14 CD8, 1 CD4, and 2 B-cell epitopes from six pre-erythrocytic *P. falciparum* antigens (12), followed by a booster with recombinant MVA encoding the same antigens, induced a geometric mean of 704 antigen-specific IFN- γ -secreting T cells per million peripheral blood mononuclear cells (PBMC) (21), most of them being CD4⁺ T cells. In a subsequent sporozoite challenge, a significant delay in time to first parasitemia detected on a thick film was observed, indicating a reduction in the liver stage parasite burden of almost 90% (5, 21). When employed in an African population, these constructs were safe (22, 25) and more immunogenic in malaria-exposed individuals than in malaria-naïve subjects (25) but ineffective at reducing the natural infection rate in semi-immune African adults (23).

Repeated priming with FP9 encoding METRAP, followed by recombinant MVA (FFM), elicited a slightly lower IFN- γ response but induced complete protection in some malaria challenge volunteers (43). Further analysis revealed that the

* Corresponding author. Present address: MRC Laboratories, Fajara P.O. Box 273, Banjul, The Gambia. Phone: 00220 4497928. Fax: 00220 4494485. E-mail: mwalther@mrc.gm.

TABLE 1. Vaccines and doses administered in VAC23

Group (no. of volunteers)	Dose (PFU, 10 ⁸)				Comment
	FP9 CS	MVA CS	FP9 ME-TRAP	MVA ME-TRAP	
1 (3)	1				Single dose
2 (3)	0.5		0.5		Simultaneous administration
3 (3)		0.5		0.75	Simultaneous administration
4 (3)	1		1		Simultaneous administration
5 (3)		1		1.5	Simultaneous administration
6 (8)	1 (doses 1 & 2)	1 (dose 3)			Doses given 4 wk apart
7 (8)	1 (doses 1 & 2)	1 (dose 3)	1 (doses 1 & 2)	1.5 (dose3)	Doses given 4 wk apart

immune response was mainly due to CD4-dependent CD8⁺ T cells (41), suggesting a preferential need for CD8⁺ T cells in conferring protection.

The CS antigen is a major sporozoite surface antigen that has been implicated in hepatocyte invasion. The RTS,S vaccine candidate bearing CS has repeatedly demonstrated protection in the sporozoite challenge model (19), in prolonging the time to infection in settings where the disease is endemic (6) and impacting disease in children in Mozambique (1). It was hypothesized that using heterologous prime-boost would generate a vigorous cellular immune response and lead to increased protective efficacy.

In the trials described here, a new construct, FP9 expressing the full-length CS antigen, was evaluated in association with recombinant MVA expressing the same CS antigen. Applying the promising FFM approach either with CS alone or in comparison with simultaneous administration of both CS and METRAP antigens, the safety, immunogenicity, and efficacy of these regimens were assessed. For the first time, these vaccines were given as intramuscular (i.m.) injections and at doses of up to 5×10^8 PFU.

MATERIALS AND METHODS

Study design. Two clinical trials were conducted with healthy adult malaria-naïve volunteers. The first (VAC23) was an open-labeled randomized phase I/IIa study evaluating the safety, immunogenicity, and efficacy of the vaccines FP9-CS and MVA-CS alone and simultaneous administration of FP9-CS plus FP9-METRAP and MVA-CS plus MVA-METRAP. In a lead-in dose ranging phase, groups of three volunteers were vaccinated intradermally (i.d.) as follows: group 1, FP9-CS alone at a dose of 1×10^8 PFU; group 2, FP9-CS plus FP9-METRAP (0.5×10^8 PFU each); group 3, MVA-CS plus MVA-METRAP (0.5×10^8 PFU and 0.75×10^8 PFU, respectively). Once safety data have been reviewed and approved by an independent safety-monitoring committee, groups 4 and 5 received a full-dose regimen as follows: group 4, FP9-CS plus FP9-METRAP (1×10^8 PFU each); group 5, MVA-CS plus MVA-METRAP (1×10^8 PFU and 1.5×10^8 PFU, respectively). After review and approval of the safety data accrued from groups 4 and 5, a decision was made to use the full dose of each vaccine for the main study groups. Volunteers in group 6 received FFM-CS alone (at 1×10^8 PFU each); in group 7, FFM CS (at 1×10^8 PFU each) plus FFM METRAP (1×10^8 PFU for F and 1.5×10^8 PFU for M, respectively) was administered. In both groups, vaccines were given i.d., 4 weeks apart (Table 1).

To assess if a higher dose or i.m. administration of the CS-based vaccines is safe and more immunogenic, a second study (VAC28) evaluated a regimen using FFM encoding CS alone, where vaccines were given as either i.m. or i.d. injections and where the dose of the boosting vaccine, MVA-CS, was increased to 5×10^8 PFU. A third group explored the impact of a longer time interval between administration of the second FP9-CS dose and the boosting MVA-CS dose (Table 2).

Figure 1 details the trial profiles and illustrates the vaccine regimens for the main groups.

Both study protocols were approved by the Oxford Research Ethics Committee and the Human Subjects Protection Committee at the Program for Appropriate

Technology in Health, Seattle, Wash. Studies were conducted in accordance with Good Clinical Practice/International Conference on Harmonisation guidelines and independently monitored. Vaccines were used after review by the United Kingdom Medicines and Healthcare Regulatory Agency (governing the use of medicinal products within the United Kingdom).

Vaccines. Recombinant viruses were prepared by *in vitro* recombination of a shuttle vector encoding the antigen with either FP9 or MVA in primary chicken embryo fibroblast cultures. All vaccines used in these studies were manufactured according to Good Manufacturing Practice by IDT, Rosslau, Germany.

MVA-METRAP and FP9-METRAP. Preparation of recombinant viruses and the malarial DNA sequence known as METRAP has been described in detail elsewhere (12, 21, 43). In brief, the 789-amino-acid-long insert includes 14 CD8⁺ T-cell epitopes, 1 CD4⁺ T-cell epitope, and 2 B-cell epitopes from six pre-erythrocytic *P. falciparum* antigens of the T9/96 strain fused in frame to the entire pre-erythrocytic TRAP sequence.

MVA-CS and FP9-CS. MVA-CS and FP9-CS both contain a synthetic gene that encodes the full-length CS protein of *P. falciparum* strain 3D7 (codon optimized, recorded toward mammalian codon bias), a T-cell epitope from *P. falciparum* liver stage antigen 1 (ls6, an epitope that may enhance efficacy in HLA type HLA-B*53 subjects), and a T-cell epitope from *P. berghei* (pb9). The CS gene has been recoded toward mammalian codon bias to facilitate antigen expression. C terminal to the CS sequence and in frame are the ls6 and pb9 epitopes, which are coexpressed with the CS protein. pb9 was included to allow potency testing of the vaccine in mice and to evaluate the immunogenicity of the recombinant viruses in murine models. The synthetic CS gene encodes a single polypeptide of 310 amino acids, and expression is driven by the vaccinia virus late/early P7.5 promoter.

Study procedures for VAC23. In the first study, all vaccines were administered as an i.d. injection into the skin over the deltoid muscle with a 27-gauge needle. Depending on the vaccine titer, each dose was given as one to three injections. The doses for the main study groups were 1×10^8 PFU of FP9-CS, FP9-METRAP, and MVA-CS and 1.5×10^8 PFU of MVA-METRAP. Where vaccines encoding CS and METRAP were administered simultaneously, these were given in separate arms. Subjects were allocated to one of the main study groups (six CS only or seven CS plus METRAP) by restricted randomization. Vaccines were given 4 weeks apart, and the challenge was performed 2 weeks after the last vaccination. Following vaccination, subjects were followed up after 1 h and on days 2, 7, and 28 (Fig. 1b). They were asked to take their temperature, to measure the largest diameters of swelling, redness, and warmth at the injection site, to grade the local pain, and to note any other local reaction, as well as any systemic adverse events, and to enter these on a daily basis into a study diary for 14 days postvaccination. At each visit, vital parameters were measured, the injection site was inspected by the investigator, and the entries on the diary card were reviewed. All local reactions were assumed to be vaccine related. The relationship and severity of reported systemic adverse events were assessed by the investigator. Table 3 explains the system used to grade the severity of adverse

TABLE 2. Vaccines and doses administered in VAC28

Group (no. of volunteers)	Dose (PFU, 10 ⁸)		Route of administration
	FP9 CS	MVA CS	
1 (8)	1	5	Intradermal
2 (8)	1	5	Intradermal
3 (8)	1	5	Intramuscular

FIG. 1. Trial profiles (a) and time course of vaccination regimens (b) for both studies. Trial 1 (VAC23) is depicted on the left, and trial 2 (VAC28) is displayed on the right.

events. Routine parameters for biochemistry and hematology were measured on the vaccination days and 7 days thereafter.

In groups 6 and 7, eight subjects per group completed the vaccine trial. One subject in group 6 was not available for the challenge.

By using a method adapted from that of Chulay et al. (8), seven subjects in group 6, eight subjects in group 7, and six nonvaccinated, malaria-naïve controls underwent a sporozoite challenge with five infectious *Anopheles stephensi* mosquitoes, each with 10^2 to 10^4 sporozoites per salivary gland.

The challenge was performed at Imperial College, London, 2 weeks after the third vaccine was administered, with a detailed follow-up of volunteers as de-

scribed elsewhere (43). Briefly, starting on day 6.5 after the challenge, all subjects were seen in clinics twice daily and screened with Giemsa-stained thick blood films and quantitative PCR for *P. falciparum* by a method developed by Andrews et al. (4). Upon the first confirmed positive thick film, subjects were treated with a full course of artemether-lumefantrine. All subjects were seen again 35 days after the challenge. Mosquitoes reared at Imperial College were used for this challenge, with a backup provided by the Walter Reed Army Institute of Research (Silver Spring, MD).

Study procedures for VAC28. To assess the effect of a high dose of MVA-CS and to evaluate how the length of time between the second and third vaccinations impacts the immune response, the first and second groups received two doses of FP9-CS (1×10^8 PFU), given 3 or 4 weeks apart, followed by a high-dose boost with MVA-CS (5×10^8 PFU) given 8 weeks (group 1) or 4 weeks (group 2) later. Vaccines were administered i.d. as described above. Subjects enrolled in a third group received i.m. injections into the deltoid muscle of two doses of FP9-CS (1×10^8 PFU), followed by a high dose of MVA-CS (5×10^8 PFU), given 4 weeks apart, with a 21-gauge needle (Fig. 1b). The vaccines for i.m. injection were diluted in 0.9% sterile NaCl to a final volume of 0.5 ml. The groups were vaccinated in a staggered fashion according to the specific regimen, and the volunteers were nonrandomized. The follow up was as described for VAC23 above, with a final follow-up 3 months after the last vaccine had been administered. In group 2, two subjects did not attend this final visit but completed the 28-day follow-up after administration

TABLE 3. Grading system used to assess severity of local adverse events

Grade	Swelling (mm)	Redness (mm)	Pain
0	0	0	None at all
1	<20	<50	Painful on touch
2	<21–50	<51–100	Partial restriction of activities of daily life
3	>50	>100	Severe, reluctance to use arm

FIG. 2. Side effects at the injection site reported for VAC23. Group 6 received FFM expressing CS, and group 7 received FFM expressing CS and METRAP in opposite arms. Frequency and severity of pain (a), redness (b), and swelling (c) over time after administration of each vaccine are shown. y axis, number of volunteers reporting; x axis, days after vaccination. Symbols: ■, grade 1; □, grade 2; ▤, grade 3. F, recombinant fowlpox; M, recombinant MVA.

of the third vaccine. In group 3, one subject was unavailable for administration of the third vaccine and was therefore withdrawn on day 35 (FF+7). There was no challenge phase in this study.

IFN- γ ELISPOT assays. PBMC were collected on the day of vaccine administration, as well as 7 and 28 days thereafter, or as indicated.

IFN- γ ELISPOT assays were performed ex vivo with fresh cells as previously described (41). In brief, 400,000 PBMC per well were plated onto ELISPOT assay plates (MAIP S45; Millipore) in the presence of 25 μ g/ml of each peptide pool and incubated for 18 h at 37°C in 5% CO₂. Spots were counted with an ELISPOT reader (AutoImmuneDiagnostics). Results are expressed as spot-forming units (SFU) per million PBMC with the individual backgrounds deducted.

TRAP-derived 20-mer peptides overlapping by 10 amino acids from the vaccine strain (T9/96) were tested in six pools. CS peptides (15-mer, overlapping by 10 amino acids) from the 3D7 strain were tested in eight pools. The epitopes comprising the ME string peptides were assayed in a single well.

Responses to phytohemagglutinin, the purified protein derivative of *Mycobacterium tuberculosis* tuberculin, and a pool of 22 known, non-vaccine-related common cytotoxic T-lymphocyte epitopes from influenza A virus, Epstein-Barr virus, and cytomegalovirus were used as positive controls.

Antibodies against MVA. Serum antibody responses to the vaccine vector were assessed by enzyme-linked immunosorbent assay. Briefly, serial threefold dilutions of serum were added to microtiter plates coated with nonrecombinant MVA or FP9 (1 $\times$ 10⁶ PFU/ml), and bound antibodies were detected with alkaline-phosphatase-conjugated antibodies specific for whole human immunoglobulin G (PharMingen). Results are expressed as endpoint titers calculated by regression of the straight part of a curve of optical density versus serum dilution to a cutoff of 2 standard deviations above the background control values. The preimmunization serum sample for each individual was used as the background control.

Statistics. Quantitative ELISPOT assay data were assessed for normal distribution. Nonparametric data are displayed as box plots. The difference between medians was compared by Wilcoxon signed rank test (paired samples) or Mann-Whitney U test (unpaired samples). Kaplan-Meier analysis for time to parasitemia between groups was performed by log rank test, all with SPSS, version 11.5 (Lead Tools). Proportions were compared by Fisher's exact test with Epi Info 6.04 (Centers for Disease Control and Prevention). Ninety-five percent confidence intervals (CIs) were calculated with CIA 2.1 (Trevor Bryant, Southampton, United Kingdom).

RESULTS

VAC23 safety. During the lead-in dose-ranging study of the new vaccine construct FP9-CS and simultaneous administration of two virus-vectored vaccines, no serious or severe adverse events were reported. The local and systemic side effect profiles were similar to those described for recombinant MVA and FP9 vaccines expressing other malaria antigens (42).

For the main study groups 6 and 7, the number of subjects in each group experiencing pain, redness, or swelling at the vaccination site is displayed for each day and vaccine in Fig. 2 according to severity. Comparing local adverse events occurring after each vaccination in response to TRAP or CS, no significant difference between antigens with regard to frequency, duration, or severity was observed. A trend toward reduced duration of redness and swelling was observed after administration of the second dose of FP9-vectored vaccines.

Apart from redness, swelling, and pain, the injection site was reported to be warm and itchy in some cases, and a total of nine reports of transient axillary lymph node swelling were documented, being evenly distributed in both groups and mainly occurring after the first and third injections.

Vaccine-related systemic adverse events consisted of symptoms of a flu-like illness, were observed in 25 to 50% of subjects, were all graded mild, and were exclusively limited to the first 24 to 48 h after vaccine administration. The majority of subjects did not take any treatment. Figure 3a and b show the

FIG. 3. Number of volunteers experiencing vaccine-related systemic adverse events at least once within 1 week postvaccination. Panels: a, group 6 (VAC23, FFM expressing CS); b, group 7 (VAC23, FFM expressing CS and FFM expressing METRAP); c, group 1 (VAC28, FFM CS i.d.); d, group 2 (VAC28, FFM CS i.d.); e, group 3 (VAC28, FFM CS i.m.). Symbols: ■, one injection; □, two injections; ▨, three injections.

FIG. 5. Summed IFN- γ ELISPOT assay responses to peptides, measured at various time points during VAC23. Panels: a, responses to CS peptides in groups 6 and 7; b, responses to TRAP and ME in group 7. Black arrows indicate vaccinations. Results are displayed as numbers of spot-forming units per million PBMC, shown as box plots with medians, with the individual backgrounds deducted. Circles, outliers; stars, extremes. Number of subjects: group 7, eight throughout; group 6, seven throughout, apart from FFM+14, where there were six.

number of subjects experiencing general adverse events at least once within 1 week postvaccination.

From this comparison, it appears that the number of subjects experiencing generalized symptoms trends higher in group 7. Within each group, no significant difference in the frequencies of adverse events caused by the vaccines administered was seen.

VAC28 safety of i.d. and i.m. administration of a high dose of MVA-CS. The nature, frequency, duration, and severity of local adverse events observed in groups 1 and 2 for i.d. administration of two doses of FP9-CS (1×10^8 PFU) were very similar to those documented in the previous trial (VAC23) with the same vaccine (Fig. 4).

Intradermal injection of a high dose (5×10^8 PFU) of MVA-CS induced a more pronounced local side effect profile, with some subjects reporting higher degrees of pain, redness, and swelling for a longer period compared to FP9-CS (1×10^8 PFU) or MVA-CS (1×10^8 PFU; Fig. 4). A minority of subjects reported warmth, itchiness, and scaling too.

Injection of FP9-CS i.m. (1×10^8 PFU, group 3) showed minimal local reactogenicity, with subjects reporting only some pain on days 0 and 1. The high dose of MVA-CS (5×10^8 PFU) given as an i.m. injection initially induced redness in five subjects and swelling in up to four subjects, lasting for a maximum of 6 and 4 days, respectively, in one and two of those individuals, respectively. The duration and severity of pain were more pronounced after administration of the high dose.

Vaccine-related general adverse events were all mild; 95.5% occurred within the first 48 h of vaccination, and all resolved spontaneously within 4 days (Fig. 3c to e). In groups 1 and 2 (i.d. injection), there was a trend of more people experiencing systemic side effects after administration of the high dose of MVA-CS.

In group 3 (i.m. injection), slightly more study subjects reported systemic side effects compared to the i.d. groups. However, the frequency or severity of systemic side effects was not

increased after administration of a high dose of MVA-CS in this group.

Throughout the studies, no clinically significant alterations in laboratory parameters were observed.

Immunogenicity of vaccine regimen. The T-cell immunogenicity of the vaccine inserts was assessed by using ex vivo IFN- γ ELISPOT assay responses to peptide pools representing the antigens engineered into the vaccines.

Background values remained low throughout the studies (median, 5 SFU/million PBMC; 95% CI, 5 to 6.25), and responses to phytohemagglutinin (median, 490 SFU/million PBMC; 95% CI, 487 to 491), purified protein derivative (median, 49 SFU/million PBMC; 95% CI, 40 to 63), and cytotoxic T-lymphocyte epitopes (median, 99 SFU/million PBMC; 95% CI, 76 to 121) were clearly positive.

VAC23 immunogenicity. The medians of the summed ex vivo IFN- γ ELISPOT assay responses to the pooled CS and TRAP peptides or the ME string are shown in Fig. 5. IFN- γ responses to CS peptides obtained in groups 6 and 7 did not differ significantly, and there was no significant difference when responses to the CS and TRAP peptides were compared. For none of the time points were the responses statistically significantly different from that obtained at baseline (day 0). In particular, following MVA boosting (FFM+7), there was no enhancement of the response to either antigen.

VAC28 immunogenicity. Figure 6 depicts the IFN- γ ELISPOT assay responses to the pooled CS peptides for each vaccination regimen. Throughout the groups, the overall responses are similarly low, irrespective of the administration route (i.d. or i.m., groups 1 and 2 versus group 3).

For groups 2 (i.d.) and 3 (i.m.), there is no evidence that the heterologous boost with 5×10^8 PFU of MVA-CS enhanced the antigen-specific IFN- γ response. Only for group 1, where MVA-CS was given 8 weeks after the second priming with FP9-CS, was a significant difference observed between the day

FIG. 6. Summed IFN- γ ELISPOT assay responses to CS peptides measured at various time points during VAC28. Panels: a, group 1; b, group 2; c, group 3. Black arrows indicate vaccinations. Results are displayed as numbers of spot-forming units per million PBMC, shown as box plots with medians, with individual backgrounds deducted. Circles, outliers; stars, extremes. Unless otherwise indicated on the box plots, $n = 8$.

the last vaccine was given (FF+28) and the time point 7 days thereafter (FFM+7), suggesting a significant boosting of the response in this case. However, its magnitude remained modest (median, 52.5 SFU/million PBMC at FFM+7), and when comparing the responses obtained for the time point (FFM+7) for both groups, where vaccines were given as i.d. injections (groups 1 and 2; 4-week versus 8-week interval between the second and third vaccine injections), no significant difference was seen.

To evaluate the effect of the dose of the boosting vaccine, IFN- γ responses to CS peptides obtained for the key time point, FFM+7, after boosting with MVA-CS at either 1×10^8 PFU (first trial, group 6 or 7) or 5×10^8 PFU (second trial, groups 1 to 3) were compared. Despite a fivefold increase in the dose of the boosting vaccine, the peptide-specific response increased only marginally (the median numbers of spot-forming units per million PBMC for groups 6 and 7 [VAC23] were 5 and 15, and those for groups 1 to 3 [VAC28] were 52.5, 34.4, and 30, respectively), with the difference becoming significant only for the comparison of group 6 versus group 1 ($P = 0.0001$) or group 2 ($P = 0.005$).

VAC23 sporozoite challenge. Two weeks after completion of the vaccine regimen, 15 of 16 subjects vaccinated in groups 6 and 7 of the first trial were challenged with *P. falciparum* sporozoites alongside six malaria-naïve, nonvaccinated controls. No significant difference in time to parasitemia was observed for either vaccine regimen compared to controls.

The mean time to parasitemia detected by slide reading was 12.2 days (95% CI, 11 to 13.4) for group 6, 11.4 days (95% CI, 10.2 to 12.5) for group 7, and 11.8 days (95% CI, 10.9 to 12.8) for controls. Kaplan-Meier analysis results are shown in Fig. 7. Taking a measure of PCR positivity as $>1,000$ parasites/ml, parasites were detected in group 6, on average, on day 11.1 (95% CI, 9.6 to 12.6), in group 7 on day 10.6 (95% CI, 9.5 to 11.6), and in controls on day 11.1 (95% CI, 9.8 to 12.4). Results of the Kaplan-Meier analysis by the log rank test were $P = 0.78$ (comparing group 6 to controls) and 0.58 (comparing group 7 to controls).

Antivector antibodies. To investigate if the lack of a boosting effect of the third vaccine observed in both regimens used in VAC23 was due to cross-reacting humoral antivector responses, sera from six subjects in group 6 and eight subjects in group 7 obtained on day 0, the day of the administration of the MVA vaccines (FF+28), and 2 weeks thereafter (FFM+14) were examined for anti-MVA antibodies. At FF+28, anti-MVA antibodies induced by the preceding vaccinations with FP9-vectored vaccines were similarly low in both groups (median endpoint titers: group 6, 140.5; group 7, 93.5; $P = 0.181$). Two weeks after the MVA vaccines were given, anti-MVA titers increased significantly to medians of 971 (group 6, $P = 0.028$) and 1,046 (group 7, $P = 0.012$), respectively. Taken together, these data indicate that priming with FP9 did not induce any relevant antivector immune response to MVA-vectored vaccines.

FIG. 7. Kaplan-Meier analysis for time to parasitemia based on microscopic slide reading. y axis, cumulative probability to remain slide negative; x axis, days after challenge. Group 6 (VAC23) received FFM CS, and group 7 (VAC23) received FFM CS plus FFM METRAP. Log rank test comparison to controls: group 6, $P = 0.82$; group 7, $P = 0.69$.

DISCUSSION

Recent work with humans has shown promising results for the use of heterologous prime-boost vaccination regimens for malaria vaccines. Priming with DNA encoding METRAP, followed by MVA-METRAP, resulted in unprecedented levels of immune responses and a significant delay in the time to patent parasitemia in a sporozoite challenge model (21). When FP9 was used as the priming vector instead of DNA in an FFM regimen, complete protection could be achieved with this antigen (43) in some individuals.

Currently, the most successful malaria vaccine candidate is RTS,S, a recombinant protein expressing the C-terminal half of the CS protein (6, 19). The rationale for the trials reported here was to combine vaccines by the successful FFM approach in an attempt to induce powerful T-cell responses to an apparently promising pre-erythrocytic antigen, the CS protein.

For both i.d. and i.m. administrations, at all doses, FP9- and MVA-vectored vaccines were generally safe and well tolerated, with no serious adverse events occurring. The safety data accrued during these trials further add to the good safety record of these vaccines (24, 25, 42). Here we describe for the first time their safety profile for i.m. administration and for doses of up to 5×10^8 PFU given i.d. and i.m.

No difference in the frequency, duration, or severity of adverse events was observed between the vaccines expressing different antigens (Fig. 2), adding support to the hypothesis that reactogenicity is mainly vector related. Overall, the quality and quantity of adverse events reported for FP9- and MVA-vectored vaccines were very similar too. As described previously (42), priming with FP9-based vaccines appears to attenuate the side effects of subsequent administration of FP9-based vaccines.

Not surprisingly, the fivefold increase in the dose of MVA-CS results in slightly more pronounced local reactogenicity after i.d. administration. Similarly, the frequency, but not the severity or duration, of generalized adverse events increases with the dose. Reassuringly, this increase in side effects is not proportionate to the increase in the dose and remained acceptable for all volunteers. The side effect profiles described here for 5×10^8 PFU, given i.d., are comparable to what has been reported for AIDS patients receiving a similar dose of recombinant MVA subcutaneously (10, 15).

In some cases, the duration of redness appears to be long after i.d. vaccination. However, irrespective of the dose, vaccine, or antigen, the acute inflammatory redness disappeared in all subjects within 4 to 7 days. Thereafter, a reddish discoloration at the injection site, not exceeding 2 to 3 mm, remained in some individuals for several weeks and accounts for the occasionally reported redness up to 28 days postvaccination.

Recombinant MVA or FP9 has been used safely as an i.m. injection in animal studies, including rhesus macaques (2, 32). However, to our knowledge, only recombinant MVA has been given as an i.m. injection in doses of up to 1×10^8 PFU to 13 tumor patients, by whom it was well tolerated (33). Here, both recombinant viral vectors were administered i.m., with MVA being given at a dose of 5×10^8 PFU. Injections were well tolerated and minimally reactogenic, apart from sporadic injection site pain. In comparison to the i.d. administration, slightly more subjects experienced systemic side effects after

i.m. vaccination, but these were all mild, mostly occurred within the first 48 h, and settled spontaneously. Importantly, neither the frequency nor the severity of systemic side effects increased after i.m. administration of a high dose of MVA-CS.

In the first trial (VAC23), vaccine-induced antigen-specific immune responses—as measured by IFN- γ ELISPOT assay—were disappointingly low; and the lack of a clear booster effect of the final vaccine was particularly surprising. To exclude the possibility of an assay failure, ELISPOT assays were repeated with frozen cells stimulated with newly reconstituted peptides, and cells from other studies with the same antigens were assayed on plates prepared for this trial. No systematic error could be detected, indicating that the results obtained were accurate. The magnitude of the immune response may explain the lack of protection observed for both regimens in the subsequent sporozoite challenge. Alternatively, the type of protective immune response required for CS is not generated by this vaccine concept.

No obvious reason for the unexpectedly low immune responses could be established. When retested after the trial, the immunogenicity of the vaccines assessed in a standardized murine system was similar, suggesting that loss of vaccine potency is very unlikely. FP9 did not induce significant anti-MVA antibodies, and ELISPOT assay responses to an HLA-A*0201-restricted T-cell epitope, conserved among vaccinia viruses (40), remained at the background level throughout the trial (data not shown). This argues against an important role of cross-reacting antivector responses induced by preceding vaccinations. Besides, results obtained with mice suggest that recombinant MVA can be used successfully even under conditions of preexisting immunity to the vector (29).

Given that in a previous trial FFM-METRAP, when used alone, induced a strong immune response after the final vaccine (41), translating into a significant delay in parasitemia in the sporozoite challenge, as well as full protection in some cases (43), the very weak immune responses to the TRAP-based vaccines seen here, when used at the same doses and time intervals but simultaneously with CS-based constructs, is not easy to explain. Antigenic competition seems unlikely, since vaccines were given i.d. at two different locations.

With regard to the CS-based construct, we have to conclude that, irrespective of the route of administration (i.d. or i.m.), the dose of the boosting vaccine, or the time interval between the second and third administrations of vaccine, the heterologous prime-boost approach with FFM failed to induce strong cellular immune responses. This could relate to the observation that people naturally exposed to malaria frequently do have rather weak cellular responses to CS despite many years of exposure (reviewed in reference 39), suggesting a low immunogenicity of this antigen. There is some limited evidence that CD4⁺ T-cell responses to CS epitopes are associated with a degree of protective immunity (18, 31) and that polymorphism of CD4⁺ T-cell epitopes might be driven by immune selection (14). An association between protection and CD4⁺ T cells, detected in a cultured ELISPOT assay, to exactly these epitopes has been confirmed recently in a very similar study (30), supporting a role for fairly low-level resting memory T-cell responses to CS in conferring immunity against the pre-erythrocytic stage of the infection.

Alternatively, the possible impact of the glycosylphosphati-

dylinositol (GPI) anchor at the N-terminal end of the CS protein on antigen processing could provide an explanation for the low immunogenicity observed in these studies. The recombinant viral vector needs to infect a human cell so that the encoded antigen can be produced and expressed by this cell. During assembly of the nascent protein within internal cell organelles, the GPI anchor needs to be added by a highly species-specific transamidase. Differences between the plasmodial and mammalian GPI signal sequences make the CS protein an inferior substrate for the mammalian transamidase. As a result, the protein may be retained within the mammalian cell and not expressed on the cell surface (26). Indeed, in the murine model, removal of the GPI signal sequence from CS-encoding DNA and adenovirus-vectored vaccines resulted in enhanced CS-specific immune responses and improved protection against a sporozoite challenge (7, 36).

In conclusion, our data suggest that i.m. injection of both FP9 and MVA vaccines is equally well tolerated as i.d. administration. For MVA-CS, this was demonstrated for doses of up to 5×10^8 PFU. Assuming that both routes would induce similar levels of immunogenicity, the i.m. route would be advantageous under field conditions.

The heterologous prime-boost regimen that induced high levels of immunogenicity and some protection (21, 43) led to lower immunogenicity with the current CS vaccine constructs encoding the full-length CS protein with a GPI anchor. Removal of the GPI sequence might provide the means to enhance the immunogenicity of DNA-based or virus-vectored vaccines encoding the CS protein.

ACKNOWLEDGMENTS

We are grateful to all of the volunteers who participated in these studies, to Trudie Lang for managerial support, and to Zia Sherrell for help with the database and compiling study reports.

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Editor: W. A. Petri, Jr.