MVI Annual Report to USAID

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Acronyms and abbreviations

ADCI	antibody-dependent cellular inhibition
CEF	chicken embryo fibroblasts
CFA	Complete Freund's Adjuvant
CRADA	cooperative research and development agreement
CS	circumsporozoite
CSP	circumsporozoite protein
DRC	Democratic Republic of Congo
EBA	erythrocyte-binding antigen
E. coli	*Escherichia coli*
ENV	envelope
EVI	European Vaccine Initiative
FDA	US Food and Drug Administration
FY	Fiscal Year
GIA	growth-inhibition assay
GLA-SE	glucopyranosyl lipid adjuvant-stable emulsion
GLURP	glutamate-rich protein
HIV	human immunodeficiency virus
ICGEB	International Centre for Genetic Engineering and Biotechnology
ICS	intracellular cytokine staining
IFN-g (or γ)	interferon-gamma
IgG	immunoglobulin G
IND	Investigational New Drug
ME-TRAP	multi-epitope thrombospondin-related adhesive protein
MVA	modified vaccinia Ankara
MVI	Malaria Vaccine Initiative
NIAID	US National Institute of Allergy and Infectious Diseases
NIBSC	National Institute for Biological Standards and Control
NIH	US National Institutes of Health
PATH	Program for Appropriate Technology in Health
P. berghei	*Plasmodium berghei*
PBMC	peripheral blood mononuclear cell
P. falciparum/Pf	*Plasmodium falciparum*
*Pf*Ripr	*P. falciparum* Rh5 interacting protein
pLDH	parasite lactate dehydrogenase
PNG	Papua New Guinea
REC	Research Ethics Committee
Rh	Rhesus
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Seattle BioMed	Seattle Biomedical Research Institute
SMC	Safety Monitoring Committee
SNP	single nucleotide polymorphism
TAC	Technical Advisory Committee (Optimalvac)
TAG	technical advisory group
TB	tuberculosis
TNF-α	tumor necrosis factor-alpha
USAID	US Agency for International Development

USMMVP	US Military Malaria Vaccine Program
vp	virus particles
WEHI	Walter and Eliza Hall Institute of Medical Research
WIRB	Western Institutional Review Board
WRAIR	Walter Reed Army Institute of Research

Executive summary

The PATH Malaria Vaccine Initiative (MVI) carried out the activities described in this report from October 1, 2011, through September 30, 2012. The cooperative agreement between MVI and the US Agency for International Development (USAID) was signed on October 8, 2004. On September 17, 2008, a cost extension was granted, which extended the term to September 16, 2013, and increased the ceiling from $8 million to $23 million.

During Fiscal Year (FY) 2012, USAID funds supported science and technical activities and policy and commercialization activities. The highlights of each are summarized below.

Science and technical highlights

Phase 1/2a clinical trial of Crucell's Ad35.CS.01/Ad26.CS.01. This project tested the ability of Crucell's adenovirus-based vaccine platform to induce humoral and cellular immune responses, directed at circumsporozoite protein (CSP), and evaluated protective efficacy in the controlled challenge model. It was the first-in-human administration of Ad26.CS and Ad35.CS in a heterologous prime-boost study. A total of 17 volunteers completed the immunization and challenge phase. Only one challenged vaccinee was parasitemia negative at the end of the observation period. There was no delay to parasitemia in the other 16 subjects. All six control subjects exhibited blood-stage parasitemia, confirming the robustness of the challenge. Based on agreed-upon MVI Go/No-go criteria, a No-go decision was reached for this approach.

Oxford VAC045 trial. This was the first of three trials in a joint effort between Oxford University and the US Military Malaria Vaccine Program (USMMVP) that aimed to assess which components of two vaccine platforms are most effective and whether the two platforms can be combined to enhance vaccine efficacy. The two programs also examined whether combinations of individual malaria vaccine antigens can enhance vaccine efficacy, in either an additive or synergistic fashion. Groups of 15 volunteers were immunized with ChAd63 expressing ME-TRAP (Group 1) or CSP (Group 2) antigen and boosted two months later with the same antigens delivered in a modified vaccinia Ankara (MVA) vector. Following challenge, two of 15 volunteers in Group 1 and one of 15 volunteers in Group 2 were parasitemia negative. All six control subjects exhibited blood-stage parasitemia, confirming the robustness of the challenge. MVI and USAID decided that the level of protective efficacy justified a Go to proceed with the second Oxford study. Preparations are underway for that trial and for one at USMMVP.

Addressing AMA1 polymorphism and optimizing AMA1 as a vaccine candidate/component. A consortium of three preclinical projects addressed the feasibility of overcoming the challenge of polymorphism of AMA1, by developing either a multivalent vaccine or an engineered vaccine that can induce highly cross-reactive responses against diverse parasite isolates.

- **Burnet Institute.** Preclinical results from an AMA1 project with the Burnet Institute provide evidence that a multi-allele vaccine that provides broad coverage of circulating parasite strains may be achievable, and a combination of three or four alleles may provide sufficient coverage. However, identifying the optimal combination of alleles that will maximize the synergistic and additive potential of cross-reactive antibodies is now a near-term challenge. Further work in this area is warranted to confirm whether a three-allele combination will be sufficient, or if a four-allele combination is required to cover the global diversity of AMA1.

- **Walter Reed Army Institute of Research (WRAIR).** WRAIR investigators proposed that by systematically mapping the broadly inhibitory epitopes on AMA1, it may be possible to rationally design a strain-transcendent vaccine. As a first step toward this goal, a prototype quadrivalent vaccine referred to as "Quadvax" was prepared using an equimolar mixture of four laboratory strains representing 3D7, FVO, W2mef, and HB3 alleles. Quadvax antibodies were tested against a total of 26 strains (23 non-vaccine strains) representative of the global diversity of AMA1, and none were found to be refractory to antibody-mediated inhibition of parasite invasion. WRAIR also performed ELISAs and invasion inhibition reversal assays using their panel of chimeric proteins displaying specific surface residues of AMA1 and demonstrated that Quadvax immunization-induced antibodies directed at conserved regions of the molecule as compared with a mono-allelic 3D7 vaccine.

EBA-175 R3-5 rabbit immunization studies with antigen combinations. This is an ongoing preclinical feasibility study with the Walter and Eliza Hall Institute of Medical Research aimed at selecting antigens from the *P. falciparum* reticulocyte binding-like (*Pf*Rh) family that elicit the highest growth-inhibition assay activity against the broadest range of parasite strains following immunization in combination with EBA-175. The goal is to select the minimum number of antigens that are needed in combination for development as a vaccine. Results from this past year showed that the combination that elicited the highest growth inhibition was EBA-175 R3-5 + *P. falciparum* Rh5 interacting protein (*Pf*Ripr) C-terminal at 2 mg/mL of total immunoglobulin G; this combination also showed the lowest IC_{50}. Based on these data, the project team made a decision to deprioritize *Pf*Rh2 for process development work at Gennova Biopharmaceuticals, in favor of EBA-175 R3-5 and *Pf*Ripr C-terminal and *Pf*Rh5. However, antibodies raised to *Pf*Ripr C-terminal alone were only weakly inhibitory, suggesting that EBA-175 R3-5 alone may be as good as the best combination. Based on these data, efforts at Gennova are now focusing on EBA-175 R3-5 and work is expected to begin in Q4 2012 on *Pf*Rh5, codon harmonized for optimal expression in *E. coli*.

Optimalvac. Antibody-dependent cellular inhibition (ADCI) and T cell assay testing took place in the final project year, in which the participating laboratories tested a panel of standard reagents using in-house protocols. For ADCI, initial testing of standard reagents highlighted several notable issues, including the importance of the method of staining (i.e., double staining) and that any parasite strain showing robust growth characteristics could be used in ADCI, not just Wellcome and 3D7. For the T cell assays, good consistency of results was observed in IFN-g ELISpot data, but a great deal of variability, some of which was due to gating strategy, was observed in intracellular cytokine staining testing.

Policy and access highlights

Malaria vaccine decision-making framework. With support from USAID and other sources, MVI has worked with national authorities and partners to organize briefing and consultation meetings to share information on progress on malaria vaccine development and to discuss processes for planning ahead of a policy decision. Highlights of 2012 include:

- A meeting held with Kenya, Tanzania, and Uganda officials in February to draft the terms of reference and objectives of the East Africa network of technical advisory groups (TAGs) for the RTS,S decision-making framework, which will be officially endorsed at the stakeholders meeting in November.
- A first report to stakeholders prepared by the Ghana and Tanzania TAGs, which will be disseminated in November.

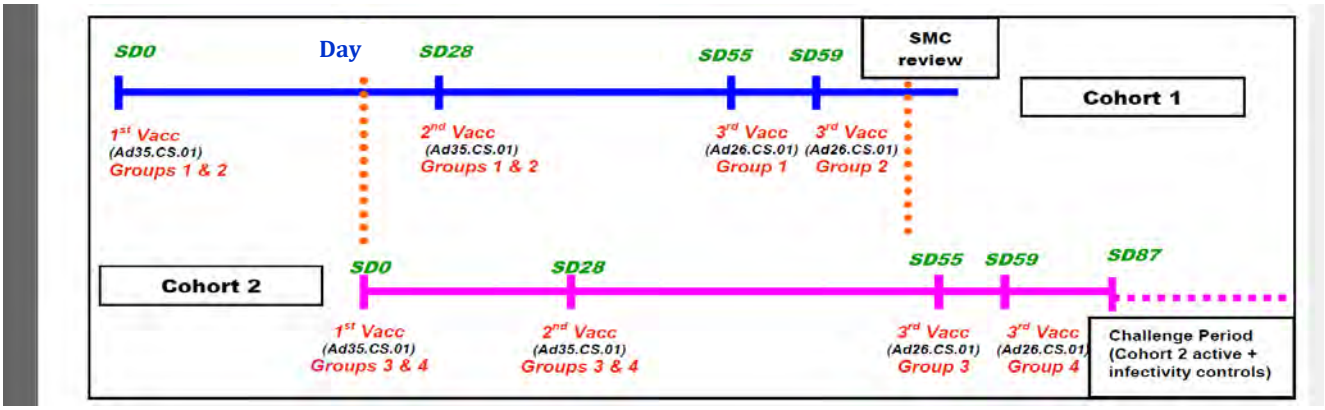
Science and technical

Crucell's Ad35.CS.01/Ad26.CS.01 program

The Ad35.CS.01/Ad26.CS.01 project is a collaboration involving Crucell, the US Agency for International Development (USAID), and the PATH Malaria Vaccine Initiative (MVI) to test Crucell's adenovirus-based vaccine platform. Crucell's strategy was to use two adenovirus serotypes, Ad35 and Ad26, expressing a codon-optimized *P. falciparum* gene encoding the circumsporozoite (CS) surface antigen in a heterologous prime-boost vaccine regimen. Ad35 and Ad26 have a lower seroprevalence in the general population compared to Ad5 and thus could potentially represent a more effective vaccine. Figure 1 illustrates the study design.

USAID funding—along with that of the Bill & Melinda Gates Foundation—was used to support Crucell's activities as sponsor of the clinical study entitled "MAL-V-A001 Safety and Efficacy of the Ad35.CS.01/Ad26.CS.01 Malaria Vaccine." This included regulatory oversight, trial support, and immunological assays. USAID funding was also used to support stability testing of Ad35.CS (CTM2) and Ad26.CS drug products. Non-USAID funding was used to support stability testing of current Good Manufacturing Practice-grade Ad35.CS (CTM4) drug product, the clinical trial activities, and the Walter Reed Army Institute of Research (WRAIR) anti-CS ELISA Reference Laboratory.

Figure 1. Study design.



The study was completed in May 2012 when the last follow-up phone call was conducted, although formal study closeout is not expected until early 2013. Subjects in Cohort 1 received two doses of Ad35.CS and one dose of Ad26.CS, each at $1x10^{10}$ virus particles. In Cohort 2, the vectors were delivered at a dose of 5×10^{10} virus particles. The high dose of Ad26.CS was not administered until the Safety Monitoring Committee (SMC) reviewed all safety data collected from the low-dose cohort and recommended proceeding with the dose escalation. In general, the vaccines were well tolerated, but there were three occasions when the study was stopped briefly after a holding rule—two grade 3 adverse events—was met. On each occasion, the study was resumed within 24 hours, following a review by the SMC. After review of the safety data following the third vaccination, the SMC recommended to proceed with challenge, with one Cohort 2 subject held pending evaluation for reported new-onset exertional dyspnea. This subject was subsequently withdrawn from the study due to exercise intolerance, at the sponsor's request. Two other volunteers were withdrawn from the study after receiving two vaccinations of Ad35.CS high dose. One of them experienced one grade 3 event after each vaccination; also, events in the subject's personal life were preventing this

person from continuing in the study. The other individual was deemed unreliable. Neurological adverse events, which were of concern following the US National Institutes of Health (NIH) Ad35.CS trial (ClinicalTrials.gov Identifier: NCT00371189), were not seen in the Ad35/Ad26 trial, and the first-in-human administration of Ad26.CS showed no significant safety concerns.

Volunteers in Cohort 2 were challenged with *P. falciparum* approximately four weeks after receiving the Ad26.CS boost. Six unimmunized control subjects were challenged at the same time. This was the first challenge study involving vaccinated volunteers conducted at Seattle Biomedical Research Institute's (Seattle BioMed) Malaria Clinical Trials Center. The challenge model at Seattle BioMed was validated in all six control subjects, who became infected between days 9 and 12 after challenge. Out of 17 vaccinees who proceeded to challenge, only one subject was parasitemia negative at the end of the observation period. There was no delay to parasitemia in the other 16 subjects, who had positive blood smears between days 9 and 13 after challenge.

Immunological assessments for antibody and T cell responses to circumsporozoite protein (CSP) were completed in 2012. The anti-CS antibody responses were comparable with the NIH Ad35.CS Phase 1 trial, in which dose levels of Ad35.CS of 1×10^9, 1×10^{10}, and 1×10^{11} were administered on a 0, 1, 6 month schedule, and no significant T cell responses were observed as measured by ELISpot. The subject who remained parasitemia negative was the only subject with CS-specific CD8 T cell responses measured by intracellular cytokine staining (ICS). The Ad35 and Ad26 neutralizing antibody levels observed in this trial were comparable to the titers observed with Ad35.TB, Ad35.HIV, and Ad26.HIV in other clinical trials, suggesting there were no problems with the immunogenicity of these vaccine lots.

On March 16, 2012, the US Food and Drug Administration (FDA) placed the study on clinical hold following Crucell's submission of a Chemistry, Manufacturing, and Controls Amendment informing the FDA of ongoing investigations into the identity, origin, and potential clinical impact of visible particles that were observed during the stability testing program for Ad35.CS and Ad26.CS drug products (at the 12- and 24-month time points, respectively). The investigation for Ad35.CS and Ad26.CS concluded that the visible particles (mainly cellulose), which were observed during stability testing, were introduced during the filling process at the contract manufacturer SAFC Pharma (formerly Molecular Medicine Bioservices, Carlsbad, CA, USA). The products were considered to be within the specification of "practically free from visible particles," and passed all other tests, including potency. All trial volunteers were notified by Seattle BioMed of the Ad35.CS/Ad26.CS-related clinical hold.

The final study visits (phone call) were conducted on May 19 and May 21, 2012. An ad hoc SMC meeting was held at the request of the principal investigator for the purpose of informing the SMC about the issue with the vaccine and the clinical hold. Crucell conducted a site closeout visit on September 11–12, 2012; however, the regulatory file will remain open until the clinical hold is lifted by the FDA (target date: January 2013). The study closure report will be submitted to the institutional review board once the hold is lifted.

A clinical hold was also placed on other studies in which Crucell's adenoviral products were used (Ad35.CS and Ad26.HIV ENV). On August 8, 2012, Crucell submitted their response to the clinical hold letter to the FDA. The response included investigation reports for all affected products (Ad35.CS, Ad26.CS, and Ad26.HIV ENV). To date, the hold has been lifted for all Investigational New Drugs (INDs) related to Crucell's products for which 100 percent visual inspection of vials has been performed (Ad35.CS and Ad26.HIV ENV). The release of the clinical hold for the Ad35/Ad26 study will be delayed because Crucell deferred 100 percent visual inspection of Ad26.CS until early

October 2012. Following the inspection, the vials with particulates will be sent for identification and the investigation report will be updated to include data from the inspection. Crucell plans to make another submission to the FDA once the investigation report and batch disposition for Ad26.CS product have been completed (target date: November/December 2012), in an effort to lift the hold from the Ad35.CS/Ad26.CS study.

Given Crucell's decision to postpone 100 percent visual inspection of Ad26.CS, which resulted in submission of an incomplete package to the FDA, MVI informed Crucell that any cost associated with the consequent delay in study closure will be Crucell's sole responsibility. This was formalized in an amendment to the clinical trial agreement, which is currently under review with Crucell.

Following 100 percent visual inspection of Ad35.CS, a new lot number was assigned to the product, and no further stability studies of this product are anticipated. The same will apply to Ad26.CS product once the visual inspection is completed.

Due to the shift of approximately six months in clinical trial program timelines, the remaining project milestones and deliverables were adjusted in December 2011and are reflected in Table 1, which outlines the status of this project. Based on the predefined and agreed-upon MVI Go/No-go criteria (No-go: <30 percent efficacy), a No-go decision was reached for this approach and it will not be supported further.

Table 1. Status of Crucell's Ad35.CS.01/Ad26.CS.01 program.

Activity	Deliverable	Status
Project decision	• Go/No-go based on clinical trial results	Completed Q4 2011
Conduct Phase 1/2a clinical study	• Interim report on clinical trial immunological analyses • Data management report, timelines/ milestones met to date • Monitoring update to include summary of interim monitoring reports submitted within the reporting period	Completed Q1 2012
Finance	• Interim financial report	Completed Q3 2012
IND for Ad35.CS.01/ Ad26.CS.01 clinical trial	• Copy of the IND first annual report to the FDA	Completed Q3 2012
Conduct Phase 1/2a clinical study	• Copy of the final closeout report from the study monitor • Final report on the clinical trial immunological analyses	Completed Q3 2012
Stability	• Stability data reports on Ad35.CS and Ad26.CS	Expected Q4 2012
Conduct Phase 1/2a clinical study	• Copy of the final clinical study report • Final statistical report • Final locked database	Expected Q4 2012/ Q1 2013
Finance	• Interim financial report	Expected Q4 2012

Oxford-USMMVP Phase 1/2a clinical trials

The Oxford-USMMVP project is a joint effort between Oxford University and the US Military Malaria Vaccine Program (USMMVP) to test and compare their vaccine platforms, both of which have

demonstrated modest protection against controlled challenge with *P. falciparum* in Phase 1/2a clinical trials. The two laboratories use heterologous prime-boost vaccination approaches in which one of the components is an adenovirus vector. The other component is either Oxford's modified vaccinia Ankara (MVA) or USMMVP's plasmid DNA. The ongoing and planned clinical trials aim to assess which components of the two vaccination platforms are most effective and whether they can be combined to enhance vaccine efficacy. They also examine whether combinations of individual malaria vaccine antigens can enhance vaccine efficacy, in either an additive or synergistic fashion.

The trials will be conducted in parallel at Oxford and USMMVP, and have been designed to address the following questions:

1. Can protection using Ad-MVA ME-TRAP be enhanced by:
   a. Substituting CSP for ME-TRAP? *(Oxford Trial 1)*
   b. Combining ME-TRAP and CSP; or ME-TRAP, CSP, and AMA1? *(Oxford Trial 2)*
2. Can protection using DNA-Ad CSP+AMA1 be enhanced by adding vectors encoding TRAP? *(USMMVP)*
3. Can ChAd63 be substituted for HuAd5 in the protective DNA/Ad regimen (CSP+AMA1)? *(USMMVP)*

Oxford trial 1: VAC045

Preparation of the VAC045 study protocol was underway at Oxford when MVI approved the project at the end of January 2012. MVI worked with USAID and Oxford to finalize the protocol and it was submitted to the Oxford Research Ethics Committee (REC) and Western Institutional Review Board (WIRB) for ethics review in mid-February. During the same period, efforts were initiated to prepare a clinical trial agreement and budget. Immunization of volunteers was initiated in late April 2012, following ethics committee approval, UK regulatory approval, approval of the budget by MVI, and execution of the clinical trial agreement. Volunteers received their boosting vaccination in June and were challenged with malaria parasites in July. Efficacy results were received in early August.

The design of the VAC045 trial is shown in Table 2. Groups of 15 volunteers were immunized with ChAd63 expressing ME-TRAP (Group 1) or CSP (Group 2) antigen and boosted two months later with the same antigens delivered in an MVA vector. All volunteers completed the full vaccination regimen. A variety of local and systemic adverse events was noted following vaccination, particularly following the MVA boost. While some of these were rated as severe, the investigator felt them to be in line with reactogenicity experienced in previous studies. There were no serious adverse events following vaccination. Volunteers were challenged by bites of *P. falciparum*-infected mosquitoes three weeks after boosting. An additional six unimmunized volunteers were challenged at the same time. Volunteers were then monitored for malaria infection over the next three weeks. By the end of the three-week period, all six unimmunized volunteers had tested positive for malaria infection, while in Group 1, 13 of 15 volunteers were positive and in Group 2, 14 of 15 were positive. The clinical site noted that one of the two protected volunteers in Group 1 had received the boosting vaccination late, resulting in a shortened interval of two weeks between boosting and challenge. Fresh-cell ELISpot results indicated that T cell responses were observed in both groups at levels consistent with previous studies. Further T cell and antibody analyses are pending.

The Go/No-go criteria, defined before efficacy results were received, specified that the results should be discussed to determine how to proceed. MVI and USAID decided subsequently that the level of protective efficacy justified a Go to proceed with the second Oxford study.

Table 2. Design of the VAC045 trial.

Group	N	Prime (Week 0)	Boost (Week 8)
1	15	ChAd63 ME-TRAP (5×10^{10} vp)	MVA ME-TRAP (2×10^{8} pfu)
2	15	ChAd63 CSP (5×10^{10} vp)	MVA CSP (2×10^{8} pfu)

Oxford trial 2: VAC052

Preparation for VAC052 is in progress. The clinical protocol has been submitted to the Oxford REC and WIRB for ethics review, and a clinical trial agreement is being drafted. A toxicology study, necessitated by the use of novel combinations of vectors, was conducted in mice at Huntingdon Life Sciences under the VAC045 agreement. No unusual observations were reported during the in-life phase of the study. Histopathological analysis is underway and a final report is expected in October.

The design of VAC052 is shown in Table 3. The trial will test the same prime-boost platform and schedule as VAC045, with volunteers being primed with ChAd63 adenovirus vector and boosted with MVA vector. VAC052 volunteers will receive two or three antigens in combination: ME-TRAP+CSP (Group 1) or ME-TRAP+CSP+AMA1 (Group 2). Go/No-go criteria will be set prior to challenge to determine whether this vaccination platform would be of further interest from either an experimental medicine or product development perspective.

Table 3. Design of the VAC052 trial.

Group	N	Prime (Week 0)	Boost (Week 8)
1	15	ChAd63 ME-TRAP+CSP (5×10^{10} vp/construct; 1×10^{11} vp total dose)	MVA ME-TRAP+CSP (2×10^{8} pfu/construct; 4×10^{8} pfu total dose)
2	15	ChAd63 ME-TRAP+CSP+AMA1 (5×10^{10} vp/construct; 1.5×10^{11} vp total dose)	MVA ME-TRAP+CSP+AMA1 (1.33×10^{8} pfu/construct; 4×10^{8} pfu total dose)

USMMVP trial

In the USMMVP trial, volunteers will be primed with three administrations of plasmid DNA mixtures encoding two or three antigens, and will be boosted with adenovirus expressing the same antigens. For this study, new clinical batches of DNA are required and will be produced by a new manufacturer using a modified process. Bridging studies will be needed to demonstrate equivalency of DNA produced by the old and new methods; results will be submitted to the FDA under an existing IND application. A new IND will be necessary for the use of ChAd63 vectors. A repeat-dose toxicology study in rabbits will be performed to demonstrate the safety of different DNA plasmids and adenovirus vectors delivered in combination.

The proposed study design is shown in Table 4. Group 1 volunteers will be primed with DNA plasmids encoding CSP and AMA1 and boosted with HuAd5 virus vector expressing the same antigens. Group 2 will use the same plasmids for priming, but boosting will be done with ChAd63 expressing CSP and AMA1. In Group 3, a third antigen, TRAP, will be included in both the priming and booster doses. Group 1 will include an additional ten volunteers who are seropositive for HuAd5 neutralizing antibody to test the question of whether pre-existing anti-vector neutralizing antibody interferes with the ability of the HuAd5 vectors to stimulate immunity against CSP or AMA1. USMMVP will pay the costs associated with the additional Group 1 volunteers with separate funding. Volunteers will be challenged by bites of *P. falciparum*-infected mosquitoes four weeks after boosting.

The manufacture of new DNA is not funded under this project. However, the project will include preclinical mouse studies to test the immunogenicity of the new DNA preparations and Oxford's ChAd63 vectors. To this end, a cooperative research and development agreement (CRADA) among USMMVP, Oxford, and MVI is being developed for preclinical activities, including the bridging study described above, a confirmatory immunogenicity study in mice, and a general safety test. The preclinical CRADA will also cover preparation of a pre-IND meeting package and a revised Chemistry, Manufacturing, and Controls section for the existing IND, and preparation and submission of the new IND.

A second CRADA, which will also serve as the clinical trial agreement, is in the drafting stage. The CRADA will cover conduct of the study and the parties will be USMMVP, Oxford, the US Army Medical Materiel Development Activity (trial sponsor), WRAIR (challenge site), and MVI.

Table 4. Design of the USMMVP trial.

Group	N	Prime (Weeks 0, 4, 8)	Boost (Week 24)
1	30	DNA CSP+AMA1 (1 mg/construct)	HuAd5 CSP+AMA1 (1×10^{10} vp/construct)
2	20	DNA CSP+AMA1 (1 mg/construct)	ChAd63 CSP+AMA1 (5×10^{10} vp/construct)
3	20	DNA CSP+AMA1+TRAP (1 mg/construct)	ChAd63 CSP+AMA1+ ME-TRAP (5×10^{10} vp/construct)

Immunomonitoring harmonization

In order to best compare immune responses from the three trials, MVI and its project partners are drafting a harmonized immunomonitoring plan that seeks to ensure that assays performed in different laboratories use methods and reagents that are as similar as they are practical. The plan also provides that serum samples be submitted for testing at the ELISA Reference Center at WRAIR and that peripheral blood mononuclear cells (PBMCs) be submitted for testing at the International AIDS Vaccine Initiative T cell Reference Center at Imperial College. The immunomonitoring plan will be appended to the VAC052 clinical trial agreement and to both CRADAs for the USMMVP trial.

USAID funding has been used to support all of Oxford's clinical trial activities beginning at the start of VAC045 immunization, including conduct of the study, regulatory oversight, immunological activities, study monitoring, and report preparation. USAID funding will be used for similar activities for the VAC052 and USMMVP trials (with the exception of costs associated with the additional USMMVP Group 1 seropositive volunteers, for which USMMVP will use separate funding). USAID funding will also be used for USMMVP pre-trial animal studies, the toxicology study, and regulatory activities. Funding from the Gates Foundation was used for Oxford's activities prior to the start of the immunization. This included preparation of the study protocol, consent forms, and other clinical documents; preparation of clinical documents for submission to WIRB; advertising for volunteers; and participation in clinical site assessments and audits of vaccine manufacturers. Gates Foundation funding is also used to support the activities of the ELISA and T cell reference laboratories. Table 5 summarizes the status of the activities and deliverables of the Oxford-USMMVP clinical trials.

Table 5. Status of the Oxford-USMMVP Phase 1/2a clinical trials.

Activity	Deliverable	Status
Intra-MVI/USAID discussions of TAG recommendations	USAID/MVI approval to proceed with negotiations, regulatory approvals	Completed Q4 2011
Regulatory submissions, VAC045	Regulatory/Ethical approval by UK Medicines and Health Care Products Regulatory Agency, Oxford REC, and WIRB	Completed Q1 2012
VAC045 clinical trial agreement	Clinical trial agreement signed by Oxford and MVI	Completed Q1 2012
Trial initiation, VAC045	First immunization	Completed Q2 2012
Volunteer challenge, VAC045	Initial efficacy data	Completed Q3 2012
Decision on whether to proceed to second Oxford study	Go/No-go decision	Completed – Go decision Q3 2012
Harmonization of immunological assays	Finalized harmonized immunomonitoring plan	Expected November 2012
Regulatory submissions, VAC052	Regulatory/Ethical approval by UK Medicines and Health Care Products Regulatory Agency, Oxford REC, and WIRB	Expected Q4 2012
Preclinical CRADA with USMMVP	Executed CRADA	Expected Q4 2012

TAG: technical advisory group.

Addressing AMA1 polymorphism and optimizing AMA1 as a vaccine candidate/component

The consortium of three preclinical projects addressing the feasibility of overcoming the challenge of polymorphism of AMA1 by developing either a multivalent vaccine or an engineered vaccine that can induce highly cross-reactive responses against diverse parasite isolates came to a close in late 2011/early 2012. The major outcomes and conclusions from these collaborative projects, which

were entirely supported using USAID funds, were presented at a meeting in December 2011. The passage below summarizes the highlights of these collaborative projects.

1. Burnet Institute: Toward the development of a multi-allele AMA1 vaccine

A mixture of rabbit antibodies (2 mg/mL) raised to monovalent vaccines of the 3D7, FVO, W2mef, and HB3 alleles inhibited 18 genetically diverse parasite isolates in a two-cycle invasion assay. All parasite isolates tested were genotyped and sequenced.

- Multi-allele competition ELISAs were used as a tool to assess the level of cross-reactivity among alleles, and to assess whether additive or synergistic effects could result by combining different alleles. More than 20 different allelic combinations were tested using serum from naturally exposed individuals from Papua New Guinea (PNG), and two combinations were identified that provided a high level of cross-reactivity of 11 different alleles; the combination of 3D7+W2mef+HB3 and D10+W2mef+FVO provided 92-100 percent and 97-100 percent cross-reactivity respectively against the panel of 11 alleles tested.
- Transgenic parasite lines were generated by transfecting W2mef with six diverse AMA1 alleles (3D7, FVO, W2mef, HB3, *Pf*2006, and XIE). These transgenic parasites were then tested in growth-inhibition assays (GIAs) with serum antibodies from 100 adults and children from PNG. Results showed that AMA1 is clearly a prominent target of inhibitory antibodies, and antibodies to 3D7, FVO, and W2mef were the most prevalent in this population.
- To identify residues important for immune escape in GIA, specific mutations of known polymorphic residues were introduced by transfection; these mutant parasites were then tested for escape of inhibitory antibodies using human, rabbit, and monoclonal antibodies. GIAs performed using these mutants suggested that major changes in residues are needed for antigenic escape to occur. The importance of the C1L region that consists of five polymorphic residues was evaluated in 3D7 and W2mef parasite lines. This analysis revealed that this region accounts for 25-30 percent of inhibitory antibody activity in 3D7, and one polymorphic residue appears important for immune escape in the W2mef line. Overall, some sequence differences between alleles are unlikely to be significant for vaccine escape, but a few play a key role. Defining the key residues that mediate immune escape using mutational strategies like this will be beneficial for informing vaccine design.
- Drawing on collective data from GIA analyses using rabbit and human antibodies, and competition ELISAs using mice and naturally exposed adults and children from PNG and Africa, the Burnet investigators built a model to depict the level of antigenic relatedness and differences between AMA1 alleles. This analysis indicated that 3D7 and FVO are distinct, with almost no overlapping epitopes; and therefore, both should be considered for inclusion in a multivalent vaccine. Also, a substantial overlap appears to exist between W2mef and 7G8, and also between FVO and 7G8, which suggests that epitopes of 7G8 may be covered by W2mef and FVO.
- The collective results from this project provide evidence that a multi-allele vaccine that provides broad coverage of circulating parasite strains may be achievable, and a combination of three or four alleles may provide sufficient coverage. However, identifying the optimal combination of alleles that will maximize the synergistic and additive potential of cross-reactive antibodies is now a near-term challenge. Further work in this area is warranted to confirm whether a three-allele combination will be sufficient, or if a four-allele combination is required to cover the global diversity of AMA1.

2. WRAIR: Broadly cross-reactive inhibition induced by a multivalent AMA1 vaccine

The AMA1 crystal structure revealed that polymorphisms on AMA1 tend to form clusters on the three-dimensional surface, and, importantly, there are regions on AMA1 that are highly conserved. Using this information, the WRAIR investigators proposed that by systematically mapping the broadly inhibitory epitopes on AMA1, it may be possible to rationally design a strain-transcendent vaccine. As a first step toward this goal, a prototype quadrivalent vaccine referred to as "Quadvax" was prepared using an equimolar mixture of four laboratory strains representing 3D7, FVO, W2mef, and HB3 alleles.

- The distant relatedness of the four Quadvax alleles was confirmed by CLUSTAL analysis of ten polymorphic residues within the C1 cluster using 229 different AMA1 sequences (polymorphic residues within the C1 cluster have been identified as the most important escape residues on AMA1). Results of this analysis were consistent with the recommendations regarding selection of alleles made by Burnet (described above).
- Quadvax immunogens were prepared by expressing (*E. coli*) and purifying AMA1 antigens from each strain. Rabbits were immunized with an admixture of equivalent quantities of each of the four alleles (25 µg each protein). Four groups of control animals received 25 µg doses of the monovalent AMA1 proteins included in Quadvax. The adjuvant used in all groups was Montanide ISA720.
    - Results of the rabbit immunization studies showed that each of the four monovalent alleles elicited a very characteristic strain-specific immune response. The mean invasion inhibition of all homologous strains was significantly higher than the mean percent invasion inhibition of all eight (heterologous) strains tested at WRAIR using a 20 percent whole serum invasion assay.
    - Quadvax-immunized rabbits, however, showed a similar high level of inhibition against all strains, including four that were not included in the vaccine.
    - To further investigate apparent synergies in co-immunization with multiple alleles, a pool of serum was prepared from animals raised to each monovalent vaccine. When this mixture of serum antibodies was tested against the same eight strains, the mean inhibition was significantly lower than that of the Quadvax-immunized rabbits ($P<0.006$), suggesting that with Quadvax immunization, the immune response generates antibodies that are more broadly cross-reactive.
- To further evaluate the breadth of the immune response elicited by the Quadvax, serum samples were sent to the GIA Reference Laboratory at the US National Institute of Allergy and Infectious Diseases (NIAID) and also to collaborators at Burnet for GIA testing against additional parasite isolates. The NIAID purified immunoglobulin G (IgG) from the sera and tested samples from Quadvax-immunized rabbits and rabbits that were given a bivalent 3D7+FVO vaccine against ten parasite strains, five of which were recent field isolates, in a one-cycle pLDH GIA. Only two of the ten strains tested by the NIAID were present in the Quadvax. Using 2.5 and 1.25 mg/mL concentrations of anti-Quadvax total IgG, the mean percent invasion inhibition against the panel of ten strains was 95 percent and 82 percent, respectively. At Burnet, purified IgG at 1 mg/mL was tested using a two-cycle invasion assay against an additional ten parasite isolates, none of which were in the Quadvax. Mean invasion inhibition against these isolates was 80 percent. At both laboratories, anti-Quadvax IgG showed a significantly higher mean percent invasion inhibition compared with the rabbits that were immunized with the bivalent 3D7+FVO vaccine ($P<0.0002$, and $P<0.003$ at NIAID and Burnet, respectfully). In summary, Quadvax antibodies were tested against a total of 26 strains (23 non-vaccine strains) representative of the global diversity of AMA1, and none were found to be refractory to antibody-mediated inhibition of parasite invasion.

- Monoclonal antibodies were generated using recombinant proteins to investigate the basis for the observed cross-strain inhibition elicited by the Quadvax. Western blots were used to structurally map mAbs that showed inhibitory activity by GIA against a panel of chimeric proteins displaying specific surface residues of AMA1 on an immunologically neutral *P. berghei* scaffold. Two mAbs, 1E10 and 4G2, showed evidence of being strain transcending, as they reacted and inhibited to seven of seven strains tested, albeit weakly. Dot blot assays showed mAb 1E10 bound to the polymorphic face on Domain 3 of AMA1, and 4G2 bound to the base of the Domain 2 loop on the conserved face. During the invasion process, AMA1 interacts with the RON complex, a set of rhoprty neck proteins, that comes together during invasion and forms a link between the erythrocyte and the parasite. To study the mechanism of invasion inhibition, assays were performed to determine if the mAbs blocked the interaction of AMA1 with RON2 or if they blocked AMA1 processing on merozoites. mAb 4G2 was one of the five mAbs that was found to block RON2 binding of AMA1. A secondary proteolytic processing of AMA1 on merozoites was blocked by mAbs that mapped to Domain 3 (1E10 and 2C6). Invasion inhibition assays were performed to determine if pairs of mAbs could function cooperatively. Interestingly, when mAb 1E10 and 4G2 were combined, they showed a synergistic inhibition (higher than expected from additive interactions), and this combination inhibited seven diverse strains better than the two mAbs individually ($P<0.05$). These data provide evidence that two different classes of AMA1 mAbs (RON2 inhibitory and processing inhibitory) acted synergistically in invasion assays. Competition assays revealed that the Quadvax elicited higher levels of antibodies to epitopes defined by the broadly inhibitory mAbs.
- WRAIR also performed ELISAs and invasion inhibition reversal assays using their panel of chimeric proteins displaying specific surface residues of AMA1 and demonstrated that Quadvax immunization-induced antibodies directed at conserved regions of the molecule as compared with a mono-allelic 3D7 vaccine. However, responses to the more polymorphic regions of AMA1 did not differ significantly between anti-Quadvax and anti-3D7 antibodies, suggesting that the mechanism of the observed cross-strain inhibitory activity is to focus the immune response to the highly conserved regions of AMA1.

3. LaTrobe University: Engineering AMA1 to overcome antigenic diversity

The aim of the LaTrobe project was to generate a mutated AMA1 that induces high-titer antibody responses to conserved rather than strain-specific epitopes. The basis for this approach is that the loop 1d region (also referred to as C1L) contains five of the nine highly polymorphic sites, and a key assumption is that these residues are largely responsible for the strain specificity of growth-inhibitory antibodies. LaTrobe investigators targeted this critical region by mutating five polymorphic residues in loop 1d to alanine or serine for 3D7 and FVO strains of AMA1. The mutants were expressed in *E. coli*, determined to be properly folded, and were used to immunize rabbits, along with wild type 3D7 and FVO as controls. Major conclusions of this effort include:

- Loop 1d can be mutated with no detrimental effects on structure or immunogenicity.
- Immunization with mutant forms of AMA1 diverts the immune response away from strain-specific antibodies and toward cross-reactive antibodies.
- The AMA1 combination of 3D7(ser) and wild type FVO induces the most cross-reactive and growth-inhibitory antibodies.
- If 3D7 is to be used in a vaccine, including the loop 1d serine mutant rather than wild type 3D7, it would induce the best cross-reactive and growth-inhibitory antibody response.

At this time, MVI is awaiting a final report from LaTrobe. Data received to date, however, indicate that this approach is inferior to the Quadvax and will not be pursued further. Table 6 summarizes the status of the activities and deliverables of the AMA1 optimization projects.

Table 6. Status of the AMA1 optimization projects.

Activity	Deliverable	Status
Walter and Eliza Hall Institute of Medical Research		
GIA using human and rabbit antibodies, transgenic parasites	• Final estimates of the prevalence of inhibitory antibodies to each allele expressed by the transgenic parasites • Summary of the breadth or specificity of antibodies generated for each allele • Recommendation on the contribution of specific C1L residues to immune escape for multiple AMA1 alleles	Completed
Competition ELISAs using single, double, and triple allele combinations	• Summary of competition ELISA results using human and mouse antibodies	Completed
Develop and refine criteria for selecting AMA1 variants for inclusion in a vaccine	• Final recommendation on which alleles to include in a vaccine	Completed
Walter Reed Army Institute of Research		
GIAs using purified IgG from rabbits immunized with the Quadvax or Quadvax including 102-1 instead of 3D7 (collaboration with Burnet and the GIA Reference Laboratory)	• GIA results against additional parasite isolates • Final results on the extent of cross-strain inhibition using the original Quadvax versus a Quadvax containing 102-1 instead of 3D7	Deferred*
Complete rabbit study to confirm initial results from immunization with 3D7-FVO and HB3-W2mef chimeras	• Comparative GIA results from immunization with bivalent chimeras versus the Quadvax	Deferred*
Determine if priming with full-length AMA1 and boosting with subdomains can focus the immune response to Domain 3	• GIA results indicating extent of cross-reactivity of antibodies generated from this prime-boost approach	Deferred*
Complete mapping of cross-reactive epitopes using mAbs	• Final cross-reactive epitope mapping results	Deferred*

Activity	Deliverable	Status
LaTrobe University		
GIAs using purified rabbit IgG raised to combinations of mutant AMA1	• Final summary of GIA results and conclusion on whether immunization with mutant forms of AMA1 results in a broadly inhibitory response	Awaiting final report
Production and purification of mutant and wild type recombinant AMA1 to use in immunization and immunogenicity assays	• Purified recombinant wild type and mutant AMA1	Awaiting final report
Inhibition ELISAs using sera from rabbits immunized with combinations of wild type/mutant AMA1	• Final inhibition ELISA results	Awaiting final report
GIAs using purified IgG from rabbits in the final combination immunization study	• Final GIA results and conclusion on the optimal combination of wild type and mutant AMA1 that induces the most cross-reactive immune response	Awaiting final report

*These activities were not specified as deliverables per the scope of work.

Assessment of the feasibility of an EBA/Rh combination invasion ligand recombinant protein vaccine

In September 2011, MVI received approval to apply USAID funds to support an ongoing preclinical feasibility study with the Walter and Eliza Hall Institute of Medical Research (WEHI) aimed at selecting antigens from the *P. falciparum* reticulocyte binding-like (or *Pf*Rh) family that elicit the highest GIA activity against the broadest range of parasite strains following immunization in combination with EBA-175. The goal is to select the minimum number of antigens that are needed in combination for development as a vaccine. This program is leveraging support from MVI's partnership with Gennova Biopharmaceuticals, where non-USAID funds are being used for process development of the leading EBA/Rh candidate antigens. WEHI began work on this new project phase in September 2011. USAID funds support the WEHI project in its entirety. During the past year, significant progress was made in the following areas:

- **Transfer of materials from WEHI to Gennova.** WEHI provided Gennova with expression constructs (all codon optimized for *E. coli* expression), proteins, and antibodies for the priority antigens identified in the first phase of the project (2010-2011). These included EBA-175 R3-5, *Pf*Rh2a/b, *P. falciparum* Rh5 interacting protein (*Pf*Ripr, N- and C-terminal fragments), and EBA-175 F2-R5. The goal is for Gennova to perform process development on the leading antigens and provide highly pure, low-endotoxin protein to WEHI for evaluation in preclinical immunization studies using the GLA-SE adjuvant.
- **Rabbit immunization studies with antigen combinations.** During FY2012, two antigen combination immunization experiments were performed using research-grade proteins that WEHI produced using Complete Freund's Adjuvant (CFA) as a preliminary immunogenicity screen. Groups of three rabbits were immunized using a three-dose schedule with EBA-175 R3-

5, delivered in combination with *Pf*Rh2 and/or *Pf*Ripr C- and/or N-terminal fragments. IgG was purified from the serum of immunized rabbits and titrated to concentrations ranging from 2 mg/mL to 0.02 mg/mL in the well and added to *in vitro* cultured parasites. Parasite growth was measured in a GIA using FACS after two cycles of invasion. GIAs were completed to evaluate the effect of IgG raised against each antigen combination on three parasite strains: 3D7 (homologous strain), W2mef, and FCR3. With respect to invasion pathway usage, W2mef and FCR3 are sialic acid-dependent strains of *P. falciparum*, whereas 3D7 is sialic acid independent. Sequence analysis of EBA-175 R3-5 across these strains showed that FCR 3 and 3D7 are in the same dimorphic group (identical in Region 3) (F-type), whereas W2mef is in the other group (C-type). In previous experiments, FCR3 was the strain least well inhibited by anti-Regions 3-5 antibodies alone. Results showed that the combination that elicited the highest growth inhibition was EBA-175 R3-5+*Pf*Ripr C-terminal at 2 mg/mL of total IgG, with mean inhibition values of 93 percent for 3D7, 86 percent for W2mef, and 91 percent for FCR3. This combination also showed the lowest IC_{50}, with a mean of 0.07 mg/mL for these three strains. Based on these data, the project team made a decision to deprioritize *Pf*Rh2 for process development work at Gennova, in favor of EBA-175 R3-5 and *Pf*Ripr C-terminal (the C-terminal fragment of *Pf*Ripr was selected over the N-terminal fragment, as initial expression results showed a better yield as a soluble protein) and *Pf*Rh5. However, antibodies raised to *Pf*Ripr C-terminal alone were only weakly inhibitory (<20 percent parasite growth inhibition), suggesting that EBA-175 R3-5 alone may be as good as the best combination. Based on these data, efforts at Gennova are now focusing on EBA-175 R3-5 and work is expected to begin in Q4 2012 on *Pf*Rh5, codon harmonized for optimal expression in *E. coli*.

- **Further investigation of EBA-175 antigens.** The cross-strain GIA comparison using W2mef and 3D7 strains suggested that the most important epitopes for parasite growth inhibition are located in Regions 4-5, which is highly conserved according to sequencing data. Collaborators at Gennova also suggested that structurally, inclusion of the F2 component of Region 2 may result in a more stable expression product, so the WEHI team worked to express both R4-5 and F2-R5 protein fragments in parallel; pending promising GIA results, either of these could be pursued as a back-up to EBA-175 R3-4 in the event of production failure.
    - WEHI synthesized codon-optimized R4-5 with both N- and C-terminal 6-His tags and expressed it in *E. coli*. SDS-PAGE showed the protein was abundantly expressed in the soluble fraction. This material was purified over Nickel agarose and injected into rabbits. GIAs performed using purified IgG from these animals showed the antibodies inhibited invasion of 3D7 and FCR3 (both 80 percent), and W2mef, albeit to a slightly lesser extent (64 percent).
    - Constructs were also synthesized with codon-optimized versions of EBA-175 Regions F2-5 with either N- or C-terminal His tags. Both forms of the protein were expressed in *E. coli* as soluble products to variable levels. The soluble form of R2-5 was found to bind erythrocytes, suggesting it is likely to be correctly folded (R3-5 constructs do not contain the receptor-binding region, R2, so cannot be assessed in the same way). The N-terminal tagged version was found mainly in the insoluble fraction, and so the C-terminal His tagged protein was used to immunize rabbits with CFA. The F2-R5 construct elicited a high level of inhibition (80 percent at 2 mg/mL total IgG) against 3D7, W2mef, and FCR3, but no inhibition of the 3D3 EBA-175 knockout line, indicating the effect is specific and dependent on EBA-175 targeting. Given that previous studies in 2010 using the EBA-175 R2 vaccine from the NIH Division of Microbiology and Infectious Diseases showed that antibodies to R2 were not very inhibitory (22 percent GIA against homologous 3D7), these results suggest that most of the inhibitory response is directed against R3-5 of EBA-175. This F2-

R5 candidate may also be considered a back-up antigen to pursue for process development in the event of R3-5 production difficulties.

- **Further evaluation of antibodies raised to EBA-175 R3-5** revealed that this protein could be targeted by antibodies whether or not the parasites are using the EBA-175-glycophorin A pathway. W2mef parasites are dependent on EBA-175 (and other sialic acid-dependant pathways) for invasion. WEHI investigators showed that when the C-terminal portion of EBA-175 is deleted downstream of R5 in this parasite, EBA-175 function is ablated and the parasite switches to a sialic acid-independent pathway. Both wild type W2mef and the W2mef EBA-175 deletion mutant parasite lines were tested in GIA with anti-R3-5 antibodies and results showed no difference in the ability of these antibodies to confer invasion-inhibitory activity. They further showed that antibodies raised to the homologous Regions 3-5 of EBA-140 are also inhibitory, although the effect is more varied among the strains tested.
- **Update on evaluation of *Pf*Rh5 and *Pf*Ripr as potential vaccine antigens.** Using funds from other sources, WEHI immunized animals with full-length *Pf*Rh5 expressed in the wheat germ cell-free expression system (in collaboration with Taka Tsuboi, Ehime University) and a full-length *Pf*Rh5 expressed in baculovirus. Unfortunately, neither of these elicited GIA activity in the rabbits; however, only results from the first bleed are available at this time. During the past year, WEHI successfully expressed a 50 kDa fragment of *Pf*Rh5 in baculovirus, and demonstrated erythrocyte-binding activity of this stable breakdown product of the full-length expressed protein, which is observed in the parasite. Animals will be immunized in the coming weeks with this material, and preparations are now being made to clone this fragment for expression in *E. coli* at Gennova.
- **Epidemiological investigations.** The WEHI investigators have access to individuals from PNG who participated in a treatment/re-infection cohort study. One objective of this scope of work was to evaluate the correlation of antibody titer to EBA-175 R3-5 with GIA and protection from clinical disease. Thus far, a small number of samples have been tested where serum and plasma are both available for each individual. This comparison has highlighted significant differences in the GIA activity between serum and plasma and efforts are underway to try to remove heparin from the plasma samples, which can affect merozoite invasion. Future work will include testing of plasma samples in ELISA for reactivity against all of the antigens and GIA testing against 3D7 and EBA-175 knockout parasite lines. Work is also in progress to source samples from other regions, including Kenya and Mozambique.
- **Assessment of antigenic diversity in potential vaccine antigens.** Thirteen laboratory strains were sequenced for Regions 3-5 and revealed a remarkable degree of conservation. No polymorphisms were found in Region 3; F-type strains (sialic acid independent) had 1-2 single nucleotide polymorphisms (SNPs) in Region 4; and C-type strains (sialic acid dependent) had one SNP in Region 4. Possible interpretations of these data with respect to interstrain variation in GIA could conclude that sequence variation does not account for variation in GIA, and/or invasion ligand usage does not affect susceptibility of different strains to antibodies to EBA-175. However, protein expression levels may vary by strain, and may thus impact susceptibility to antibodies. SNP data from PlasmoDB were also compiled for EBA-175 R3-5, *Pf*Rh2, *Pf*Ripr (C- and N-terminal fragments), and *Pf*Rh5, and revealed very few polymorphisms in the predicted antigenic regions.
- **Structural studies.** This activity is dependent on the availability of highly pure proteins, and will begin once protein is transferred to WEHI from Gennova. Crystallography of EBA-175 R3-5 will be pursued, and these studies will inform how this protein is structured in relation to the erythrocyte-binding domain and how it is exposed to the immune system. If Gennova is

successful at producing a stable, purified *Pf*Rh5, WEHI also plans to co-crystallize domains of *Pf*Rh5 and *Pf*Ripr to closer examine how this complex is structured.

Table 7 summarizes the status of the EBA/Rh project activities and deliverables.

Table 7. Status of the EBA/Rh project.

Activity	Deliverable	Status
Protein expression and rabbit immunization with EBA-175 constructs	Expression and immunogenicity results of EBA-175 F2-R5 and EBA-175 R4-5	Completed
Perform combination immunization studies of EBA-175 R3-5, *Pf*Rh2, and *Pf*Ripr (N- and C-terminal fragments)	GIA results from combination immunization studies	Completed
Technology transfer of materials to Gennova	Report of materials transferred and reproduction of protein expression data using WEHI protocols	Completed
Perform invasion assays using purified merozoites	Invasion inhibition results to confirm the mode of action of antibodies to EBA-175 R3-5	Expected October 2012
Perform assessment of antigenic diversity in potential vaccine antigens	Sequencing results of EBA-175 R3-5, SNP database analysis of other antigens	Completed
Epidemiological investigations	GIA assay results using clinical samples	Ongoing
Structural studies of lead antigens	Crystallography analysis of EBA-175 R3-5 and *Pf*Rh5	To begin Q4 2012/Q1 2013

Assay harmonization

USAID has been supporting assay harmonization efforts through the consultancy of Dr. Patrice Dubois since 2009. He provided expert technical assistance in the oversight and coordination of two work packages aimed at harmonization of critical antibody and T cell assays supported by the European Union-funded Optimalvac project, which was coordinated by the European Vaccine Initiative (EVI) based in Heidelberg, Germany. This three-year project concluded on May 31, 2012.

Antibody-dependent cellular inhibition assay harmonization

In the past year, a single round of testing occurred in which the participating laboratories tested a panel of standard reagents using in-house protocols. Four participating laboratories were expected to send results, but only the University of Edinburgh and the International Centre for Genetic Engineering and Biotechnology (ICGEB) sent data by the close of the project.

Identification of reagents for testing

The standard reagents used were identified as follows:

- **Sera from endemic areas.** The National Institute for Biological Standards and Control (NIBSC) gave ethical approval for the use of serum samples testing positive in antibody-dependent

cellular inhibition (ADCI) assay as reference reagents, and the following sera were shipped to NIBSC for distribution to laboratories participating in the work plan:
  - Sera from Institut Pasteur (collected in Côte d'Ivoire and testing positive for MSP3 and other *Pf* antigens by ELISA).
  - Sera from Radboud University Nijmegen Medical Centre (collected in Gabon and testing positive for MSP1, MSP2, AMA1, and GLURP by ELISA). These sera were not distributed due to relatively small volumes and the need for viral testing before distribution.

- **Monocytes.** All laboratories used their own source of cells, following acceptance criteria and guidance documents agreed upon by the group.
- **Parasites.** All laboratories used their own source of parasites. The strains used by the two testing laboratories were 3D7 and Wellcome.
- **A monoclonal antibody, RAM 1** (human monoclonal antibody anti-*P. falciparum* MSP3), developed by Pierre Druilhe and known to display specific activity in ADCI, was used as a positive control. MVI supported production of a lot of this antibody for use by Optimalvac participants using non-USAID funds. The antibody completed quality control testing at Institut Pasteur. Functional activity as measured by the ADCI assay was lower than observed in previous lots; however, the level of activity was deemed acceptable for use as a control in this harmonization exercise. NIBSC developed a vialing process (microtubes with caps) that does not alter RAM 1 activity.

NIBSC completed distribution of reagents by the end of 2011. Testing was performed in early 2012 following guidance documented in the report generated by Dr. Dubois on the development and rationale of acceptance criteria for assay performance. This report defined minimal acceptance criteria for key assay reagents—such as parasite cultures, monocytes, and serum—and requirement for their performance in assays, as well as optimal ADCI readout.

While receipt of only two data sets was disappointing, several issues were highlighted:

- The method of staining is critical. Double staining, such as Hoechst 3342 and anti-CD45, enables the determination of the proportion of monocytes versus lymphocytes in each sample. Use of two nucleic acid-binding dyes is required, especially in the case of samples with low parasitemia, to avoid non-infected red blood cells appearing positive. While the US Centers for Disease Control and Prevention and ICGEB use hydroethidine as their main parasite detection method, comparison of slide-reading and flow cytometry using other dyes was suggested to find the most accurate method for determining parasitemia.
- Parasite strains other than Wellcome and 3D7 may be used. Pierre Druilhe stated that any strain showing robust growth characteristics could be used in ADCI.
- Non-specific inhibition was observed in one laboratory when testing the Côte d'Ivoire sera against the Wellcome strain. This inhibition may have resulted from several freeze/thaw cycles, resulting in oxidization of serum lipids which are toxic in ADCI.

Future efforts were discussed. The group agreed to a second testing with their own reference reagents, in order to compare the Optimalvac-supplied reagents with the in-house reference reagents. Since the ADCI assay is required for ongoing clinical trials supported by the EVI, hands-on training of staff is necessary. The suggestion was made to test pediatric MSP3 samples from vaccinated children showing protection to evaluate whether ADCI correlates with protection. Pierre Druilhe was requested to develop an ADCI strategy plan to seek further funding, as no funding remains from Optimalvac.

The Technical Advisory Committee (TAC) recommended that more funding is needed to establish more robust ADCI assays and it remains unresolved whether a central ADCI reference laboratory or two or three labs should perform further assay optimization. More comparative data between labs need to be generated and to be published in order to increase support for the ADCI assay by the community. Table 8 contains the milestones and status of ADCI project activities.

Table 8. Status of ADCI project activities.

Activity	Deliverable	Status
Discussions on assay parameters	Consensus SOP for ADCI	Completed
Discussions on assay acceptance criteria	Acceptance criteria for assay performance and requirements for key components identified	Completed
Collection/Generation/ Identification of reagents suitable for ADCI testing	Standard reagents, sera, and IgG fractions available for distribution and testing	Completed
Conduct ADCI testing and reporting of results	Report/Publication on interlaboratory variability	Completed

T cell assays

The objective of this project was to harmonize T cell-mediated immune assays (IFN-γ ELISpot and ICS) at malaria vaccine development laboratories using standard assays that detect responses to cytomegalovirus, Epstein-Barr virus, and influenza virus (CEF).

Standard reagents to be distributed

The following standard reagents were distributed to all laboratories for testing:

- **PBMCs.** Dr. Giuseppe Pantaleo's laboratory at University of Lausanne Hospital supplied PBMCs for inclusion in ICS and ELISpot panels, in an effort supported by MVI with non-USAID funds. The laboratory performed leukaphoresis on five healthy volunteers and characterized the PBMCs for responses to tetanus toxoid and CEF peptides. The PBMCs were distributed to participating laboratories for testing.
- **Tetanus toxoid peptide antigens.** A supply of tetanus toxoid (provided by Serum Institute of India) for use as an antigen was distributed to all participants.
- **CEF peptide antigens.** CEF peptides were purchased and qualified by NIBSC from JPT Peptide Technologies GmbH in Berlin and distributed to all participants.

On the advice of the Optimalvac TAC in 2011, existing standard operating procedures (SOPs) for IFN-γ ELISpot and ICS were obtained from Duke University. Based on these SOPs, NIBSC generated guidance documents for performing critical aspects of the assays and distributed these to participating laboratories. In the first round of testing, the laboratories were instructed to follow their own procedures altered to incorporate the guidance document. The first round of testing occurred in early 2012. ELISpot data were received from five labs, and ICS data from four.

Analysis of the IFN-g ELISpot data revealed reasonable consistency of results. In all data sets received, the preliminary analysis was that true positives were deemed positive and true negatives

were deemed negative by the participating labs. Therefore, imposing a defined procedure and select reagents seemed to work well. Where variability existed, most was attributed to the serum. One suggestion for the future was to use serum-free media. It was also suggested that labs run an extra experiment comparing their in-house protocol to the external SOP.

In the ICS harmonization exercise, CD4+ T cells expressing IFN-g, IL-2, and TNF-α, and CD8+ T cells expressing IFN-g and TNF-α were evaluated. The ICS data were less consistent between labs, compared to ELISpot. Gating strategy guidelines were provided to all labs, but were not necessarily followed. In other ICS harmonization studies, gating strategy has been shown to be the most common source of variability between results; and therefore, labs were instructed to send their raw data files to Oxford for centralized gating in order to determine whether gating strategy had such an impact in this study. Oxford completed the analysis, and found that centralized gating removed some of the variability.

Five African laboratories were identified for participation, but they received their shipments of reagents near to the end of the project and their results are not yet available. Labs identified were the Malaria Research and Training Center (Mali), the Medical Research Council (The Gambia), Albert Schweitzer Hospital (Gabon), the Noguchi Memorial Institute for Medical Research (Ghana), and Centre National de Recherche et de Formation sur le Paludisme (Burkina Faso).

The TAC congratulated the consortium on the good consistency of the ELISpot results, and offered suggestions. They recommended that plates be sent to NIBSC for centralized reading. The ICS data were quite inconsistent, and the TAC recommended that participants integrate into existing ICS harmonization networks for continued improvement. Table 9 contains the milestones and status of the T cell assay project activities.

Table 9. Status of T cell assay project activities.

Activity	Deliverable	Status
Discussions on assay conduct and protocols	Key parameters, acceptance criteria, reference cell preparation, and reagents identified	Completed
Identification of possible repositories and selection of suitable repository	Repository of cells and reagents established	Completed
Generate guidance document based on Duke SOPs	Harmonized SOP available to all participants (modified to guidance document)	Completed
Training of relevant lab staff on assay procedures	Training of personnel completed	Completed
Perform testing and report results	Results of intra- and interassay variability available	Completed

Policy and access

Framing the malaria vaccine introduction decision

MVI's Policy and Access unit has been working since 2006 with African policymakers to prepare for a timely decision on the introduction of a malaria vaccine, when ultimately licensed, as an additional tool for malaria control, to avoid unnecessary delays in making a decision about its use—as has been the case with other lifesaving interventions.

With support from USAID and other sources, MVI has worked with national authorities and partners to organize briefing and consultation meetings to share information on progress on malaria vaccine development and to discuss processes for planning ahead of a policy decision. Such meetings have been organized at least once a year in 12 countries. MVI has also participated in regional and subregional meetings on malaria and immunization to share information and raise awareness and support for the need to plan for a decision. In addition, MVI has worked with some countries to actually put in place mechanisms to prepare their decision-making frameworks. Burkina Faso, Ghana, Tanzania, and Uganda have established technical advisory groups (TAGs) to compile the evidence that will inform policymakers on a decision about a malaria vaccine. Other countries—the Democratic Republic of Congo (DRC), Gabon, Kenya, and Rwanda—have initiated a similar process.

Our original proposal for use of USAID funds was to complement existing funding for the organization of a regional meeting on the decision-making framework to develop with countries an outline of a policy brief that could be adapted later by national TAGs. Because this activity has been postponed to 2013, when the next set of results from the RTS,S trial is available, MVI requested—and USAID agreed—that USAID funds instead be used to cover a portion of the Kenya-based senior program officer's time—the staff person who is leading the decision-making framework project. The following activities (in Table 10) were carried out as part of the 2012 work plan.

Table 10. Status of decision-making framework-related activities.

Activity	Deliverable	Status
Support existing TAGs in their work and support four additional countries to act on their needs for decision-making on RTS,S	Report of TAG activities	• **Completed: Ghana and Tanzania TAGs** have prepared a first report to stakeholders, which will be disseminated in November 2012 • **In process: Burkina Faso and Uganda TAGs** are hiring local consultants to assist in some activities • **Not yet completed: DRC, Gabon, Kenya, and Rwanda** TAGs have yet to develop their activity plans
Support establishment and functioning of networks for experience-sharing and collaboration between national TAGs on their decision-making frameworks	Report of the launch of TAG networks	• **Completed:** Meeting held between Kenya, Tanzania, and Uganda in February to draft the terms of reference and objectives of the East Africa network of TAGs for the RTS,S decision-making framework, which will be officially endorsed at a stakeholders meeting in November • **Nearing completion:** A meeting for English-

Activity	Deliverable	Status
		speaking West African countries will take place in November 2012 in Accra, Ghana
Work with regional and international partners to organize subregional technical meetings on the development of policy briefs to inform future decisions on RTS,S	Report of the meeting	**Postponed:** MVI decided to postpone this activity until after the next set of key data become available