MALVAC Meeting 2004: Evaluation of malaria vaccines

Pre-clinical Evaluation Group: Optimizing the developmental pathway from the lab to the clinic

Report from a technical consultation at WHO/IVR Malaria Vaccine Advisory Committee meeting

Montreux, Switzerland,

11-14 October, 2004

Contents

EXECUTIVE SUMMARY **4**

1.1 INTRODUCTION **9**

1.2. KEY ISSUES IN THE PRODUCTION, CONTROL AND EVALUATION OF VACCINES: WHO PERSPECTIVE **11**

1.3. REVIEWING THE DEVELOPMENTAL PATHWAY OF LEAD MALARIA VACCINE CANDIDATES **15**

1.3.1 CONSIDERATIONS 15
1.3.2 CURRENT MALARIA VACCINE CANDIDATES - VACCINE DEVELOPMENT RATIONALE 16
1.3.2.1 Pre-erythrocytic vaccines - *16*
1.3.2.2 Blood-stage vaccines *19*

1.4. RODENT MODELS **25**

1.4.1 CONSIDERATIONS 25
1.4.2 ADVANTAGES 26
1.4.3 LIMITATIONS 27
1.4.4 CONCLUSIONS AND POTENTIAL IMPROVEMENTS 28

1.5. NON-HUMAN PRIMATE MODELS **29**

1.5.1 CONSIDERATIONS 29
1.5.2 NEW WORLD MONKEYS 29
1.5.2.1 Advantages *30*
1.5.2.2 Limitations *30*
1.5.3 RHESUS MACAQUES 31
1.5.3.1 Advantages *31*
1.5.3.2 Limitations *31*
1.5.4 CONCLUSIONS AND POTENTIAL IMPROVEMENTS 31

1.6. IMMUNOASSAYS IN MALARIA VACCINE DEVELOPMENT **33**

1.6.1 CONSIDERATIONS 33
1.6.2 GROWTH INHIBITION ASSAY (GIA) 34
1.6.3 ANTIBODY DEPENDENT CELLULAR INHIBITION (ADCI) ASSAY 36
1.6.4 USING ASSAYS TO DEVELOP AND DEFINE CORRELATES FOR MSP1-42 - THE WRAIR EXPERIENCE 37
1.6.5 ADVANTAGES 39
1.6.5 LIMITATIONS 39
1.6.6 CONCLUSIONS AND POTENTIAL IMPROVEMENTS 40

1.7. IMMUNO-EPIDEMIOLOGICAL ASSOCIATIONS AND POTENTIAL CORRELATES OF NATURAL IMMUNITY **42**

*1.7.1 **CONSIDERATIONS*** 42
1.7.2 IMMUNO-EPIDEMIOLOGICAL RATIONALE BEHIND THE CLINICAL DEVELOPMENT OF MALARIA VACCINE MSP3 43
1.7.3 IMPROVING AND STANDARDIZING THE IMMUNO-EPIDEMIOLOGICAL APPROACH - EFFORTS OF THE AFRICAN-IMMUNOASSAY(AIA) NETWORK 45
1.7.4 ADVANTAGES 47
1.7.5 LIMITATIONS 47
1.7.6 CONCLUSIONS AND POTENTIAL IMPROVEMENTS 47

1.8. FUTURE NEEDS AND WAYS FORWARD **48**

*1.8.1 GENERAL **CONSIDERATIONS*** 48
*1.8.2 **WAYS FORWARD - IMPROVING MODELS AND ASSAYS*** 49
*1.8.3 **EXAMPLES OF WAYS FORWARD IN THE DEVELOPMENT OF NEW MODELS*** 50

1.9. PRE-CLINICAL EVALUATION GROUP: CONCLUSIONS 54

1.9. PRE-CLINICAL EVALUATION GROUP: RECOMMENDATIONS 55

ANNEX 1. WHO MALVAC COMMITTEE MEMBERS, 2004/2005 57

ANNEX 2 LIST OF PARTICIPANTS: MEETING I- OPTIMIZING THE DEVELOPMENTAL PATHWAY FROM THE LAB TO THE CLINIC 59

ANNEX 3 REFERENCES 62

Executive Summary

A broad-range of candidate malaria vaccines derived from diverse novel technologies exist, the majority of which have been discovered and developed based on results from experiments in animal models and in vitro assays as well as epidemiological associations of clinical protection and immune responses in the field. The uncertainty around the issues of relevance and predictive value of these imperfect screening tools and immune response associations means that ultimate proof-of-concept for most candidates will require evaluation in clinical efficacy trials. Optimizing the tools and criteria for evaluation of candidate vaccines means decisions made in R&D will be based on 'best practices' that provide high quality scientific evidence to support sound vaccine development rationales, and focus on well selected candidates for clinical testing.

Specific scientific and technical challenges in the evaluation of malaria vaccines were discussed at the 2004 meeting of the WHO Malaria Vaccine Advisory Committee (MALVAC), in Montreux, Switzerland on 11-14 October, 2004. In addition to the committee members, and representatives of the major malaria vaccine funding agencies, the meeting also gathered experts in immunology, parasitology, malaria epidemiology, vaccine development and clinical trials from diverse malaria vaccine research laboratories, clinical trial sites and industry. The meeting participants were divided into two groups, addressing either pre-clinical/non-clinical evaluation or clinical evaluation. This report presents the discussions and outcomes of the preclinical/ nonclinical group.

' Optimizing the developmental pathway from the lab to the clinic'

Meeting participants analysed and evaluated the use of in *vitro* assays, animal model data and immuno-epidemiological associations in non-clinical evaluation of candidate vaccines in order to identify what needs to be done to optimize the development rationale of malaria vaccines, and make specific recommendations to WHO on steps to take to address these needs.

It was emphasized that early consideration of issues surrounding production, control and evaluation of vaccines is essential for successful development. Translation of discoveries from research labs into licensed products require that the development process meets the stringent requirements of a safe, consistent, well characterized vaccine.

The demonstration of consistency of the production process and the importance of high-quality characterization is critical. Poorly defined candidate vaccines will lead to uncertainties regarding whether differences in immunogenicity, protective efficacy or safety are due to unintentional variations, suboptimal vaccination schedule, poorly designed trials or differences in the target populations.

Vaccine development rationale and the evaluation of a candidate antigen, may differ for different candidates, depending on the particular hypothesis about protective

effector mechanisms justifying vaccine potential. However, absence of reliable immune correlates means that current development is to some extent empiric. This should not preclude that evaluation tools are improved and remain coherent for a candidate vaccine throughout its development pathway. This strengthens the empiric method and as further understanding of protective immunity develops, the strive towards a more rational approach should continue in parallel. For example, an important means of validation of the existing surrogates is the back validation by on-going clinical efficacy trials, which should be promoted and included in trials plans.

A review of the developmental pathway of current leading candidates showed that the diverse tools and criteria used for decision-making, making comparisons between candidates impossible. Given the broad range of vaccine concepts being developed for candidate malaria vaccines, judgment based on the best science available will form the rationale of the type and extent of non-clinical evaluation of candidate vaccines. 'The best science available' should therefore be reflected in the development pathway of a candidate from the researcher's bench to the clinic.

In reviewing model systems, it is clear that conceptual insights gained from model systems develop and improve on vaccine design but there is a critical need for a systematic and continuous re-evaluation of current knowledge and uses of these systems, which were primarily developed for pure research purposes. This is to ensure their rational application along the development pathway to help optimize performance.

Rodent models

These models have historically played a key role in providing the first clues of an antigen's immunogenic and protective potential, and will most likely continue to do so, for quantitative and financial reasons.

In terms of selection of antigenic targets for candidate vaccine development, the demonstration of protective efficacy in murine models (orthologue), characterization of biological function of the antigen, and the role of gene knock-outs and knock-ins are used increasingly in the prioritization process. Further roles in preclinical development include the identification and prioritization of vaccine technologies, development of optimal formulations to take to further development, and investigation of optimal vaccine technologies.

Limitations that can affect the relevance of these results that were discussed included that these models are unnatural host parasite combination that have characteristics particular to each species (and each line or clone). The acute, often lethal infections established in these hosts are very different from the chronic infection in natural host parasite combinations and this needs to be kept in mind. In addition, comparability is difficult as models are numerous (large number of parasite species or strain -mouse strain combinations) not standardized and route, method of challenge and outcome measures vary greatly. A coherent strategy to systematically and critically compare and evaluate the relationship between read-outs in mice versus non-human hosts and then the target hosts should be a followed so that the relevance of the model and its predictive value will eventually be determined.

There should be support for research efforts to develop more relevant models using recent advances and knowledge. The development of new models such as the P.falciparum-SCID mouse model and the cytoadherence rodent model which allows

for investigation of the *in vivo* effect of a given immune response could be very useful in investigating immune and disease mechanisms, correlates of protection, and material derived from early clinical trials. Advances in the creation of transgenic mice and chimeric parasites where replacement of a mouse antigen by the corresponding human parasite antigen is another example of model improvement that should be supported.

Non-human primate models

Given their morphological and genetic similarities to humans, non-human primates appear intuitively to be the best host for studying human disease. Despite debate and uncertainty over the relevance of non-human primates as a surrogate of either human malaria or immunity, they remain an important means to directly test the immunogenic potential of malaria vaccine candidates before injection into human volunteers.

Currently, the most widely available and utilized non-human primate models are the New World Monkeys (the *Aotus* and *Saimiri* species) and the rhesus monkeys (*Macaca mulatta*). The former are most useful due to their receptivity to*Plasmodium falciparum, Plasmodium vivax* and *Plasmodium malariae*. Beyond safety and immunogenicity, trials in New World primates could thus provide efficacy data to help make decisions on antigen forms, expression systems, and formulations, and thus improve trial preparedness for collection of safety data.

However fundamental biological differences manifested by various parameters including fast acquisition of effective immunity by infection(as compared to humans) and the development of life-threatening anemia make any extrapolation from data obtained from these models to predictability and relevance to humans difficult.

The widely available rhesus macaque model is more homologous to humans than New World primates and has been useful in assessment of immunogenicity, formulation selection and safety testing of malaria vaccine candidates. These models, refractory to human malaria species are susceptible to *P.knowlesi* sporozoite or blood stage induced infections, can develop chronic infections and semi-immune states, and demonstrate antigenic variation. The large size of these monkeys also allow adequate sera to be collected for analysis.

On-going clinical trial experience must now help to retroactively determine which models can best fit the needs and goals of the research and development pathway. The models could be used to investigate correlates related to vaccine-induced responses including *in vitro* assays. However, cost and availability considerations as well as the increasing pressure to limit animal testing will ultimately lead to increasingly stringent cost-benefit analysis of non-human primate testing versus human trials where true efficacy data can be assessed.

In vitro Assays

The *in vitro* environment does allow for better and easier control of host factors relative to animal models., and other variables such as amount of parasites, antibodies or cells. However *in vitro* conditions are “fragile” and artifactual inhibition of growth is more easily obtained than in *vivo*.

Assays that reflect *in vitro*, immune defense mechanisms underlying either naturally acquired or vaccine-induced protection status, could when validated, greatly help

rationalize and accelerate vaccine development. Key assays that have the potential to provide correlates or surrogate markers of protective immunity should be objectively evaluated, optimized and standardized and made available to the malaria vaccine research and development community.

At the meeting, two functional assays in malaria vaccine research were discussed, the growth inhibition assay (GIA), and the antibody-dependent cellular inhibition (ADCI) assay, both of which have played an important role in developing the lead blood-stage candidates. The GIA measures the capacity of antibodies to limit invasion or subsequent growth of *P.falciparum* parasites in red cells. The biological activity demonstrated in this assay has been widely used as proof of vaccine potential for several blood stage vaccine candidates, such as merozoite surface protein-1 (MSP1) and apical membrane antigen-1(AMA1).

The ADCI (antibody-dependent cellular inhibition) activity assay measures parasite growth inhibition resulting from the cooperative mechanism between monocytes and IgG. This is set up to compare with positive controls (IgG from pooled sera of immune African) and negative controls (IgG of healthy non-immune controls). Adding to the parallel demonstration of correlation of *in vivo* protection with *in vitro* ADCI activity is the finding in further studies that this activity is highly dependent on cytophilic antibodies.

None of these or other current assays can be taken as reliable surrogate markers of protection. This constitutes a major hurdle in rational vaccine development that deserves to be addressed rapidly.

Immuno-epidemiological associations

These data have the paramount value of being collected in humans. However, determinants of the quality and significance of immuno-epidemiological association studies often rests with the design of these studies. Aspects such as study methodology, especially case definitions of malaria attacks and frequency ,way of collecting clinical data and bio-statistical analysis differ among studies and therefore making extrapolating and developing robust correlation between the measured immune parameter and protection challenging. Longitudinal, prospective studies, consisting of follow-up of subjects in areas where malaria is endemic or seasonal, and studying the development of malaria and immune status of these subjects are a more appropriate means of studying potential correlations between the immune response of interest and state of protection. Differences in case definitions and intensity of follow-up, methods of data collection and statistical analysis, and ways to handle potential confounding factors (eg age), presently affects comparability of results between studies.

The large field diversity of malaria parasites and the large number of parameters measured, make that some associations found between a given immune response and protection can be due to chance. Statistically significant correlation in one study must be confirmed in similarly designed, and, if possible, larger scale studies in distinct malaria endemic regions. The cost, logistical and operational difficulties as well as potential ethical issues of conducting multiple immuno-epidemiological studies of high quality (i.e. longitudinal, daily follow-up from birth) to investigate and dissect the development of protective immunity to malaria were discussed. It was generally

agreed upon that the whole malaria research and development field, not just malaria vaccinology would benefit from information gathered through such studies.

Conclusions and Recommendation

Participants emphasized the need for a systematic and continous re-evaluation of current knowledge and uses of animal models and assays, which were primarily developed for pure research purposes and concluded that it was critical to ensure that what knowledge generated through on-going basic preclinical research, early product development and vaccine trials be applied towards the reiterative process of improvement of current tools and the development of new ones.

Priority should be directed towards an approach optimizing or developing models with *P falciparum* as the target parasite (or target antigen in transgenic parasites). Technical advances have allowed greater manipulation of these models where methods that allow for tracking and analyzing host-parasite interactions on a cellular level are being developed and models can be developed to study specific mechanisms of defenses. Support is also needed to make assay development and optimization a strategic priority. This is particularly essential because these efforts are costly time-consuming and poorly rewarding for researchers, yet essential at rationalizing and accelerating vaccine development. A prerequisite to the success of projects aimed at immunoassay optimization, standardization and validation is availability of standards and reference methods.

The meeting participants recommended that international collaborative working groups be formed and supported to address issues related to the evaluation of malaria vaccines. Two specific working groups were recommended to address assay development, optimization, validation, and standardization and animal model issues. Investments to improve novel models could be instrumental in accelerating vaccine development

A database could be established to collect and make accessible knowledge acquired through on-going research including data from the analysis of sera from clinical trials should be applied towards validation, continuous reevaluation and hopefully improvement of currently used models and assays.

The participants strongly recommended that these initiatives be fully developed and supported as a coordinated effort among the agencies and institutions that fund malaria vaccine research and recommended that WHO should help ensure that the assays and animal model SOPs optimized and standardized through this effort are accessible for technology transfer efforts among the global malaria vaccine R&D community.

1.1 Introduction

Experiments in animal models and in vitro assays as well as immuno-epidemiological associations provide the justification for the development of current candidate malaria vaccines. The absence of a robust animal model, faithfully mimicking the human immune system and human-host parasite interaction is a limitation to development. Knowledge regarding both the protective immune responses in humans as well as the model combinations is lacking and often contradictory, leading to unresolved debates on the usefulness and relevance of experimental outcomes in models.

Imperfect, of questionable relevance and even more uncertain predictive value these systems may be, data generated from experimentation in these systems have helped push candidates through the pipeline into human trials. Robust, vaccine-induced protection has not yet been convincingly demonstrated in human trials, thus correlation of models to human malaria protection is deficient. However, laying the groundwork for future model validation and development of improved and more relevant models require that currently used models are critically analyzed to better understand the relevance and significance of measured parameters, and build knowledge on the advantages and limitations of specific model systems. The quality of protection data in animal models will also be influenced by various components of the system, such as the challenge route, technique and outcome measure used to define efficacy or protection. These differences and lack of standard approaches make comparability of data generated in animal model experiments challenging.

In vitro assays are critical in the analysis of materials obtained from animal or human studies, as well as from immuno-epidemiological studies in search of correlation between an immune response and a state of protection. Responses associated with exposure to the antigen alone (i.e. total IgG) can be meaningless, unless shown to be highly correlated to protection. Functional and cellular response assays which demonstrate biological activity are of greater significance, but similarly remain to be validated. As with in vivo models, standard methodology and assay components are needed to improve the quality and comparability of the read-outs.

The aims of the meeting were twofold. The first aim was to conduct a review and critical analysis of animal models (rodent and non-human primate models), *in vitro* assays, and immuno-epidemiological associations used along the developmental pathway of current malaria vaccine candidates from the bench to the clinic. The second aim was the identification of critical needs in the area around which specific activities could be organized that would have an impact on improving the quality of evaluation of malaria vaccine candidates.

Experts from key malaria vaccine research laboratories, malaria vaccine development program managers, and representatives from major funding agencies some with years of experience in the development and use of these model systems, discussed the model

systems and reviewed their strengths and weaknesses as tools for the screening and evaluation of candidate malaria vaccines.

This report details the issues considered, as well as the advantages and limitations of the model systems presented and discussed at the meeting. In addition, the meeting participants were also guided by the goal of the overall need to improve future evaluation of malaria vaccine candidates from the discovery and selection process to clinical trials and the report reflects two recurring themes; first, the importance of relevance in these assessment tools and second, the need to ensure that the most relevant model systems measuring the most appropriate parameter are used (or developed) to evaluate antigens, vaccine concept, or strategy.

The meeting concluded with recommendations for the WHO to address key areas that were identified as critical towards optimizing the developmental pathway from the bench to the clinic. These are described in further detail in the recommendation section of the report.

1.2. Key issues in the production, control and evaluation of vaccines: WHO perspective

1.2.1 General Considerations

Many of the initial discoveries leading to vaccine development of an antigen occur in academic research labs. In reality, the majority of these innovations never reach the market. It is estimated for instance, that in the United States, out of 100 drugs submitted for Investigational New Drug applications, approximately 20 will be licensed. The differences between aims of academic research (output measured by publications) and pharmaceutical industry (output measured by licensed products) contribute somewhat to this.

Translation of discoveries in research labs into licensed products require that the development process meet the requirements of standardization, quality control, and efficacy and safety evaluation. General principles and standards exist that help guide development of safe and high quality vaccines, compliant with regulatory and licensure requirements. These include GLP[1], Clinical Evaluation of Vaccines: Regulatory Expectations [2]and Non-clinical Evaluation of Vaccines[3]. These principles can be applied to the production, control and evaluation of malaria vaccines. Early consideration of determinants of good practice in the development process such as production consistency, product characterization, safety assessment as well as regulatory and licensing issues is essential to meet these challenging requirements.

1.2.2 Consistency of production

The demonstration of consistency of the production process where the product does not differ from vaccine lots shown to be safe and immunogenic in clinical trials is a crucial component of vaccine evaluation, licensing and batch release. Product manufacturers should make every effort to characterize the clinical lots and maintain some lots for future reference, if needed.

Ideally, preclinical testing should be done on the same lots meant for clinical trials or if not should be comparable and prepared under GMP-like conditions to obtain clinical grade material.

Clinical data to demonstrate consistency is not usually required except in circumstances when clinical data may help to demonstrate manufacturing consistency.

1.2.3 Vaccine characterization (physicochemical and functional) and formulation .

The importance of high-quality characterization of vaccines cannot be over-emphasized. The well-defined candidate vaccine offers the best chance of success, since, if it is shown to be protective in clinical trials, it must then be scaled-up and made to the exact same specifications as the clinical trial product. If the materials and resulting product are poorly defined, it is never certain whether differences in immunogenicity, protective efficacy or safety are due to unintentional variations, suboptimal vaccination schedule, poorly designed trials or differences in the target populations.

Full characterization and non-clinical testing should be done on the formulation intended for clinical trials. Changes in formulation or other production methods or scale-up after licensing will require additional product characterization to demonstrate equivalence.

Malaria vaccine development is at the forefront of using novel adjuvants and formulations with the aim to augment immunogenicity and induce the desired immune response. The assessment of a novel adjuvant should be undertaken as required for a new chemical entity. The compatibility of the adjuvant with all antigenic components of the vaccine should be evaluated. If no toxicological studies exist for a new adjuvant, these studies should be conducted on adjuvant alone. Adsorption of all antigenic components should be consistent on a lot-to-lot basis. Potential desorption of the antigen during the shelf-life of the vaccine may affect both the immunogenicity and safety of a product and should be determined as part of product stability studies. The establishment of working standards with and without adjuvant, is essential for proper evaluation. When developing immunogenicity and safety profile of antigen/adjuvant formulation, the formulation used should be the one intended for clinical testing.

Clinical evaluation of vaccines should first establish the full characterization of the vaccine candidate as previously described. Secondly, the type of production system must be fixed and stable (DNA, live attenuated, synthetic peptide, etc). Thirdly, standardized methods for ensuring quality control of vaccines and evaluation of immune response must be developed. For example for live attenuated vaccines, the issue of shedding and potential genetic recombination must be addressed.

A comprehensive characterization of the initial batches of the product should be undertaken to establish consistency with regard to identity, purity, potency and other product characteristics. These data should be completed by the end of Phase III trials. Following licensing, the vaccine is then subject to batch release by the regulatory authorities. There is a clear distinction between comprehensive characterization of a vaccine during development and the specific tests used for the purpose of batch release

1.2.4 Non clinical testing of clinical grade material: Characterization of immune response for Proof-of-concept

For the different malaria vaccines being developed, there is no single pathway towards proving the vaccine concept, given that multiple approaches are being investigated for different stages of parasite immunity and therefore determination of criteria for selection of a candidate for clinical development can vary.

Data on immunogenicity and efficacy in animals should be well-documented before commencing clinical trial, if appropriate and relevant animal models for the evaluation of

immunogenicity and efficacy exist. If these models are not available, relevant data from alternate approaches or *in vitro* testing that are justified need to be documented and considered for proof of concept to support a proposed clinical development plan. The appropriateness of current tests must be evaluated i.e. determining the desired immune response to measure and ensuring that it is truly being measured in the tests. In view that antibody response may merely reflect exposure and for some vaccines, it may not be the best indicator of protection and in some cases, there may be no correlation between antibody titers and protection.

The relevance of *in vivo* assays such as protection models in mice should be addressed, along with the lack of comparability of data generated in different animal models. Efforts should be made to establish standardized assays and standards. The use of animal models as well as trial sera to define and develop correlates of protection should also be pursued. Improvement of future evaluation should result from addressing these considerations in a systematic and consistent way.

1.2.5 Clinical testing: Trial design

The design of clinical trials is critical in the clinical investigation of the vaccine in the field. Particularly for malaria, accurate baseline knowledge regarding the epidemiology of the parasite and disease in the target population is critical. The clinical spectrum of illness must be defined for various populations along with high risk groups (where vaccine should have most impact). Laboratory values and sero-prevalence studies will also further characterize the population.

1.2.6 The role of standards and reference materials

The standardization of methods used to evaluate vaccines and to evaluate immune responses to vaccine antigens depend on the establishment of standards and reference materials. A WHO expert committee on biological standards is tasked with the development, review and maintenance of written and measurement standards used in the production and control of vaccines, in order to:

- Provide measurement standards for quality control of clinical lots
- Provide measurement standards and standardized assays for the measurement of the desired immune responses in clinical trials
- Establish written standards for production, control and evaluation of vaccines
- Coordinate and convene international experts to provide objective technical advice on the review of a specific candidate

WHO International Standards and Reference Reagents form the primary standards and individual regulatory agencies and manufacturers establish their own national and working standards that are calibrated to the primary standards.

1.2.7 Early consideration of safety and regulatory issues

Safety of vaccines is addressed through testing at different levels of production i.e. control of starting materials, in-process control, final bulk and final lot consistency. An objective, competent and independent regulatory body should assess the safety testing

conducted on a vaccine through the licensing and batch release process as well as subsequent post-marketing surveillance. Several questions should guide these appraisals such as the degree of knowledge of safety of the vaccine at the various production stages, whether current safety assessments of vaccines containing novel adjuvants are adequate and need improvement, and whether regulators need to be more specific and define their expectations of novel vaccines. At the same time, it is important to consider regulatory issues early in the development process to ensure compliance with requirements for clinical trial approval, licensing, batch release and post-marketing surveillance and avoid unnecessary delay in vaccine development

Regulators need to play an active role as well to assist the developers with regulatory compliance through:

- Establishment of clinical trial regulation standards
- Interaction with other relevant bodies i.e. ethical review committees
- Provision of expert advice and guidance on what is expected of vaccine quality and safety assessment
- Establishment of early dialogue with researchers/vaccine developers/manufacturers

A strong national regulatory agency with established written guidelines on these processes and clear pathways to trial approval and licensing will ensure the definition of vaccines of assured quality for use in the population.

1.2.8 Conclusion

In conclusion, given the broad range of vaccine concepts being developed for candidate malaria vaccines, judgment based on the best science available will form the rationale of the type and extent of non-clinical evaluation of candidate vaccines. 'The best science available' should therefore be reflected in the development pathway of a candidate from the researcher's bench to the clinic and aim to address the following considerations:

- Define vaccine candidate and characterize it as much as possible
- Select the parameters essential for consistency demonstration
- Choose adjuvant based on a scientific rationale
- Identify and justify relevant animal models
- Determine animal models for demonstration of safety
- Characterize desired vaccine induced immune response
- Standardize key assays and develop and establish working standards
- Consider product profile and target population

1.3. Reviewing the developmental pathway of lead malaria vaccine candidates

(Contributors: Carter Diggs, Ann Stewart, Evelina Angov, Alan Thomas, Pierre Druilhe; session chaired by Carter Diggs)

1.3.1 Considerations

Knowledge on how protection is induced and what are the critical effector mechanisms in the development of immunity against malaria should lead to studies that identify sensitive and specific surrogate markers of this protective immunity. The availability of surrogate markers could allow screening and prioritizing candidate antigens, design optimal formulations, select efficient production processes, determine robust pre-clinical tests and confirm efficacy in field trials.

In reality, this knowledge is lacking, and there are today no reliable and undisputable correlates, and actually little research devoted to that end.. For the most part, antigens have been selected and promoted to the status of “candidate vaccines” based on their ability to induce some measurable efficacy in one of the numerous and diverse existing in vivo or in vitro models, e.g. identification of possible effector mechanisms 'likely' to play a role in protective immunity , “promising “immune response in animal models or assays. Thus,, any proof-of-concept, on whether the response is protective or not, remains to be demonstrated at large-scale efficacy trials.

Given the number of potential candidates, and the need to compare performance among them, a review and comparison of vaccine development rationale for each candidate would be informative and could help identify ways to improve quality of evaluation and comparability of data.

Meeting participants reviewed the experimental *in vitro* and *in vivo* models that have played a critical role along the development pathway of current vaccine candidates; from discovery, selection, concept development, formulation optimization, pre-clinical evaluation and early proof-of-concept, clinical trials and product refinement. They also considered the contribution of immuno-epidemiological data in determining an antigen's vaccine potential. How this knowledge has been used for some candidates, currently in or about to enter clinical trials, was presented and analyzed.

These considerations are particularly critical for blood-stage vaccine development, whereas the evaluation of pre-erythrocytic vaccines has been facilitated through the human sporozoite challenge model. Phase 2a challenge trials can demonstrate efficacy at an early stage and although the predictive value of this model in terms of both efficacy

and duration of efficacy is not consistent, it has acted as a valuable screening measure. In contrast for blood-stage vaccines Phase 2a models have yet to be designed , and if they can be developed, then optimized and validated.

1.3.2 Current Malaria Vaccine Candidates - vaccine development rationale

Ideally, a rational approach to the development of vaccines could follow the proposed framework:

A) Determine key protective immune responses and their correlates or surrogates markers; develop quantitative and qualitative measures of the markers

B) Employ those surrogates to

a) Discover antigens of vaccine potential

b) Screen and prioritize candidate antigens

c) Optimize and select vaccine formulations designed to induce maximal desired immune response

d) Select optimal production processes

e) Develop and standardize assays that measure the surrogates and validate their potential predictive value in clinical trials

f) Defining end-points that maintain coherence of the developmental pathway, and will be validated in clinical trials

However, absence of immune correlates means that current development is empirical. Vaccine development rationale and the evaluation of a candidate antigen, can differ depending on the particular hypothesis about protective effector mechanisms justifying vaccine potential. The current empiric approach is necessary, but does not preclude that evaluation tools remain coherent for a candidate vaccine throughout its development pathway. The empirical methods should also be refined as further understanding of protective immunity develops, and the strive towards a more rational approach as proposed above should continue in parallel.

1.3.2.1 Pre-erythrocytic vaccines -

Observations that sterile protection could be artificially induced through vaccination with irradiated sporozoites have played a critical role in the emphasis on the research and discovery of the few major antigens being developed as candidate vaccines. These are expressed either at sporozoite , or liver stage. The liver stage has attracted considerable attention as it is the only phase of the malaria life cycle where the parasite lies in a host cell expressing MHC molecules.

The first target antigen discovered , the circumsporozoite protein (CS), which coats the surface of the sporozoite, has attracted the largest number of clinical studies.
The recent discovery of a peptide sequence that binds to the surface of liver cells in a sporozoite invasion inhibition assay was thought to play a critical role in liver invasion[4,5]. In clinical trials with synthetic subunit vaccine constructs, however, the protein was poorly immunogenic, and few subjects have been protected[6,7]. Various modifications to enhance immunogenicity were attempted, none of which correlated with improved

efficacy of the antigen until a breakthrough in development occurred with the creation and formulation of the virus-like particle known as RTS,S with a proprietary novel adjuvant, described below.

A hybrid molecule recombinantly expressed in yeast, consisting of the circumsporozoite protein, $(NANP)_{19}$ central tandem repeat epitopes, and carboxyl-terminal regions are fused to the N terminal of the S antigen of hepatitis B virus (HBsAg) in a particle that also includes the unfused S antigen. The lyophilized antigen is reconstituted in AS02A adjuvant (proprietary oil in water emulsion with the immunostimulants monophosphoryl lipid A [MPL; Corixa, Seattle, WA, USA] and *Quillaja saponaria* fraction 21 [QS21; Antigenics, New York, NY, USA] previously known as SBAS2).

A Phase 2a challenge trial (with SBAS2) , briefly protected 6 of 7 participants, with various formulations inducing high levels of CS protein-specific Abs and proliferative T-cells responses which did not correlate with protection [8,9,10]. Assays have been used to analyze the vaccine-induced responses and explore biological function of the vaccine but results are not conclusive. Primate experiments showed induction of robust antibody responses and strong antigen-specific delayed hypersensitivity, and mice experiments showed proliferative and cytolytic T-cell responses.
Mice and primate immunization studies have shown that specific antibodies opsonize *P. berghei* and *P. knowlsei* sporozoites to promote their phagocytosis by macrophages for subsequent intracellular degradation. An in vitro assay set-up to detect and compare opsonizing activity of pre- and post-immune plasma from RTS,S/AS02 vaccinees, showed that CS protein-specific antibodies formed Ag–Ab complexes with soluble fluoresceinated- *P.falciparum* CS protein (Fl-Pf CS protein) and that these complexes were endocytosed by the THP-1 monocytic cell line. Electron microscopy revealed that exposure to immune plasma promoted phagocytosis of intact *P.falciparum* by the THP-1 cells, demonstrating the in vitro opsonizing capability of RTS,S-induced antibodies, and suggesting a role for antibody-mediated opsonization of infectious sporozoites in the protection induced by the RTS,S/AS02 vaccine[11]. In another experiment,
IFN-γ responses against a pool of *P. falciparum* CS protein peptides were compared in 20 participants vaccinated with RTS,S. The ELISPOT assay detected differential IFN-γ responses of CS protein-specific T cells that distinguished protected from nonprotected subjects. Moreover, both CD4 and CD8 T cells secreted IFN-γ specifically in response to CS protein peptides, and T cell reactivity was sustained in 2 out of 7 subjects with protracted protection[12].
This is the first demonstration that a possible relationship exists between RTS,S recombinant protein malaria vaccine induced protection from *Plasmodia* sporozoite challenge study and peptide-specific CD4 and CD8 T cells producing IFN-γ. In addition, the persistence of elevated IFN-γ responses observed in 2 subjects with protracted protection suggests that memory T cells may have been induced by RTS,S. However, past studies of experimental malaria vaccines have observed a robust induction of Ag-specific T cell response marked by either IFN-γ or CTL in the absence of protection. Field-based malaria vaccine studies are in progress to validate the establishment of this cellular response as a possible in vitro correlate of protective immunity to pre-erythrocytic stage malaria vaccines.

The role for assays in terms of selection of different RTS,S formulations was discussed. The ELISA and IFN- γ and IL-5 ELISPOT assays have been used to evaluate different formulations of RTS,S with Adjuvant System 02A, 01B and 05. Results showed superior responses for the RTS,S/AS02B formulation. Six months later, a follow-up tuberculin skin test to evaluate response to the antigen showed the maximal reaction of the same formulation. Currently, this formulation is in early clinical trials.

Another pre-erythrocytic vaccine candidate discussed was LSA3 which was discovered through screening a subset of clones with sera from protected humans(natural protection and induced by irradiated sporozoites) and animals to identify antigens of vaccine potential. The LSA3 molecule was preferentially recognised and this observation was further extended using 3 peptides derived from the initial DG729 clone[13].
The vaccine potential of this molecule has been substantiated by the animal immunization heterologous challenge studies.

Various animal immunization-challenge models have demonstrated protection against heterologous challenge:-
a) *P. yoelii* challenge in mice[14]
b) *Aotus trivirgatus grisemembra* monkeys against a *P. falciparum* sporozoïte challenge[15]
c) chimpanzees against several successive *P. falciparum* sporozoite challenges[16,17]- more than 80 challenges were performed in both LSA3 immunized and control animals, with some animals undergoing 2,3 or even 5 successive *P. falciparum* challenges with large numbers of sporozoites (10^5-$8x10^6$). Finally, protection was obtained in challenges made up to a year post-immunisation, both in chimps and *Aotus*.

These challenge models also play a role in screening various delivery systems to optimize vaccine design which showed the four antigen delivery systems that induced protection against *P. falciparum*, namely: a) micro particles without adjuvant[18], b) lipopeptidic formulations without adjuvant[19,20], c) recombinant proteins or LSP's adjuvated by GSK-ASO2[21], d) DNA genetic immunisation[14].

To further dissect the vaccine-induced immune responses, different regimens of LSA-3 DG729 as well as the combination of the N-term DG729 with the repeats called NN and the C-term called PC, as well as the Long Synthetic Peptide GP1 were studied in experiments involving over 1000 mice. These mice experiment showed heterologous species protection against a live *P.yoelii* sporozoite challenge[22]. The protection achieved by this interspecies immunisation showed that the same extent of protection was induced using either Freund complete adjuvant, ASO2, Montanide, as well as lipopeptide formulations, microparticulate formulations without adjuvant, or genetic immunisation. Prime–boost regimens of various kinds did not show any advantage over single formulation regimens.
The above vaccines formulations induced various types of immune responses including moderate antibody responses and strong T-cell responses in C3H mice as well as Balb-C or C57/Bl6, , except for DNA immunization, which induced only ELISPOT responses. Protection was associated with high interferon-γ production and usually moderate to low antibody and T-cell responses..

1.3.2.2 Blood-stage vaccines

Clinical disease results from parasite multiplication and growth by merozoite invasion of erythrocytes. Therefore, the identification of the molecular mechanisms of merozoite invasion of red blood cells has been a logical target of blood-stage malaria vaccine research. Multiple blood-stage vaccine concepts can be clustered around putative mechanisms of vaccine induced protection - i.e. induction of antibodies that inhibit parasite growth directly or indirectly, or through Th1 responses - demonstrated via in vitro assay systems or protection in relevant animal models.

The leading blood stage vaccine candidate and one of the most studied antigens is the merozoite surface protein 1 (MSP1). A 195 kDa protein found on the surface of merozoites, it undergoes processing by proteolytic cleavage to a 42 kDa fragment and further to a 19 kDa fragment that has been implicated in the invasion of erythrocytes by the merozoite[23]. Evidence of vaccine potential has been accumulated through various experiments with the tools under consideration and multiple versions of this antigens with different expression and delivery systems and formulation is currently being developed.

A recombinant version of MSP1 has been produced at the Walter Reed Army Institute of Research, FMP1 (Falciparum merozoite protein 1), as a lyophilized $MSP1_{42}$ produced in and purified from *E. coli* bacteria. The antigen is the 42 kDa carboxy-terminal end of MSP1 comprising 392 amino acids and contains both T-cell and B-cell epitopes. It is derived from the 3D7 clone of *P. falciparum* malaria parasite, and is expressed as a fusion protein to which six histidine residues are added to the N-terminus to facilitate purification.

The role of assays and animal models in characterizing the induced immune response and helping down-select various antigen formulations[24]is described below. The criteria used for selection of this antigen are summarized below:

- Epidemiological correlations between immune sera recognition of antigen by malaria-exposed individuals and a reduction in parasitemia and morbidity
- Surface localization on merozoites and susceptibility to antibodies - monoclonal antibodies raised against native parasite MSP1 recognize correctly folded conformational disulfide-bonded epitopes within the recombinant 42 kDa antigen.
- Ability of antibodies raised against antigen to exert an anti-parasitic effect *in vitro*- induction of MSP-1 fragment specific antibodies; and induction of antibodies that demonstrate acivity in growth inhibition and process inhibition assays.
- Ability of the antigen, as immunogen, to exert a protective effect in animal models of malaria- protection demonstrated against homologous challenge strain
- Ability to manufacture to cGMP
- Safety and immunogenicity in Rhesus monkeys, rabbits and mice- the formulation has been shown to be safe and highly immunogenic in pre-clinical testing conducted in monkeys and mice.

The vaccine is formulated in the same adjuvant system used in the RTS,S vaccine, AS02A. A comparison of total antibody response[25] in rhesus monkeys between alum with AS02 formulations led to the selection of the AS02 formulation based on a higher titre. Like RTS,S, the $MSP1_{42}$ antigen is manufactured as a lyophilized product and reconstituted just prior to injection. The vaccine has undergone 3 phase Ib safety and immunogenicity studies, in two areas of different malaria transmission intensity (high/western Kenya, and low/Mali). A phase 2b pediatric trial is being planned for spring 2005.

Another highly investigated candidate is the apical membrane antigen 1 (AMA1), an 83-kDa type I integral membrane protein with an ectodomain organized in three domains stabilized by eight disulfide bonds. Initially located in the merozoite apical organelles, it is processed to a 66-kDa form that relocates to the surfaces of mature merozoites. Its stage specificity and location suggest The ability of antibodies directed against AMA1 to inhibit invasion of RBCs *in vitro* lends support to the hypothesis of its' involvement in the process of invasion of host red blood cells (RBCs).. The vaccine potential of this candidate is supported by the following findings:-

d) The antigen has a ready homologue in all malaria species

a) Strain and species specific protection is induced in animal (NHP, rodents) immunization challenge model studies[26,27,28]

b) Human affinity-purified antibodies, and those raised from rabbits inhibit merozoite invasion of erythrocytes[29,30,31]

b) The protein is thought to be essential protein - evidence from gene knockout testing

c) AMA1 sequence analysis shows strong diversifying selection, likely in response to immune pressure[32, 33]

d) Correlation of pre-existing AMA-1 antibody levels with disease outcome, association of point mutations with degree of disease severity[34, 35]

An outline of some critical steps in the preclinical and clinical development plan of the AMA1 candidate being developed by BPRC was presented at the meeting, and described below:

- The vaccine molecule conformation as the protective efficacy of this molecule requires its native confirmation.
- Stability of formulation (SDS-PAGE)
- Safety and immunogenicity in rabbits
- Induction of invasion-inhibitory antibodies in rabbits and rhesus (quality and quantity)
- Animal immunization challenge efficacy model - *Aotus* monkeys with antigen formulated in Complete/Incomplete Freund's Adjuvant

- Safety and immunogenicity in Rhesus monkeys comparing three formulations with Alum, ISA720 and AS02
- Stability, Potency and Toxicology studies on the selected formulation

In the planned Phase Ia trial of the vaccine, along with the standard primary end-points of safety and reactogenicity, secondary evaluation criteria will include ELISA for IgG and IFA read outs, as well as analysis of proliferative responses with T cell proliferation and ELISPOT assays for IFN- γ and IL-4. Furthermore, assays will also be conducted to analyse the quality of humoral response through isotype, domain specificities and avidity analysis. Finally functional assays to measure the biological activity thought to be mediating protection will be measured, including growth inhibition, strain specificities, blocking and processing inhibition assays.

The *Plasmodium falciparum* Chimeric Protein 2 (PfCP-2.9) is a hybrid molecule made up of the C-terminal regions of two leading malaria vaccine candidates, domain III of apical membrane ag-1 (AMA-1) and 19-kDa C-terminal fragment of the merozoite surface protein 1 (MSP1), derived from the k1 line[36]. It is a 26.86 kDa of chimeric protein consisting of 241 amino acid residues and contains eighteen cysteine residues, of which six are located in AMA-1 (III) and the rest in MSP1-19, to form nine intramolecular disulfide bonds. Produced by *Pichia pastoris*, analysis of conformational properties of the chimeric protein showed that all critical conformational epitopes were retained.

Anti-PfCP-2.9 sera from both rabbits and rhesus monkeys almost completely inhibited in vitro growth of the *P. falciparum* FCC1/HN and 3D7 lines when tested at a 6 to 7-fold dilution. It was shown that the inhibition is dependent on the presence of Abs to the chimeric protein and their disulfide bond-dependent conformations. Moreover, the activity was mediated by a combination of growth-inhibitory Abs generated by the individual MSP1–19 and AMA-1(III) of PfCP-2.9.

PfCP-2.9 was found to be highly immunogenic in rabbits as well as in rhesus monkeys (*Macaca mulatta*). The chimeric protein induced both anti-MSP1–19 and anti-AMA-1(III) Abs at levels 11- and 18-fold higher, respectively, than individual components did.

Merozoite Surface Protein 3 (MSP-3) is an antigen identified by a novel approach where the protection which could be passively transfered by African IgG into naive infected subjects[37,38] was used to identify a mechanism of defense. The latter, an antibody-dependent cellular inhibition (ADCI) mechanism of parasite killing mediated through monocytes[39], was used to screen a *P. falciparum* genomic expression library and to eventually identify an antigen, MSP-3, as being the target of antibodies with protective effect in human beings[40].

The decision to move from pre-clinical investigations into clinical trials with a MSP-3 based vaccine resulted from convergent data from a series of studies which all strengthened the association between protection and antibodies directed to this particular antigen, namely a) anti MSP3 antibodies, either naturally occurring or elicited by immunisation, could achieve parasite killing in the presence of normal monocytes, either under *in vitro* conditions[40]or b) *in vivo* by passive transfer in *P. falciparum* infected immunocompromised mice[41]; c) similar results were obtained with a human recombinant anti-MSP3 monoclonal antibody (Dziegel et al submitted); d) IgG3 anti-MSP-3 antibodies

were associated with protection in the 2 African villages of Dielmo and Ndiop[42]and in the village of Oo-do in Asia[43] e) IgG3 anti-MSP-3 antibodies were associated with an improved prognosis of drug treated cerebral malaria[42]; f) a strong protection was induced by MSP-3 in Cebidae monkeys against a *P.falciparum* challenge[44] further arguments were g) the full conservation of the C-terminal part of the antigen containing the epitopes targetted by ADCI[14 15]and h) the immunogenicity of all proteic and peptidic formulations tested and particularly a synthetic polypeptide covering 3 B-cell epitopes targetted by protective antibodies [42](and Oeuvray et al unpublished material).

A first synthetic vaccine construct was derived from regions fully conserved among various strains and containing B-cell epitopes targeted by human antibodies (from malaria immune adults) that are able to mediate a monocyte-dependent, parasite killing effect. The corresponding long synthetic peptide was administered in Phase I trial to 36 volunteers, with either Alum or Montanide as adjuvant. Both formulations induced cellular and humoral immune responses. With Alum, the responses lasted up to 12 months. The vaccine-induced antibodies were predominantly of cytophilic classes, ie. able to cooperate with effector cells. *In vitro*, the antibodies induced an inhibition of the *P. falciparum* erythrocytic growth in a monocyte-dependent manner, which was in most instances as high or greater than that induced by natural antibodies from immune African adults. *In vivo* transfer of the volunteers' sera into *P. falciparum* infected humanized SCID mice abrogated parasitemia. These inhibitory effects were related to the antibody reactivity with the parasite native protein, which was seen in 60% of the volunteers, and remained in samples taken 12 months post-immunisation.

The glutamate rich protein (GLURP) belongs to a group of soluble antigens known as exoantigens and like MSP3, has *in vitro* ADCI activity of cytophilic antibodies, and the strength of immunoepidemiological associations between protection status and high responses to the antigen as the basis of its vaccine potential[45,46].

Immuno-epidemiological association of anti-GLURP antibodies and protection against clinical disease has been demonstrated in numerous studies in Africa and one in Asia[43]. Predominance of cytophilic subclasses against GLURP in protected individuals was demonstrated in independent immunoepidemiological studies. Affinity purified antibodies against nonrepeat as well as repeat epitopes of GLURP inhibit parasite growth *in vitro* in an ADCI manner. Evidence has accumulated that cytophilic antibody responses to the glutamate-rich protein (GLURP) play a role in protection against *P. falciparum* malaria[47].

Four B-cell epitopes have been identified in the nonrepeat R0 region as targets of ADCI-active human antibodies[48]. They are almost completely conserved within 44 tested isolates and 14 laboratory lines of *P. falciparum*[49]. Of these, P3 may be potentially the most important epitope since affinity-purified human antibodies against P3 mediated the strongest ADCI effect *in vitro*.

Variations of the P3 epitope have been produced as long synthetic peptides(LSP) and humoral and cellular immune responses to the LSPs in sera of different populations exposed to natural infection were evaluated.

Level of cytophilic antibodies against the LR67 LSP was significantly correlated with protection. Proliferative responses showed that LR67 contains T-cell epitopes recognized by malaria exposed individuals.

Immunogenicity studies with mice confirmed the results from human T-cell studies. The relatively high prevalence of T-cell responses in immune individuals, as well as the strong immune responses consistently elicited by LR67 in five strains of mice with different *H-2* haplotypes suggests that there may be little genetic restriction against this immunogen.

These results support the choice of LR67 as a valuable synthetic molecule for further immunogenicity studies.LR67 has been tested in a Phase Ia vaccine trial comparing the antigen formulated with Alum and Montanide ISA720.

The SERA based vaccine is a recombinant protein molecule SE36 (resulting from removal of serine repeats from SE47); produced in *E.Coli* with synthetic DNA
The SERA/P126 protein was first identified using a parasite inhibitory mouse monoclonal antibody, 43E. With 989 amino acids including a repetition of 35 serine residues, it is the largest parasite protein that accumulates in the parasitophorous vacuole of trophozoites and schizonts and is processed into three fragments (47kDa, 50kDa, 18kDa) [50].

Cross-sectional studies conducted in Uganda [51], Solomon Islands and Brazil [52] have shown an association between responses to SERA based constructs and lower parasitemia. An association was also observed between higher levels of cytophilic antibodies (IgG1 and IgG3) responses and lower parasitemia.

In vitro assays of antibodies raised (in rats) or affinity purified human antibodies against SERA constructs - Antibodies raised in rats by immunization with recombinant *E.Coli* produced SE47 inhibit *in vitro* parasite growth [53]. Isotype analysis showed that antiserum produced contained SE47 specific IgG2 and IgG3 that were highly inhibitory. The addition of guinea pig complement enhanced this effect which was absent if complement was heat inactivated. Incubation of schizonts with the IgG2 and IgG3 and complement resulted in abnormal schizonts with degenerated nuclei- suggesting that complement mediated lysis may play a role in the enhancement of parasite inhibition [54].

In a comparison of direct and indirect mechanism of action of SERP using mouse and human antibodies, monoclonal antibodies specific for 2 epitopes from N terminal of SERP and recognizing the 126kDa protein on western blots of *P falciparum* asexual stage extracts showed significant ADCI activity; with parasite growth inhibition dependent on antibody concentration. In contrast, no significant direct inhibitory effect was seen [55].

SE47 (residue 17-382) a recombinant protein was used to affinity purify specific antibodies from immune adults (Ivory Coast and Uganda). The resulting anti-SERP antibodies had no direct parasite growth inhibitory effect but had high ADCI -mediated inhibitory effect in a dose dependent manner

In a study of 40 Ugandan adults serum samples comparing ELISA reactivities to N terminal domains of SERA proteins, and MSP1-19: SERA3,-4-5-6, showed all SERA

proteins were highly immunogenic; anti- SERA5 titres positively correlated to serum inhibition of parasite growth; this was not seen with MSP 1-19 [56].

In animal model immunization challenge studies protection(1,000 fold reduction in peak parasitemia) following immunizations with two recombinant proteins from N terminal region of SERP was observed in four out of six *Aotus* monkeys following challenge with blood stage *P.falciparum* parasites. However, the level of protection was not strictly correlated with antibody levels [57,58]. Protection following immunizations with recombinant SE47 expressed in *E.Coli* was observed in two out of four *Saimiri* monkeys following challenge with blood stage *P.falciparum* parasites. The humoral responses were noted to be boosted after challenge [59]. Protection induced after immunization with recombinant SE36/Alum in two monkeys following challenge, which boosted anti-SE36 titers.

This candidate vaccine will be in Phase Ia trials in Japan in January 2005.

1.4. Rodent Models

(Contributors: Denise Doolan, Mats Wahlgren, Pierre Druilhe; Session chaired by Mats Wahlgren)

1.4.1 Considerations

Rodent models have played an important role in the acquisition of current knowledge in malaria vaccine research and development. Among the available laboratory rodent models, affordability, accessibility and the availability of diverse combinations of strains and species mice model systems have made these models the workhorses of malaria vaccine development as tools of immunological and genetic experiments.

Immunization-challenge models from sporozoites or parasitized RBC of *P. yoelii, P. berghei,* and *P. chabaudi* provided the rationale for further clinical development of some candidate antigens or delivery platforms. In actual fact, comparability is difficult as models are numerous (large number of parasite species or strain -mouse strain combinations) not standardized and route, method of challenge and outcome measures vary greatly. Yet the first clues of an antigen's immunogenic and protective potential required evaluation in these modelsleading to conclusions that drive candidates further down the pipeline.

In immunization-challenge models, the outcome measure used to define 'protection' is an important consideration. For pre-erythrocytic protection, the absence of blood stage infection or delay in patency is the usual outcome measure in the sporozoite challenge model. In addition, liver stage parasite burden can be assessed by measuring the reduction in the number of late liver stage parasites following challenge in the pre-erythrocytic sporozoite challenge model.

For protection against blood-stage challenge, using either sporozoites or parasitized RBCs, a reduction in peak parasitemia, prolongation of the pre-patent period, and protection from mortality are usual outcome measures using lethal and non-lethal strains of parasites.

For the evaluation of immunogenicity, outcome measures depend on whether humoral, cellular or functional immune responses are being measured. Humoral responses that can be characterized in these models are either parasite specific assays like immunofluorescence assays/IFA against sporozoites, liver-stage and/or blood-stage parasites or antigen specific like ELISA subclasses against recombinant proteins or synthetic peptides).

Cellular immune responses defined by CD8+ and CD4+ T cells, Th1 and Th2 responses can be characterized through a variety of assays such as ELISPOT, intracellular staining, multi-parameter flow, tetramer, CTL responses etc.

Outcome measures of biological or functional activity can also be assessed by *in vitro* assays such as the GIA, and ADCI. Additionally, parameters that can be assessed and

compared include the frequency and magnitude of the response, the kinetics and duration and breadth of CD8+ and CD4+ cells and antibody response. Furthermore, delineation of not just the quantity or magnitude but the quality or fine specificity of the antibody response can be characterized by identification and analysis of the immunodominant, subdominant or conformational epitopes as well as comparisons of the proportion of responder against non responder activation.

The approach taken by the malaria vaccine research group at the US Naval Medical Research Center in using these models as tools for evaluation of either vaccine potential of an antigen, formulation or delivery platform was discussed. The importance of maintaining a detailed experimental plan describing the number of groups, number of animals per group, vaccine dose and immunization regimen, control parameters, predefined outcomes and go/no go criteria as well as a statistical analysis plan was emphasized. This was essential to ensure systematic and valid analysis of the experimental process.

An initial screening study is designed to evaluate capacity of the vaccine concept or technology to enhance immunogenicity and/or protective efficacy, followed by a confirmatory study if the results from the screening study are significant. These studies can be further expanded in a systematic way to conduct a comprehensive evaluation that includes dose-ranging considerations; multiple antigens form multiple stages, and even designed to evaluate capacity to overcome genetic restriction.

Critical to the approach are predefined go/no go criteria. For example, the outcome measure in a prime-boost approach is a $p<0.05$ and a more than 10-fold enhancement after viral boost in mice of select T cell responses against pre-erythrocytic antigens, PfCSP, PfSSP2, PfLSA1 or antibody responses against erythrocytic stage antigens PfMSP1, PfAMA1, detected in at least 4 of the 5 antigens tested, and the absence of significant inhibition in the other antigens. In the event that these predefined criteria are not met, the development of the candidate vaccine is halted.

All this ensures an optimized approach to screening and evaluating maximum number of potential concepts or vaccine technologies with minimum number of animals while minimizing the risk of incorrectly rejecting useful approaches.

1.4.2 Advantages

Meeting participants discussed the fact that, affordability, relative ease of handling, and wide availability of these models meant that they have played a crucial role in both the discovery research as well as the preclinical development of current malaria vaccine candidates, despite the acknowledged limitations of relevance. For example, in terms of selection of antigenic targets for candidate vaccine development, the demonstration of protective efficacy in murine models (orthologue), characterization of biological function of the antigen, and increasingly, the role of gene knock-outs and knock-ins are used in the decision-making process to prioritize. Rodent models may also allow a more thorough investigation of immune mechanisms such as the characterization of immune responses induced by vaccination, the identification of immune correlates of vaccine induced protection and the investigation of host or parasite genetic factors in susceptibility or resistance.

Further roles in preclinical development include the identification and prioritization of vaccine technologies, development of optimal formulations to take to further development, and investigation of optimal vaccine technologies.

Some participants noted that these models may also help overcome the challenge of how to combine multi-antigen, multi-stage antigens by providing a cost-effective and efficient mechanism for early evaluation of these combinations. The investigation into these multi-antigen combinations, including for possible interference as well as the optimization of immunization regimen with regards to the route, and interval can hardly be investigated in any other models. All these studies may be crucial steps in the critical path of product development (i.e. towards the application for an investigational new drug (IND) file) of the clinical vaccine material and lead to well-defined parameters for quality assurance and quality control, release criteria, vaccine formulation and product characterization, lot to lot variation, in vivo potency assays, safety and toxicology studies that meet regulatory and licensing requirements.

From the discovery and selection of the antigen, these models play a role in further experiments seeking evidence or data to prove a novel vaccine concept and may answer important questions related to the concept such as effect of pre-existing immunity to *Plasmodium* pre-erythrocytic or blood stage antigens, and the effect of pre-existing immunity to viral vectors. These models can also be used for investigating approaches to overcome pre-existing immunity issues and the investigation of protective efficacy and immune mechanisms of vaccine candidates in neonates versus adults.

1.4.3 Limitations

Despite their advantages, a major limitation discussed was that that these models are unnatural host parasite combination that have characteristics particular to each species (and each line or clone). The acute, often lethal infection established in these hosts are very different from the chronic infection in natural host parasite combinations. Also discussed was the fact that host specific antigenic targets may not be relevant to natural infection as these may differ from natural host parasite combination - i.e. antigenic targets in a chronic infection (selection for chronicity or fine molecular tuning) may likely differ from those of an acute infection. In addition the mouse immune system is obviously far from that of humans : some vaccine candidates can be highly immunogenic in rodents and poorly immunogenic in humans. Therefore, the use of these models, in particular for determination of the vaccine potential of an antigen, was questioned.

In addition, some participants noted that determination of the vaccine dose per kilogram body weight is not helpful for dose determination in clinical trials because it does not correlate with human doses.

It was suggested that the interpretation of the role of mice models can be as negative predictor (*vs.* Positive Predictor) where it appears that vaccine technologies or strategies that do not work in mice will not work in humans BUT those that work in humans do work in mice. Therefore rodent models can be considered to have a negative predictive value for potential success in the clinic. However this still will not eliminate the risk of rejecting a potentially useful concept or approach.

1.4.4 Conclusions and Potential Improvements

These models have historically played a key role in the discovery and evaluation of malaria vaccines and will most likely continue to do so, for quantitative and financial reasons. Models do not have to faithfully mimic every aspect of the human system; but measured outcomes in the experiments should be related back to the real system to demonstrate relevance. Improved interpretation of the output from experiments in these models to evaluate candidate vaccines require a systematic effort to better define these models and delineate the relevance of current models.

Participants discussed that it would be useful to develop a coherent strategy to compare and evaluate the relationship between read-outs in mice versus non-human hosts and then the target hosts so that the relevance of the model and its predictive value will eventually be determined.

There should be support for research efforts to develop better, improved more relevant models using recent advances and the current knowledge. The development of new models such as the P.falciparum-SCID mouse model which allows for parallel investigation of the *in vivo* effect of immune response with the corresponding *in vitro* assay response can be very useful in investigating immune mechanisms and correlates of protection. Advances that have led to the creation of transgenic mice and chimeric parasites where replacement of corresponding mouse antigen with human parasite antigen contribute to the improved specificity of the resulting mouse model is another example of model improvement that should be supported. Examples of new models were addressed in a separate session at the meeting contained in this report under -Future needs and ways forward.

1.5. Non-human primate models

(Contributors: John Barnwell, Anne Stewart; session chaired by Myriam Arevalo-Herrera)

1.5.1 Considerations

Given their morphological and genetic similarities to humans, non-human primates appear intuitively to be the best host for studying human disease. However, some primates are not as close to humans in physiology, cell surface antigens and immunological response as one would assume. In view of the practical and ethical issues surrounding experimentation with non-human primates, the rationale for using them to acquire information to help guide vaccine research and development decisions should be clear and specific.

Lack of reliable surrogates of protective immunity mean that decisions in vaccine development are made based on a series of assumptions that can be validated only after an efficacious vaccine has been developed. Therefore, despite debate and uncertainty over the relevance of non-human primates as a surrogate of either human malaria or immunity, they remain the only means to directly test the immunogenic potential of malaria vaccine candidates before injection into human volunteers.

Primate hosts and simian models for *Plasmodium falciparum* and *Plasmodium vivax* include humans, chimpanzees, New World Monkeys (the *Aotus* and *Saimiri* species), rhesus monkeys (*Macaca mulatta*) and African cercopithicine monkeys (baboons etc.) Chimpanzees, in addition to their genetic similarity to humans, are also fully susceptible to mosquito borne malaria and are amenable for detailed immunological investigations of malaria vaccine candidates of various stages (liver-stage, blood-stage, transmission-blocking). However, scarcity, cost and strong ethical constraints will always limit their use. Currently, the most widely available and utilized non-human primate models are the New World Monkeys (the *Aotus* and *Saimiri* species) and the rhesus monkeys (*Macaca mulatta*)

1.5.2 New World Monkeys

The *Aotus* malaria models have been most useful in studying blood-stage infection for *Plasmodium falciparum, Plasmodium vivax* and *Plasmodium malariae*. In addition, these models can also be used for mosquito transmission and susceptibility studies, and sporozoite induced infections and liver-stage studies. The *Saimiri* monkey models are most useful in similar studies for *Plasmodium vivax* infections, although they can be used to study *Plasmodium falciparum* blood stage infection as well.

In evaluation blood stage infection and corresponding antigens, *A. nancymai, A. vociferans, A. l. lemurinus,* A. *l. grisemembra* and *Saimiri. b. boliviensis* are the most suitable hosts. Splenectomy is usually necessary in *Saimiri sciureus* for *P. falciparum* or *P. vivax* blood stage infection models.

Among the numerous parasite strains adapted and suitable for use in these models are the following: FVO (Vietnam), FUP (Uganda), Malayan Camp (Malaysia), FMG (Gambia), Santa Lucia (El Salvador), FCH/4 (Philippines), Geneva (West Africa), and Indochina I (South East Asia).

In evaluation of pre-erythrocytic vaccines, the best host-parasite combinations are *Aotus l. grisemembra* with *P.falciparum,* or *P. vivax* and *Saimiri b. boliviensis* with *P. simium.* As very few *P. falciparum* strains are suitable for use in these models and host splenectomy is necessary, the utility of these models are limited. Similarly, the fact that few *P. falciparum* strains are suitable and mostly one produces gametocytes, affects the evaluation of transmission-blocking vaccines in these models

1.5.2.1 Advantages

The susceptibility of these primates to human malaria is the main advantage as it generates efficacy data. Comparisons of parasite proteins with suspected vaccine potential in New World primate challenge trials such as *Aotus nancymai* monkeys could generate the efficacy data to help make decisions on antigen forms, expression systems, and formulations. Most of the meeting participants agreed that this data was useful before investment in clinical grade vaccine production, though the larger number of candidates and the shortage in new world primates, make them less used than in the past. Finally, as with any artificial model, including the mice models, argument remained over the relevance of this data.

There are also contrasted opinions on the role of these models to better define and dissect vaccine-induced immune responses seen in early efficacy clinical trials. New World primate trials can be designed and conducted to gather additional data needed for further refinement of the test vaccine. The models could be used to investigate correlates related to vaccine-induced responses and ultimately these trials could provide data for developing, defining or validating *in vitro* assays to use as correlates of immunity induced by the candidate vaccine. In addition, like with rodent models, NHP trials could be potentially useful in the early assessment of combination vaccines.

The participants also discussed the significance of safety data in these models. Trials in New World primates could provide useful observations of unexpected events prior to human trials. Although these observations may or may not have relevant safety or biological implications, they could nevertheless highlight a signal or trend to observe for in terms of detecting potential harms during human trials, and thus improve trial preparedness for collection of safety data.

1.5.2.2 Limitations

The availability of the primates and cost accrued due to primate acquisition, housing, veterinary care and disposition according to current animal experimentation standards are among the major practical and ethical limitations described by some participants.

In addition, scientific considerations that act as limitations are due to the fundamental biological differences given that there is more than a 40 million year evolutionary difference between humans and New World primates and this is likely manifested in the following features of the models:

- Inconsistent/variable parasitological parameters
- pre-patent period/infection rate.
- tolerance of high parasitemia.
- fast acquisition of effective immunity by infection(as compared to humans).
- development of life-threatening anemia.

These idiosyncratic responses make any extrapolation from data obtained from these models to predictability and relevance to humans difficult. There is also a limited set of *Plasmodium falciparum* and *P. vivax* isolates and antigenic types available and adapted to the models (with the limitation of the 3D7 isolate not adapted) and a paucity of analytical immune reagents. The lack of biological function of target antigens in these primates also is a limitation in the assessments.

1.5.3 Rhesus macaques

The rhesus macaque model has been useful in immunogenicity, formulation selection and safety testing of malaria vaccine candidates. These models have the major drawback over New World monkeys to be refractory to human malaria species, but are highly susceptible to P.knowlesi sporozoite or blood stage induced infections, can develop chronic infections and semi-immune states, demonstrate antigenic variation, frequent recrudescence and relapse infection patterns. These useful features allow the study of specific questions about the P.knowlesi ortholog of a candidate antigen's biological function and the immunological responses induced.

1.5.3.1 Advantages

Genotypically, these monkeys are more homologous to humans than New World primates and could be assumed to have more relevant immune responses to study.

The large size of these monkeys compared to other models allows adequate sera to be collected for safety and immunogenicity analysis and provides for large amounts of reagents for immunological assays.

The availability of the monkeys and extensive historical data accumulated on these models are added advantages.

1.5.3.2 Limitations

Similar cost considerations limit the utility of these models and the fact remains that these models cannot be used as challenge models for *Plasmodium falciparum* malaria.

1.5.4 Conclusions and Potential Improvements

Cost and availability considerations as well as the increasing pressure to limit animal testing will ultimately lead to increasingly stringent cost-benefit analysis of non-human primate testing versus human trials where true efficacy data can be assessed.

As stated previously on rodent models, it is not necessary that a model faithfully mimics every aspect of the real system in order to provide useful information. However, in the case of non-human primates, particularly with experiments evaluating safety and immunogenicity, it is crucial that the features that are truly mimicked are identified,

characterized and ultimately, validated. Clinical trial experience must help to retroactively determine which models can best fit the needs and goals of the research and development pathway.

There is room for improvement of the quality and relevance of the data generated in these models. The experience and expertise in methodological, experimental and analytical processes should be shared to promote comparability of the outcomes. As with mice models, technological advances leading to the transformation of simian parasites by the exchange of *Plasmodium falciparum* or *Plasmodium vivax* protein antigen domains in orthologous candidate vaccine antigens of simian malarias - creating transgenic chimeric simian malaria parasites - can allow for even more detailed analysis of these candidate antigens in these infection models and increase the usefulness of these models.

1.6. Immunoassays in malaria vaccine development

(Contributors: Carole Long, Pierre Druilhe, Evelina Angov, Anne Stewart; Session chaired by Martin Friede)

1.6.1 Considerations

Immunoassays are widely utilized in malaria vaccine research and development and can generally be considered under the following categories-:

- **Structural and physical assays** - In order to characterize the antigen or protein, these assays are developed and used to determine protein purification, concentration, heterogeneity and endotoxin content. For the evaluation of clinical grade material, these assays should be designed and conducted in a manner that will meet regulatory and licensing requirements.
- **Antigenicity assays**- These assays use either panels of monoclonal antibodies or human antibodies or leukocytes, to test the reactivity of selected candidate antigens, map B and T-cell epitopes, and have played an important role in the selection of most of the current blood-stage candidate vaccines.
- **Immunogenicity and Efficacy assays** - These assays study and characterize the resulting humoral, cellular and functional immune response from experimental immunization trials of small animals such as mice, rabbits and non-human primates or humans trials with selected antigens or proteins to assess total antibody or functional antibody activity. Assays measuring total and type specific antibody response include i) , immunoflorescence assay (IFA), and Western Blots upon native parasite proteins, and ii) upon synthetic proteins ELISA, competition binding to monoclonal antibodies (fine specificity of antibodies), and the ELISA for IgG subclasses. Examples of assays that measure specific functional antibody activity include invasion or growth inhibition assays (GIA), MSP1/AMA1 process inhibition assay, antibody-dependent cellular inhibition (ADCI) assay, and assays measuring inhibition of sporozoite invasion of hepatocytes. In addition, cellular immune responses are measured by a wide variety of assays designed to measure the capacity of cells to secrete cytokines.

For the session, the discussion was focused on immunoassays that characterize humoral and functional responses of candidate antigens.

It was acknowledged that most of the assays have been developed by individual research laboratories within the context of the vaccine discovery efforts, with identification of measurable processes for parasite growth and virulence to test specific antigens. Resulting assays are stage, sometimes strain and even process specific, hampering credible and valid comparability of results due to these critical differences in methodologies and essential assay components such as parasites, cells and reagents. The participants agreed that although none of the current assays has been robustly validated, and over-interpretation of its relevance should be avoided, the optimization and

standardization of some of the assays that have played a key role in the selection and development of current vaccine candidates will lead to better comparability of results and a more transparent evaluation system. This would help avoid scepticism and distrust of the results that in turn generate controversy and uncertainty about the efficacy of the vaccines and rationale of the development pathway

At the meeting, two functional assays in malaria vaccine research were discussed, the growth inhibition assay (GIA), and the antibody-dependent cellular inhibition (ADCI) assay. In addition, the participants also considered the work by the malaria vaccine research and development group at WRAIR on the standardization of an MSP1-42 specific ELISA and the development and definition of humoral correlates of protection to the MSP1-42 vaccine candidate.

1.6.2 Growth Inhibition Assay (GIA)

This assay measures the capacity of antibodies to limit invasion or subsequent growth of *P.falciparum* parasites in red cells. Antibodies are mixed with late stage parasites and normal red blood cells in culture and the reduction of new infections measured. The biological activity demonstrated in this assay has been widely used as proof of vaccine potential for several blood stage vaccine candidates, namely merozoite surface protein-1 (MSP1) and apical membrane antigen-1(AMA1).

For example, the assay has played a critical role in the preclinical evaluation of AMA1 based constructs for further clinical development in the NIAID Malaria Vaccine Development Unit (MVDU) program. The AMA1-C1 vaccine consists of recombinant proteins from the FVO and 3D7 clones and is currently in a Phase Ib clinical trial. Preclinical experience plus data from other AMA1 trials has led to concern over parasite polymorphism and implications of narrow, strain specific protection in an AMA1 vaccine. For instance, in preclinical experiments with AMA1-FVO or AMA1-3D7 in rabbits, each recombinant induced a significant antibody response to the homologous protein, but a markedly reduced antibody response to the heterologous protein. However immunization with a combination of AMA1-FVO and AMA1-3D7 induced similar responses to both proteins. Growth inhibitory activity in the assay mirrored this response, in that the antibodies significantly inhibited growth of the homologous parasite, with degree of inhibition being much less in the heterologous parasite. Again, combining the two recombinant proteins induced antibodies that inhibit both parasites to a similar degree. Therefore, the results from the GIA suggest that combining the two strains could possibly provide broader protection in the field, justifying the combination approach for the AMA1-C1 candidate.

The assay method discussed at the meeting was developed and is being optimized and validated in the lab of Carol Long, MVDU, NIAID. In brief, parasites (synchronized in culture) and the test serum (which has undergone heat inactivation and preadsorption with normal RBCs; and then diluted with normal serum) are mixed in triplicate (0.3% parasitemia, 1% haematocrit) and incubated at 37 C for 40 hours. Cultures are then harvested, frozen and then thawed before the LDH assay is conducted on the sample.

Different laboratories and groups utilize different protocols and reagents for the conduct of this assay, subjecting the assay to external variability on top of the inherent variability contained within a biological test system. Two major variables in the assay are the read-

out methodology and the demonstration of specificity of the resulting inhibition (i.e. that inhibition is due to the IgG fraction of the test sera corresponding to the test antigen). The multiple ways of analyzing parasite growth include using slide microscopy to count parasitized red blood cells, using monoclonal antibodies, incorporation of radioactive isotopes, flow cytometry analysis of stained cells, and biochemical assays that involve the measurement of the enzyme lactate dehydrogenase (LDH). In the GIA method presented at the meeting, the assay read-out method of choice is LDH. A comparison of curves (% inhibition on anti-AMA1 rabbit old pool sera) resulting from read-outs using either LDH or SYBER Green DNA dye had shown smoother curves from the LDH assay read-outs. This stable cytoplasmic enzyme, present in all cells, is rapidly released into the cell culture supernatant upon damage of the plasma membrane. The reagents used in the assay preferentially bind to parasite LDH and not LDH from red blood cells. Therefore the inhibition of parasite growth will be detected by the decrease of LDH in culture with the test sera in comparison to pre-immune sera. Efforts at intralaboratory validation has shown good reproducibility in six independent tests, with a mean percentage of co variation of 3.5% (N=150).

The demonstration of antigen specificity is also being addressed in the optimization of this assay. Modifications have been made to address the confounding factors present in sera that can account for both non-specific growth-inhibitory activity as well as growth-promoting activity. Firstly, IgG fractionation of the test sera for use in the test, demonstrates that the inhibition is mediated by antibody. This purification step is critical to remove non-specific inhibitory activity. In addition, antigen specificity of the inhibitory activity can be demonstrated by the ability of the antigen in question to reverse the inhibition when it is added to the assay set-up.

Furthermore, the ability to quantify this specific inhibitory activity is now possible, at least for MSP1-19, with the development of chimeric parasites. These MSP1 chimera are constructed to differ only in the MSP1-19 portion, with one transgenic line expressing the *P falciparum* domain and the other, expressing the *P chabaudi* domain. Therefore, this comparison of inhibitory activity against these two lines can be made to demonstrate the specificity of the inhibition. It was shown that growth inhibitory activity due to IgG from rabbits immunized with MSP1 protein was largely reversed when MSP1-19 was added to the assay. When IgG from rabbits immunized with MSP1-42 protein was tested against the two chimeras described above, it was shown that the resulting growth inhibitory activity was largely effective upon the *P falciparum* line.

The assay is also used to study and define GIA correlation with ELISAs on sera resulting from various animal immunization-challenge experiments. When using purified IgG from rabbit anti-AMA1 sera at various concentrations, the growth inhibitory activity seen is a function of the ELISA titer, significant by Spearman Rank Correlation($p<0.0001$). In an experiment where 5 rhesus monkeys immunized with 25 µg of AMA1/Montanide ISA720, sera from the monkeys recognize both FVO and 3D7 proteins equally by ELISA. The antibodies were then tested for growth inhibitory activity by the assay and a significant correlation was demonstrated between GIA and ELISA.

1.6.3 Antibody Dependent Cellular Inhibition (ADCI) Assay

The ADCI (antibody-dependent cellular inhibition) activity assay was founded on the basis of clinical trials where passive transfer of IgG from immune African adults to non-immune infected children led to a marked decrease in parasitemia. Investigations to unravel the underlying protective mechanisms behind this transfer of immunity led to the identification of the ADCI mechanism as one of the mechanisms of naturally acquired immunity to malaria. These studies found a lack of direct effect of malaria immune IgG on parasite growth *in vitro*(and frequently an increased growth by clinically effective IgG). However the addition of monocytes with the immune immunoglobulin led to significant and dramatic inhibition of parasite growth, in a parasite strain independent manner.

Thus, the assay assesses the parasite growth inhibition resulting from the cooperative mechanism between monocytes and IgG. This is set up to compare with positive controls (IgG from pooled sera of immune African) and negative controls (IgG of healthy non-immune controls).

Adding to the parallel demonstration of correlation of *in vivo* protection with *in vitro* ADCI activity is the finding in further studies that this activity is highly dependent on cytophilic antibodies. The acquisition of clinically immune malaria status has been correlated to the level of cytophilic antibodies, IgG1 and IgG3 in immuno-epidemiological studies. Recent studies have also demonstrated that cytophilic antibodies binding to blood monocytes via the Fc-gamma-RIIa receptor triggers the release of tumor necrosis factor. This correlation is further supported by studies showing that allelic polymorphism in this receptor is associated with differential immune status to P falciparum malaria.

These observations collectively support the role of the antibody-dependent monocyte mediated mechanism of parasite elimination as an important mechanism underlying protective malaria immunity. As with all bioassays, this assay as conducted classically suffers from the variability and unpredictability of a fully biological system and some difficulties in the transfer and reproducibility of the assay has limited its conduct and use to very few research groups.

The assay method discussed at the meeting was developed and is being optimized in the lab of Pierre Druilhe, at the Biomedical Parasitology unit of Pasteur Institute, who also discovered the assay mechanism. In brief, *P falciparum* parasite cultures, is synchronized through sorbitol treatments and floatations on plasmagel, to schizont stage with 0.2-0.5% parasitemia in 2-2.5% hematocrit. Adherent monocytes, carefully prepared from healthy donors, are distributed in a 96-well plate and co-cultured with P falciparum synchronized schizont-infected red blood cells added at a ratio of 200 RBC per one monocyte. Purified IgG from the test/control sera is added at a concentration of 2mg/ml, corresponding to about 10% of concentration in sera. Affinity purified antibodies are added at a final concentration of 5 µg per ml. Following 96 hours of incubation, the parasitemia is estimated from microscopic examination of thin smears. Control wells consist of 1) parasites alone, 2) parasites and control IgG from malaria-naive healthy volunteers, 3) parasites and monocytes 4) parasite and test antibodies without monocytes 5) parasites, control IgG and monocytes. Dialysis of sera with RPMI to prepare purified

IgG or affinity purified antibodies is critical in excluding non-specific inhibitory effects of other materials in the test sample. In addition, these possible growth inhibitory or growth promoting effects is further accounted for in the formula expressing the ADCI effect, which considers the effects of controls 2,4, and 5 in the calculation of the specific growth inhibitory index (SGI):

SGI = 1 - ([percentage of parasitemia with monocytes and test IgG/ percentage of parasitemia with test IgG]/[percentage of parasitemia with monocytes and naive IgG percentage of parasitemia with naive IgG]) X 100 / calculating the specific growth inhibition index

Modifications to improve the assay include the two step ADCI - where the first step is the initial culture with monocytes incubated with test IgG and synchronized, mature schizonts(5-10% parasitemia), to induce infection and rupture of RBCs and release of merozoites. The second step and culture is with the centrifuged supernatant resulting from step 1, incubated with p falciparum cultures at 0.5-1 % parasitemia, 5 % haematocrit for 48 hours. In this modified assay, the read-out is from a liquid scintillation counter of the uptake of ^{3}H hypoxanthine, added at the 36^{th} hour of incubation.

Current efforts at optimizing the assay are aimed at improving the key assay components subject to the most variability. These are the effector cells (the monocytes), the read-out method and the availability of reliable and standard source of reagents. and controls. To make the assay more robust, research is ongoing to improve standard methods of monocytes preparation (through improved cryopreservation protocols, and use of a standard cell line), optimal read-out technique and standard source of reagents. The goal is to have an optimized assay conducted according to a standardized protocol with standard cells and reagents that can be used as a screening and vaccine development tool.

1.6.4 Using assays to develop and define correlates for MSP1-42 - the WRAIR experience

Work at WRAIR in the development of MSP1-42 includes the definition of immune correlates for MSP1-42 in model systems and in vitro and comparing the results of these experiments with results from similar experiments with sera from vaccinated humans. A formulation of this antigen with the adjuvant AS02, known as FMP-1, has undergone Phase 1 clinical trials. The development of assays for this antigen was presented under two broad categories of assays, biological assays and immunochemical assays. The biological assays used were the *Aotus* immunization-homologous challenge experiments, the growth inhibitory activity by GIA, and inhibition of secondary MSP1-42 processing (PIA). The immunochemical assays were domain-specific responses by ELISA to specific epitopes p42, p33, p19, EGF-1, EGF-2. In addition WRAIR has also standardized the ELISA for this candidate vaccine formulation.

Developing assay correlates for MSP1-42

In immunization challenge studies of soluble $MSP1_{42}$ (FVO) expressed in *E. coli, Aotus nancymai* monkeys were immunized and then challenged with homologous *P. falciparum* erythrocytic-stage malaria parasites. The trial included two control groups, one vaccinated with a sexual-stage specific antigen of *P. vivax*, Pvs25 as a negative control, and the other with baculovirus-expressed $MSP1_{42}$ (FVO strain) as the positive control.

The immunogen, formulated with Freund's Adjuvant appeared protective and four out of six monkeys self-cured with two requiring treatment for anemia. In contrast, all controls were infected by day 14 post-challenge. Protective immunity correlated with antibody fine specificity against the p19 fragment and the epidermal growth factor (EGF)-like domain 2 fragment of $MSP1_{42}$, but not the $MSP1_{42}$ protein itself or the EGF-like domain 1 fragment.

In immunization challenge experiment in monkeys with FMP-1/AS02, the resulting sera produced growth inhibitory activity that correlated with antibody fine specificity. In rabbits, the GIA was shown to be inversely correlated to EGF2 antibody fine specificity. In a comparison of GIA activity in sera from different species vaccinated with FMP1/AS02, the strongest growth inhibitory activity was seen in rabbits, humans, rhesus monkeys and Aotus, respectively.

Standardization of MSP1-42 ELISA

In the absence of known correlates of protection, there is a risk of over-interpretation of *in vitro* assays to predict success or failure when only *in vivo* clinical testing may be able to determine this. In order to improve the quality and reproducibility of the assay, for use in clinical trials, and to meet regulatory and licensing requirements, a validated, standardized assay is needed. This requires that assay components like the antigen, test sera, secondary antibody conjugate and substrate be standardized. Other critical considerations include the plate antigen selection, secondary antibody issues, and the influence of unknown epitopes on assay read-outs. An essential aspect of standardizing the assay is the selection of a standard for the assay where it is essential to consider issues such as the source and availability along with storage requirements. The ideal standard should be available in large volume, should be of average affinity and be able to produce a polyclonal matured response. It should be uncontaminated and uniform and parameters for freezing, thawing and storage clearly and easily defined.

For the standardized ELISA assay of MSP1-42, the plating antigen used is MSP-1p42 (3D7). Negative control sera are pooled naïve pre-screened defibrinated clinical plasma (obtained from plasma previously designated for operative procedures but not utilized). Two positive controls are pooled endemic pre-screened defibrinated clinical plasma and pooled naïve post-third-vaccination defibrinated clinical plasma

This second positive control used for the assay is produced from a pool of high titer defibrinated plasma from two weeks post third vaccination that is mixed with a negative pool to give a standard medium titer in the assay. The assay gives a quantitative estimate of mg/ml antigen-specific antibody. Both positives run on every plate as part of the quality control measures of the assay. The positive control sera are used as reference sera for comparative purposes.

A selection of samples is maintained that are used for quality control purposes. This QC panel includes low, medium, and high titer medium volume pools from different sites, on which one sample run is conducted at 1:500 and at 1:5000 as a QC test on additional dilutions. These samples are used then to monitor assay performance as well as to validate assay performance by different technicians.

In addition to MSP1-42, the same approach is also being applied to standardization of ELISA assay for AMA-1.

1.6.5 Advantages

The meeting participants discussed the potential advantage of assays as providing an alternative or support to animal model experiments that with increasing knowledge, could possibly lead to the reduction in the use of animals. In addition, the *in vitro* environment does allow for better and easier control of complicating and uncontrolled host factors relative to animal models. Other variables such as relevant dose of biochemical components of the assay, including amount of parasites, antibodies or cells, are also far easier to control and monitor *in vitro* versus *in vivo.*

In addition, assays that can reflect *in vitro*, immune defense mechanisms underlying either naturally acquired or vaccine-induced protection status, could help rationalize and accelerate vaccine development, in the following ways -

1- Antigen identification - identification of the target molecules of the defense mechanism reflected by the *in vitro* assay

2- Antigen selection - a robust, reliable and predictive assay can be a tool for the selection of the most promising molecule among the multiple molecules with vaccine potential (i.e. the molecule that performs better in the assay under the same conditions)

3- Antigen design refinement at a molecular level - identification of specific epitopes that induce the desired immune response

4- Vaccine formulation - selection of formulation using the same parameters of assay performance

5- vaccine performance monitoring during clinical trials - in early safety and immunogenicity assessment, a robust and reliable assay could assess if the vaccine did indeed induce the 'expected effectors' and therefore is a measurable vaccine performance parameter. Such an assay could be useful if correlated with ELISAs and therefore can be used in larger field trials.

Such a rationale has been applied to the development of the MSP3 candidate vaccine, using the ADCI assay, and awaits validation in field efficacy trials.

A similar approach of back-validation by results from clinical trials of the in-vitro assays employed for other candidates, such as MSP1 and AMA1, appears a valuable strategy towards improved, clinically validated, in vitro assays.

1.6.5 Limitations

The participants agreed that the lack of solid knowledge on mechanisms of protective immunity in malaria as well as the current lack of a vaccine that has induced solid protection in humans means that none of the current assays can be taken as reliable correlates or surrogate markers of protection. This constitutes a major hurdle in rational vaccine development. In addition, current evidence indicates there are different types of immunity to malaria and even for each specific stage of the parasite such as the blood-stage; there can be potentially different mechanisms of immunity at play. Therefore this

increases the complexity of predicting efficacy of one antigen that induces one mechanism of immune response thought to contribute to protective immunity.

Participants also discussed the technical challenges of comparing results when the quality of the assay components such as purity of the reagents, handling of the parasite cultures or effector cells, as well as differences in methodologies can greatly influence the results of the assay in ways that have nothing to do with the true biological effect meant to be measured by the assay.

1.6.6 Conclusions and Potential Improvements

There was consensus that the development and standardization of assays that can be used to assess immunological end-points predictive of protective immunity as well as protective vaccine efficacy are critical needs in the field. Key assays that have the potential to provide correlates or surrogate markers of protective immunity should be objectively evaluated, optimized and standardized and made available to the malaria vaccine research and development community.

The participants also highlighted that although the development, optimization and standardization of immunoassays do involve pure scientific research, it is not perceived as competitive research activities for individual research laboratories in the context of publication appeal or grant and funding applications. It was suggested that because these efforts would actually contribute towards improving the quality of laboratory results and ultimately the quality of the research, agencies and organizations that support the research should make it a strategic priority to provide additional funding to research laboratories to undertake these types of quality assurance activities. This is particularly essential because these efforts are costly and require an investment in both time and effort for researchers. Collaboration and expertise from the pharmaceutical industry could contribute to this effort.

Specific to efforts to standardize key *in vitro* immune assays, the meeting participants discussed the role for WHO as the facilitator or coordinator, to work in collaboration with interested research groups and partner funding agencies on identified key assays following the suggested approach outlined below:

- Optimization: identification of key variables and improving assay components to minimize variability;

- Standardization: identification of key assay parameters, development of standard protocol, establishment of standard reagents and controls, detailed record-keeping

- Validation: initiation and design of interlaboratory comparison studies of a standard assay using standard reagents and controls to further define and validate the performance characteristics of the assay

- Technology transfer and capacity building among interested parties
- Relevance : comparative assessment of results from assays with the outcome from efficacy clinical trials

This approach towards standardization of assays is aimed at facilitating the validation of assays from phase I to Phase III trials and most importantly for potential assays measuring correlates of vaccine induced protective immunity, could allow these assays to be used confidently in these pivotal clinical trials. The participation and support of the pharmaceutical industry should likewise be encouraged in these efforts.

A prerequisite to the success of projects aimed at immunoassay optimization, standardization and validation is availability of standards and reference methods. In the case of peptide and protein antigens, the heterogeneity of the antigens occurring in biological fluids will always be an issue in standardization efforts. In the optimization process therefore, this inherent heterogeneity could be either identified or characterized for a specific assay, and standardization efforts set at a realistic, achievable level, which would still constitute a marked improvement of the current state of standardization of most immunoassays used in malaria vaccine development. Availability to standard methods, protocols and reagents for specific assays will be essential to any effort at standardizing and validating assays.

Another critical component to support this effort will be a database of the primary data and statistical analytical methods used in the process of development, standardization and validation of key assays

1.7. Immuno-epidemiological associations and potential correlates of natural immunity

(Contributors: Aissatou Balde-Touré, Hasnaa Bouharoun-Tayoun, Daniel Dodoo; Session chaired by Sandra Chang)

1.7.1 Considerations

Immuno-epidemiological research studies the different outcomes of host-parasite interactions under natural conditions of malaria transmission in search of correlation between an immune response and a state of protection. The most robust naturally acquired immunity in malaria is that of premunition, which is the protective immune status acquired through repeated exposure to the parasite over a period of years, progressively reducing the risk of severe and mild malaria disease without completely eliminating the risk of infection. Passive transfer studies have defined antibodies as the major component of this protection against disease, or blood-stage immunity. Although the nature of the underlying mechanisms is not fully understood, making it a challenge to determine the key parameters of the immune response to be measured, the significance and use of immuno-epidemiological associations in malaria vaccine development has been substantial. In particular, taking into consideration the questions surrounding relevance of animal model systems, these associations do provide information regarding the final target of the vaccine studies, the human being.

A variety of immune responses directed against numerous parasite antigens have been identified in through immuno-epidemiological association studies, some of which are associated with parasite killing *in vitro* (example: antibody-dependent cellular inhibition, ADCI). In addition, although the measurement of total IgG in past studies did not yield consistently significant correlations, the discovery of the association of specific IgG isotypes with protection status and the reproducibility of this association in multiple studies has made the IgG isotype and subclass responses important parameters to measure in these types of studies.

Thus, the role of various blood-stage antigens in the development of blood-stage protective immunity and the underlying effector mechanisms may be understood by studying the characteristics of the relationship between concentration, isotype and function of naturally induced antibodies and the clinical outcome of host-parasite interaction (non-severe vs. severe disease).

In the assessing the value of reported associations of an immune response to an antigen as evidence of the antigen's vaccine potential, it remains that statistically significant associations between the response and protection only become truly significant if the associations are reproducible in independently conducted studies, similarly designed, in

different endemic regions. The lack of information of this nature in the face of the large field diversity of malaria antigens has confounded the use of these immuno-epidemiological associations as reliable surrogate markers of protection.

For example, multiple studies report significant association between humoral responses to specific antigens such as MSP1, MSP3, GLURP and PfEMP1 and protection from clinical malaria. Critical determinants of the quality and significance of these associations rests with the design of these studies. Aspects such as study methodology, especially case definitions of malaria attacks and frequency ,way of collecting clinical data and bio-statistical analysis differ among studies and therefore judicious comparisons of the information gathered is difficult. In general, the methodology applied to explore associations between the antigen of interest and induced immune responses can be considered under the following two headings:

- Cross-sectional serological data - these studies have been useful for providing a 'snapshot' of the prevalence of the serological outcome of interest, but cannot be used to make conclusions on temporal associations or relationships between serological response and disease
- Prospective/ Longitudinal studies of naturally developing protective immunity where potential serological correlates of protection. are measured in a cohort that is then followed for the incidence of non-severe disease and severe disease to better explore their relationship

Cross-sectional studies comparing two cohorts of children with malaria with asymptomatic children or susceptible children with immune adults can be difficult to interpret. Longitudinal, prospective studies, consisting of follow-up of subjects in areas where malaria is endemic or seasonal, and studying the development of malaria and immune status of these subjects are a more appropriate means of studying potential correlations between the immune response of interest and state of protection. Differences in case definitions and intensity of follow-up, ways to handle potential confounding factorsd (eg age), will still affect comparability of results between studies.

In this session the participants considered the use of immuno-epidemiological data to discover, select, and optimize the MSP3 antigen as a leading vaccine candidate. In addition an effort to standardize the underlying conduct and methodology associated with these studies was also discussed.

1.7.2 Immuno-epidemiological rationale behind the clinical development of malaria vaccine MSP3

Immuno-epidemiological studies have played an essential role behind the discovery and the rationale for development of MSP3 as a vaccine candidate. The in vitro assay, ADCI was used to screen a P. falciparum genomic library for potential targets of the protective antibodies (hyper immune African sera) with the resulting identification of MSP3.

The results from two longitudinal surveys studying the association of antibody responses to MSP3 and protection, conducted in two regions with different malaria transmission levels were compared and discussed. The first was conducted in Dielmo, Senegal, an area with intense perennial transmission and an EIR of 200. 224 individuals were actively followed-up daily for clinical data for two years and blood sampling conducted at the end

of the first year. The second survey was conducted in OoDo, Myanmar, an area of low seasonal transmission and an EIR of 10. 116 individuals were similarly followed up for one year, and blood sampling conducted at the beginning of the year.

For the first survey, 'protection' was defined by absence of malaria attacks during the year before and the year after blood sampling. For the second, it was absence of malaria attacks during the year following blood sampling. Overall, the prevalence of protected individuals was higher in Dielmo than OoDo across the age groups. However the trend of increasing prevalence of protected individuals with increasing age groups was similar with the highest prevalence being in ages 13 and above.

In the analysis of the evolution of IgG subclass response to MSP-3b in Dielmo, highest response levels were seen for IgG1 followed by IgG3 and IgG2. The increasing prevalence of the responders with increasing age mirrored the trend seen in the prevalence of protected individuals. In both sites, there appeared to be a strong correlation of anti- MSP-3b IgG3 responses to clinical protection. The mean ratio of cytophilic/non-cytophilic anti-MSP3 antibodies in protected individuals was significantly higher than non-protected individuals across the age groups. Similarly, in OoDo, there was a statistically significant difference between mean ratio of anti-MSP3 IgG1 and anti-MSP3 IgG3 antibodies and this remained when the data were adjusted for age.

In addition, analysis of MSP3 and GLURP specific antibody responses simultaneously in protected individuals was presented briefly, showing that in those with low GLURP R0-specific IgG3 responses, there was significantly higher ratios of specific anti-MSP3 IgG3 antibodies and vice versa. This complementarities of the responses to these two antigens has provided the rationale for a hybrid vaccine formulation currently being developed.

A study of the above described associations in pregnancy and severe disease i.e. cerebral malaria was also presented. In comparing a cohort of women during and after pregnancy to study the association between the level of various antibody responses and protection status of the mothers and their infants, it appeared that the incidence of malaria attacks is lowest when the anti-MSP3 IgG3 responses are highest and increase with decreased levels of responses. The responses were higher overall for women without attacks. When comparing the infants experiencing early attacks with first parasitemia with those in whom clinical attacks were delayed despite parasitemia (protection) - the protected infants had higher anti MSP3 IgG3 in the cord blood.

When comparing the outcomes of cases of cerebral malaria, although there was no significant difference between anti-MSP3b IgG1 between the survival groups compared to death, there was a highly significant difference between anti MSP3b IgG3 responses between the two groups.

Data on the dynamics of antibody responses and acquisition of protective immunity to Plasmodium falciparum resulting from a ten-year follow-up study of children in Dielmo, Senegal was also presented. The Dielmo project, initiated in 1990, is a multidisciplinary study of the characteristics of naturally acquired protective immunity against malaria infection in a population living in a malaria endemic area. The components include:

- Epidemiological studies based on intense active case detection and close clinical follow-up of study cohort

- Entomological studies to characterize vector species and densities and determine entomological inoculation rates

- Parasitological studies to determine population of Plasmodium species and densities

- Immunological studies to evaluate humoral and cellular responses and investigate potential correlations with protection

These long-term investigations have been conducted on 250 residents of this village, where malaria is holoendemic. The primary vector is Anopheles gambiae and EIR ranges from 100-300. The spleen rate in children age 2-9 years is 85%. In this cohort, 50 children were followed from age 3 to 12 years for the number of malaria attacks, and for antibody responses in yearly collected serum samples, to several candidate vaccine antigens, including MSP3.

Age was significantly associated with decreased incidence rates of malaria attacks (6 and 12 months after blood sampling). Responses to MSP3 showed high levels of response (IgG and IgM), which continued to rise over time (IgG), and low ratio of IgM antibodies with increasing age. In the analysis of the dynamics of isotypes responses, a higher level of anti-MSP3 IgG1 and anti-MSP3 IgG3 was seen compared to anti-MSP3 IgG2 or anti-MSP3 IgG4. The increasing levels were particularly marked between the ages of seven and eight years of age, which correlate to the evolution of clinical attacks.

The relationship of antibody responses and the risk of malaria attacks until 6 or 12 months after blood sampling was analysed with a Poisson regression model. Decreased risk was associated with IgG1 and IgG3 responses. When looking at individual responses to MSP3 and GLURP antigens, individual variation in the responses to the antigens was remarkable, with some individuals developing responses at an early age compared to others who acquire them later; or individuals who respond well to the various antigens compared to those who have low or no responses to various antigens.

Recent investigation of the different regions of the highly conserved C-terminal part of the molecule was also discussed. Six different regions of the C-terminal portion were defined and corresponding peptides (MSP3a, MSP3b, MSP3c, MSP3d, MSP3e, and MSP3f) were designed and used to analyse the prevalence of antibody responses in Dielmo, Senegal and the relationship to protective immunity. Three out of six regions were found to be associated with B-cell epitopes targeted by protective antibodies; and four out of six regions appear to be associated with the induction of a Th1 response. This information has contributed to refining the design of the MSP3 vaccine construct.

1.7.3 Improving and standardizing the immuno-epidemiological approach - efforts of the African-ImmunoAssay(AIA) Network

The AIA network represents a concerted effort, sponsored by the African Malaria Network Trust (AMANET) that aims to develop standardized assays using the same

reagents and statistical tools in order to decrease variability and improve the quality of the data and comparability of results from immuno-epidemiological studies.

To illustrate the problems with interpreting and comparing results from different studies; the results of a study to look at correlates of antibody mediated immune responses against three *P falciparum* blood stage antigens were presented. In the study, antibody responses to MSP1-19, GLURP and PfEMP1 were measured in pre-malaria season samples of a 300 cohort of Ghanaian children aged 3 to 15 years. This cohort was clinically and parasitologically followed for 2 malaria transmission seasons and subsequently categorized as those who develop clinical malaria and those who did not. The data indicated that antibody responses against GLURP and PfEMP1 correlated statistically with protection against clinical malaria, unlike responses to MSP1-19. In addition, further analysis of antibody subclasses and isotype responses found that IgG1 and IgG3 were significantly associated with protection from malaria in these children. These associations remained significantly associated even after being adjusted for the effects of age.

However, there have been contradictory reports on the association of antibody responses to specific antigens and protection from malaria, particularly in studies involving MSP1-19 antigen. It is impossible to compare results of these studies because of they were conducted in areas of different transmission and study designs, and utilized different reagents and statistical analytical methods.

Therefore, the AIA network is attempting to address this incomparability by:

- Developing standardized ELISA assay that measures isotype and IgG subclasses to malaria antigens
- Correlating the ELISA values to immune status of populations studied in Africa, living under various transmission intensities
- Standardizing and introducing a regression analysis method that corrects for age-related exposure to malaria infection
- Standardizing *in-vitro* parasite inhibition assays, for use in the field as possible markers of acquisition of clinical malaria immunity.

These assays will comparatively assess the relationship between acquisition of malaria specific antibody responses to four malaria vaccine candidate antigens (GLURP, AMA1, MSP1-19, and MSP-3) and protection from clinical malaria seen in the field. Studies with similar longitudinal cohort designs in six different geographical and epidemiological settings varying from low to high malaria endemicity are being conducted to obtain the samples. These sites include Burkina Faso, Gabon, Senegal, Ghana, Tanzania and Zimbabwe. Standardized SOP for all aspects of study design and conduct are being developed and applied, along with standardized assays using the same reagents. In addition, appropriate statistical tools will be utilized in a standard statistical analytical plan.

The optimization of key assay parameters in the AIA standard ELISA assay involves the optimization of antigen coating concentration for each individual antigen, dilution of subclass specific antibodies, and investigation of cross-reactivity and range of stability of antigen coated plates and serum dilutions. To support the implementation of this project, various activities have been conducted, including training workshops, supply of reagents

for site-specific tests, utilization of standard statistical tools and development of statistical models for analysis, and data management support.

This should lead to forming a set of criteria for the validation of malaria vaccine candidates in addition to providing essential baseline information to improve the design of clinical trials. In addition, this effort aims to also build on-site quality assured laboratory capability.

1.7.4 Advantages

As these are studies of interactions between the target host of vaccine studies (humans) and the parasite *P falciparum*, data from well-designed studies and significant associations has the potential of providing highly relevant information that the animal models and current *in vitro* assays cannot.

The availability of surrogate markers or correlates of protection could help to rationalize malaria vaccine development as well as accelerate the development process.

1.7.5 Limitations

The large field diversity of malaria antigens and therefore a large number of possible immune parameters to measure, make it likely that any association found between a measured immune response and protection is pure chance. In addition another major confounding factor in the analysis of immune response and protection in malaria is age. Statistically significant correlation in one study must be confirmed in similarly designed probably larger scale studies in different malaria endemic regions.

1.7.6 Conclusions and Potential Improvements

The difficulties in extrapolating and developing robust correlation between the measured immune parameter and protection was discussed with respect to the limitations previously mentioned.

The cost, logistical and operational difficulties as well as potential ethical issues of conducting multiple immuno-epidemiological studies of high quality (i.e. longitudinal, daily follow-up from birth) to investigate and dissect the development of protective immunity to malaria were discussed. It was generally agreed upon that the whole malaria research and development field, not just malaria vaccinology would benefit from information gathered through such studies.

1.8. Future Needs and Ways forward

(Contributors: Carole Long, Pierre Druilhe, Mats Wahlgren; Session chaired by Carter Diggs)

1.8.1 General Considerations

In malaria vaccine development, the challenge remains in developing a model that truly reflects the human-plasmodium interactions that lead to protective immunity. The non-natural host-parasite combinations of most utilized animal models display acute infections with subsequent greater acquired resistance due to a greater number of parasite molecules being antigenic and stimulating far greater immune responses. This is in sharp contrast to the chronic infections typical in natural host-parasite combinations, where antigenic parasite molecules have likely been selected over thousand of years of interactions to establish chronicity and ensure survival of both host and parasite.

Table 1. Comparison of susceptibility and resistance between non-natural and natural host-parasite combinations
(Adapted from Druilhe et al. Trends in Microbiology Vol 10 No 10 Supp; 2002)

	Host Parasite combination		
	Non-natural		Natural
Innoculation	Balb/C and *P berghei*	C57B16	Thamnomys and *P berghei*
Minimal number of sporozoites needed to infect	> 10,000	50	5
Minimal number of irradiated sporozoites needed to immunize	Single immunisation by 1,000	Three immunisation by 30,000	Unprotected despite 3 immunisation by 100,000

Stark differences clearly exist between patterns of susceptibility and resistance between natural and non-natural host-parasite combinations (Table 1). Infection patterns depend on the choice of the host and the same parasite in different hosts will likely induce unique specific defences.

All this does not preclude the use of the models, but the key differences that do exist between diverse models as well as between model responses and the real system should be clearly investigated and defined. Of particular concern in terms of relevance is that the use of models in antigen discovery may lead to identification of molecules that are not likely to be relevant in the induction of protective immunity. Protection studies in

these non-homologous models should be interpreted with caution because potentially large numbers of molecules can induce protection than in a natural host, and a molecule that may appear highly immunogenic, epitope specific and induces protective immune responses in an abnormal host could still fail when tested in humans.

1.8.2 Ways forward - improving models and assays

In either optimizing or developing models, priority should be directed towards an approach with *P falciparum* as the target parasite, such as chimerical parasites or *P falciparum* 'adapted' to the animal host. Although this does not fully overcome the fundamental molecular mismatch, it would be an improvement over other non-natural models.

In addition, advances in the knowledge of the immune and hematopoeitic systems of humans and model animals, has led to creation of functional human immune systems within animal models (e.g. SCID mice models). These 'humanized' models, grafted with RBCs, lymphocytes or hepatocytes, then infected with malaria parasites, have great potential to be utilized as more reliable predictors of vaccine performance. Additionally, technical advances have allowed greater manipulation of these models where methods that allow for tracking and analyzing host-parasite interactions on a cellular level are being developed and models can be developed to study specific mechanisms of defenses. These improved and more specific models need to be further optimized and their full potential explored and realized. Two examples of advances in the development of novel mice models were discussed at this meeting.

Models represent a distinct host-parasite combination with distinct immunological features. Their merits, limitation and relevance should be determined to improve our ability to interpret data generated and make conclusive decisions. A database could be established to collect and make accessible knowledge acquired through on-going research using these models which could then be used to determine and compare the relationship between immunogenicity results in different models (i.e. mice versus primates). The relevance of the model system to the human-*P falciparum* system should be defined and the parameters being best mimicked (as opposed to those poorly mimicked), be identified.

Data from the analysis of sera from clinical trials should be applied towards validation, continuous reevaluation and hopefully improvement of currently used models and assays. Improvement in clinical trial design and conduct should aim to maximize the information and materials that can be gathered from these efforts. Information on immunogenicity and biological efficacy of a candidate vaccine should be obtained, and this underlines the importance of developing and designing assays for correlates of protection.

Similar considerations apply to the optimization of *in vitro* assays. New advances such as hepatoma cell lines susceptible to P falciparum liver stage development and development of cell lines as reliable effectors in functional assays i.e. the monocytic cell line for a high-throughput ADCI assay are developments that could greatly improve the conduct of these assays. Optimization of current assays and confidence in a standard approach will also allow for comparisons between different versions of the same candidate antigen within different assay systems (ADCI, GIA, ELISA) that have been validated. These

comparisons may become essential as more and more candidates are developed and require evaluation and prioritization.

1.8.3 Examples of ways forward in the development of new models

1.8.3.1 A rodent model for the study of anti-sequestration/ anti-adhesion mechanisms of defense in Plasmodium falciparum malaria (Contributor: Mats Wahlgren)

One vaccine concept being developed is based on the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) variant antigen, a polypeptide encoded by the *var* gene family present in roughly 60 copies per genome. This antigen is the dominant antigen and adhesin on the *Plasmodium falciparum*-infected red blood cell (iRBC) surface. Sequestration of iRBC and uninfected erythrocytes (RBC) in post-capillary venules of the brain, the lungs and other organs leads to the excessive binding of erythrocytes in the micro-vasculature, and is thought to be the major biological process underlying severe malaria. An observation that has led to the development of PfEMP1-based vaccine concepts is that serum antibodies that disrupt erythrocyte rosetting, auto-agglutination or endothelial binding are rarely found in the small proportion (<5%) of children who develop severe malaria but they are frequent in children with mild disease.

A DBL1α domain (located in the N-terminal of each PfEMP1) of a prototypic virulent parasite (FCR3S1.2) is being investigated for its vaccine potential. DBL1α mediates adhesive features associated with severe malaria including blood group antigen A, heparan sulfate and CD35 (CR1) binding, receptors that participate in the biological processes outlined above. This domain has previously been found to be well recognized by the sera of children living in endemic areas. Roughly 50% of Gambian children with mild malaria carry antibodies that disrupt the rosettes of FCR3S1. Additionally, the iRBC surface of FCR3S1.2 is often recognized by sera of Gabonese and Kenyan children and it displays the multi-adhesive phenotype associated with iRBC obtained from children with severe malaria. These observations support the rationale that vaccination of children with one or a few DBL1α domains may prevent the development of severe malaria.

A rodent model, which has an intact immune system, has recently been developed to study the effect of anti-rosetting antibodies induced by a test immunogen, such as DBL1α, on the *in vivo* sequestration of *P. falciparum* iRBC s of FCR3S1.2.

Essentially an *in vivo* adhesion blocking assay, it requires the injection of middle to late stage FCR3S1.2 trophozoite-infected RBC (purified by a magnet-based method and radioactively labeled with $^{99m}T_{c)}$ into the tail-vein and subsequent monitoring of the sequestration in the lung vasculature with the help of a gamma-radiation sensitive screen. The sequestration can be abolished by pre-treatment of the iRBCs with a low dose of trypsin. The relative number of parasites sequestered in the lungs was calculated based on the activity of each cell and the total activity of the lungs and that of the whole animal.

In vitro binding assays with FCR3S1.2 parasites to human lung endothelial cells (HLEC) and to rat primary lung endothelial cells and rat lung cryo-sections confirm that DBL1α,

CIDR1α and DBL2δ domains are involved in the sequestration process through binding to human and rat receptors including heparan sulfate, CD36 and CD31. Using this model, it was observed that minimal sequestration of FCR3S1.2 iRBCs in the lung vasculature was seen in all the rats immunized with DBL1α constructs compared to the extensive binding seen in animals immunized with control constructs. This is seen as evidence that the DBL1α domain mediates the sequestration of iRBC and that anti-DBL1α antibodies block the interaction between PfEMP1 and host receptors *in vivo* (heparan sulfate and blood group A antigen in this case). The data from *in vivo* model suggest the binding between DBL1α and rat heparan sulfate to be dominant over the interaction between the other domains and their receptors.

This inexpensive and robust model can be used for study of mechanisms related to sequestration in blood vessels and can be relevant in both vaccine and drug related studies. The ability to study the histological effects is also an advantage. However, it is a heterologous system and the short duration of *in vivo* testing (therefore excluding the ability to study important protective mechanisms that may develop under longer exposure), and these limitations must be considered in the interpretation of results.

Chen Q et al. Immunization with PfEMP1-DBL1alpha generates antibodies that disrupt rosettes and protect against the sequestration of Plasmodium falciparum-infected erythrocytes.
Vaccine. 2004 Jul 29; 22(21-22):2701-12.

1.8.3.2 A SCID mouse model for the study of blood and liver stage mechanisms of defense in Plasmodium falciparum malaria - a potential screening tool for antigen selection and prioritization. (Contributor: Pierre Druilhe)

Severe combined immunodeficient (SCID) mice have been used to develop models for falciparum malaria studies. Two SCID mice models were discussed, the first, grafted with *P. falciparum* parasitized human red blood cells (*P. falciparum* -huRBCs) for studying blood-stage malaria and the second, grafted with human hepatocytes for studying liver stage malaria.

Asexual stage immunity has been shown to be mediated primarily by antibodies that suppress parasite multiplication and growth. The first model has allowed for the analysis of the *in vivo* effects of different components of the human immune system on *P. falciparum* (NF54) parasitemia. In the model, tissue macrophages and polymorphonuclear neutrophils were pharmacologically reduced to allow for the successful grafting of *P. falciparum* -huRBCs and establishment of parasitemia. Stable parasitemia has been established in this model (up to 4 months) at levels and patterns similar to that seen in untreated patients. In addition, the resulting morphology of the *P. falciparum* parasite and the responses to currently employed antimalarial drugs were remarkably similar to those observed in humans.

It has been previously demonstrated that in parasitemic but immunocompetent individuals, passive transfer of immunoglobulin G (IgG) from adult Africans that are

clinically immune to malaria (hyperimmune IgG or HI-IgG) led to a reduction in parasitemia. In contrast, similar passive transfer experiments in the immunodeficient mice showed no effect of HI-IgG on the course of parasitemia. Neither did the injection of mice monocytes, human monocytes or peripheral blood mononuclear cells (huPBMC) alone. However, in studies with several immune effectors (HI-IgG, huPBMCs, and human monocytes) that were injected in varied sequences, enriched human monocytes, as well as huPBMCs, together with HI-IgG showed an inhibitory effect on parasite growth, irrespective of the order of injection. A reduction to a level of low grade parasitemia was seen, similar to that observed in previous clinical passive transfer experiments.

The findings support parallel *in vitro* assay developed as a result of the clinical passive transfer experiments - the antibody-dependent cellular inhibition (ADCI) assay - which showed that HI-IgG alone had no effect on *P. falciparum in vitro*, but an inhibitory effect observed only with the presence of human monocytes.

The model was then used initially to evaluate the protective effect of antibodies with defined specificity, namely affinity-purified antibodies from the HI-IgG pool, on epitopic peptides (MSP3b and the RESA peptide), derived from the two vaccine candidates MSP3 and RESA, respectively (specificity and titers of the purified Abs were verified against both the original peptides and the native parasite antigens prior to injections).

The *in vivo* effect of the purified antibodies was investigated using the sequential procedure described above, where each mouse served as its own control. There was no effect on parasitemia in the mice following injections of either antibodies or human monocytes alone. However, injections of anti-MSP3antibodies followed by human monocytes three days later, led to a marked decrease(>10-fold/48 h) in parasitemia, which was on average more than that seen with total HI-IgG, and as fast as that observed when using chloroquine.

No effect was seen using anti-RESA antibodies and human monocytes. The same mice, when injected with anti-MSP3antibodies and human monocytes, drastically cleared their parasitemia. In contrast to similar experiments with HI-IgG and human monocytes, where reduction of parasitemia is sub curative, anti-MSP3antibodies together with human monocytes rapidly and completely cleared the parasitemia in most mice in the experiments. The lack of inhibition obtained with anti-RESA antibodies does not rule out a role for this molecule, particularly since these were purified on a short peptide. Subsequent experiments with affinity-purified antibodies against several other vaccine candidates, including EBA-175, MSP1, and MSP-2, showed similar lack of inhibitory activity on parasitemia, at the antibody concentrations employed.

The *in vivo* parasite growth inhibitory effect of low amounts of anti-MSP3 Abs supports previous observations made under in vitro conditions and immuno-epidemiological association studies. In addition, the total HI-IgGs used contained the same amounts of anti-MSP3b antibodies that were present in the purified preparation and yet did not achieve an equal effect. This observation indirectly implies that other antibodies present

in HI-IgG might block the inhibitory effect of anti-MSP3 antibodies. Such a blocking effect is reminiscent of that observed with anti-MSP1 antibodies or could also be related to non cytophilic antibodies directed to other merozoite surface antigens. This observation suggests that immunization by selected malarial antigens may elicit stronger protective responses than those resulting from exposure to all malarial proteins. In other words, the immunization by molecules which are proven targets of protective mechanisms may induce a stronger protection than that developed by natural exposure.

The second model, utilizes mice with genetic deficiencies affecting hepatocytes, and B and T cells, and then pharmacologically-induces reduction of mononuclear phagocytes and NK cells, allowing for the successful grafting of human hepatocytes. Albumin and alpha-1-anti-trypsin secretions are measured and used to monitor graft outcome and survival. Injections of P. falciparum sporozoites, conducted 3-6 weeks post graft resulted in development of liver stage parasites, detected by light microscopy , IFAT with anti-CS, anti-LSA1 antibodies, and RT-PCR. The parasitized hepatocytes have also a size and a morphology similar to that described in humans and chimpanzees. The model provides a powerful tool for demonstrating in situ events following immunizations, i.e. in LSA3 immunizations, at a cellular level.

These in vivo SCID mice models offer several significant advantages over non-human primate (NHP) models such as the Aotus and Saimiri in terms of cost and availability. In contrast to the adaptation required of *P falciparum* in order to establish viable infection in the NHP host, the humanized SCID mice establish infection with fresh parasite isolates, leading to parasitemia and sometimes gametocyte production as well. The parasitemia is long-lasting and stable, as opposed to the acute, but short-lived parasitemia seen with the NHP model. In addition, the model offers human host cells (homologous model) as opposed to the heterologous host-cell in the NHP which leads to poor definition of the immune system that may affect the comparability and relevance of results. The ability to establish immune components of the human immune system and test the effectors of immunity in the system makes the interpretation of results in this system likely more relevant.

Finally, the ability to evaluate small amounts of antibodies with defined specificities to investigate the protective role of a given antigen expressed by malarial parasites is another advantage over non-human primates. Some limitations that need to be considered are the highly demanding technical specifications in the establishment, maintenance, and analysis of these models. In conclusion, this model could be used for rapid in vivo investigations of the protective role of antigen-specific Abs. It is apparent that antigen-induced antibodies may differ in their protective effect from protective antibodies resulting from natural exposure due to differences in their fine epitope specificity or isotype profile. Given the expanding number of malaria antigens with vaccine potential being investigated, this model could be optimized to be used for screening and prioritizing these candidates. *Badell E et al. Human malaria in immunocompromised mice: an in vivo model to study defense mechanisms against Plasmodium falciparum. J Exp Med. 2000 Dec 4; 192(11):1653-60.*

1.9. Pre-clinical Evaluation Group: Conclusions

The meeting participants concluded that although humans remain the best 'models' in which to test vaccines, information from experiments with model systems play an essential role from the first steps of antigen discovery and selection and throughout the production and clinical development phase. Conceptual insights gained from these systems produce, formulate and improve on the vaccine design and their rational application along the development pathway should help optimize performance.

The advantages and limitations of the model systems discussed at the meeting underlined the need for a systematic and continous re-evaluation of current knowledge and uses of these systems, which were primarily developed for pure research purposes. Today, their real and respective value for vaccine development purposes requires urgent and stringent assessment, particularly in view of the large number of vaccines going through those pre-clinical steps. The participants concluded that it was critical to ensure that what knowledge generated through on-going basic preclinical research, early product development and vaccine trials be applied towards the reiterative process of model improvement as well as the development of new models. Knowledge on mechanisms of both natural and vaccine-induced protective immunity that is being acquired this way should feed back into the process and guide the selection and development of models most appropriate or relevant to the vaccine concept being evaluated. To improve the quality of the information acquired through experiments in model systems, steps needed to be taken to improve the comparability of results as much as possible. Despite the challenges due diversity of antigens and approaches and the state of uncertainty of relevant immune responses, international collaborative action was needed from the malaria vaccine community to develop mechanisms for sharing methods and materials utilized in these models systems among researchers in a systematic and reliable manner. Steps like setting up collaborative networks committed to sharing of information and transfer of knowledge and materials (assay reagents, standardized protocols, statistical tools) will lead to improved comparability of results between candidate antigens being developed on the basis of the same assumptions (i.e. that it induces GIA activity, for example).

Research towards the development and standardization of these tools could eventually lead to 'gold-standard' evaluation tools can help generate vaccine specific evaluation profiles or plans. Stepwise algorithms based on established parameters of qualitative and quantitative measures of vaccine performance throughout preclinical and clinical evaluation could be developed which would improve comparability between multiple candidate vaccines and novel approaches. The generation of an evaluation platform or plan will also ensure greater coherence along the development pathway.

1.9. Pre-clinical Evaluation Group: Recommendations

Knowledge gaps and scientific uncertainties require a review of current information, and thoughtful, expert scientific input in order to improve decision-making in vaccine development. Following the discussions, the meeting participants recommended that international collaborative working groups be formed and supported to address issues related to the evaluation of malaria vaccines.

Two specific working groups were recommended:

0) A collaborative working group to address assay development, optimization, validation, and standardization - Initial issues that could be addressed by this group include:

    . The development of antibody reference preparations to be used in key assays- ELISA, GIA, and ADCI.
    . The establishment of a malaria vaccine repository for reference antigens and sera (whether human or rabbit) against which results can be compared by research laboratories conducting the specific assays.
    . The support of production and distribution of specific standardized reagents:

    - Sera from human vaccine trials
    - Rabbit or monkey sera from immunogenicity trials
    - Panels of well-characterized mouse or human monoclonal antibodies, particularly against conformational epitopes

    . The development and distribution of SOP and standard methodology for specific assays
    . The establishment of a systematic, valid database to record and maintain the primary data that is generated on the specific assays involved in the optimization and standardization effort.
    . As much as possible, comparative assessment of methods developed by different groups in technical workshops

0) A collaborative working group to address animal model issues - Some issues mentioned that could be addressed by the group include exchange of SOP and reagents among groups working on similar models and the development of a standardized SOP for similar models in order to improve results comparability. Potentially, investments to improve novel models that can be instrumental at speeding up vaccine development

The participants strongly recommended that these initiatives be fully developed and supported as a coordinated effort among the agencies and institutions that fund malaria vaccine research. They suggested that WHO should help ensure that the assays and animal model SOPs optimized and standardized through this effort are accessible for technology transfer efforts among the global malaria vaccine R&D community.

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