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Ozwara Suba, Hastings

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DEVELOPMENT AND APPLICATION OF A *PLASMODIUM KNOWLESI* TRANSFECTION SYSTEM

PROEFSCHRIFT

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Hastings Ozwara Suba
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Promotor:	Prof. Dr. A.M. Deelder
Co-promotor:	Dr. A.W. Thomas (Biomedical Primate Research Centre, Rijswijk)
Referent:	Dr. J. Langhorne (National Institute of Medical Research/Medical Research Council, Londen, U.K.)
Overige leden:	Prof. dr. R.W. Sauerwein (Universitair Medisch Centrum Radboud, Nijmegen) Prof. dr. J.T. van Dissel Prof. dr. E.J.H.J. Wiertz Dr. A.P. Waters

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Cover illustration: butterfly pupa, transfection construct, GFP expressing *Plasmodium knowlesi* and *in vitro* culture adapted *P. knowlesi*.

Voor Hague, Stacy en Florence

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CHAPTER 1

General introduction

General introduction

1.1 Aim of this study

Transfection of malaria parasites is a technology that provides modern genetic tools for investigating the regulation of gene expression, expressing transgenes as well as elucidating the function of proteins by disrupting, modifying or replacing the genes encoding them. The technology now permits the experimental evaluation of molecular techniques for developing attenuated malaria parasite vaccines.

The difficulty of working with human malaria parasites has meant that a significant amount of research has been performed on different animal models of malaria. Furthermore, these models offer the only means to experimentally investigate natural host-parasite interactions *in vivo*. *Plasmodium knowlesi*, a natural parasite of macaques, is a comparative model for understanding many of the important aspects of human malaria. The entire life cycle of the parasite is accessible to experimentation suggesting that all aspects of malaria, from biology to host-parasite interaction can be studied under controlled conditions. In addition, an *in vivo* transfection technique has been developed for the parasite and the entire genome has been sequenced. This enables investigation of the parasite at a mechanistic level and identification of new targets for chemotherapeutic or immunological intervention. In order to fully benefit from the advantages offered by malaria transfection technology, the protocols for transient, stable episomal and integration dependent transfection of *P. knowlesi* are required.

The goal of the studies described in this thesis was to develop transient, stable episomal and integration dependent transfection protocols for *P. knowlesi* and to use the system to evaluate transgenic expression of an immunomodulatory host cytokine as a strategy for generating immunopotentiated malaria parasites. In addition, the current procedures for *in vitro* culture of *P. knowlesi* do not allow long term culturing and are insufficient for generating sufficient amounts of parasites for analysis. It was important that long term culture procedures are optimised in order to develop a versatile *in vitro* transfection system for *P. knowlesi*. Finally, a new baboon model was developed for *in vivo* determination of host-parasite interaction of the genetically modified parasites.

1.2.1 Malaria: the problem

Malaria is caused by protozoan parasites of the subphylum Apicomplexa belonging to the genus *Plasmodium*. The disease is transmitted by female mosquitoes of the genus *Anopheles*. Human

malaria is caused by four parasite species namely *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. *P. falciparum* is the most lethal accounting for over 90% of malaria associated deaths. Malaria is a major disease of mankind. It is estimated that there are 300-500 million cases world wide and up to 2.7 million deaths occur annually. Africa accounts for over 90% of the deaths, mainly children aged below five. Of all the parasitic diseases, malaria is not only the greatest killer but is also the most widely spread. It is a threat to approximately 40% of the human population in over 90 countries in the tropics and sub-tropics [1].

Efforts to control malaria are made through treatment of infected people and by physical and chemical strategies to control the mosquito vector. However, it is to be expected that, due to the rapid increase in multi-drug resistant parasites and reduced investment in developing new drugs, the number of malaria cases and casualties will rise in the coming years. These factors necessitate the development of new and alternative approaches to resolve the current malaria crisis.

The development of new and alternative strategies for malaria control is hampered by the complex life cycle of the parasites and their interactions with human hosts and insect vectors. Therefore, a more rational approach to resolving the malaria crisis requires a deeper understanding of the biology of malaria parasites.

1.2.2 Malaria vaccine development

To complement existing means of controlling malaria, effort is being put into the development of a vaccine. Vaccine development against human malaria currently focuses on three types based upon aspects of the parasites life cycle. Transmission blocking vaccines are directed against sexual stages. They target to interrupt transmission to the mosquito. Vaccines directed at sporozoite and/or liver stages (pre-erythrocytic stages) are aimed at protecting against onset of blood stage infection and blood stage vaccines are to prevent malaria related pathology and erythrocyte invasion [2].

Current vaccine efforts largely focus on the development of single parasite molecules and on combinations of parasite molecules either delivered as recombinant proteins or through DNA/live vector approaches. So far the protection induced in volunteers from endemic and non-endemic areas have not been optimum for manufacturing a vaccine (reviewed in [2]). This partly results from the polymorphic nature of many malaria vaccine candidates. It seems likely that any ultimate malaria vaccine will comprise multiple components directed against different parasite stages.

Two observations suggest that an attenuated malaria vaccine may be achievable. First for people that survive malaria infection, immunity develops slowly as a consequence of exposure. As few as one or two infections may be sufficient to protect against severe non-cerebral malaria [3] although clinical immunity may not be fully developed until adolescence. Second, immunisation with

attenuated sporozoites induces sterile protection in mice and humans [4, 5] mediated via IFN-γ dependent mechanisms and recently published data [6] shows that chimpanzees can be protected. Hence there is justification for development of a vaccine based upon attenuated malaria parasites.

1.2.2.1 Attenuated malaria vaccines

An attenuated vaccine is a weaker, related or modified version or lower inoculum of a pathogen that stimulates protection against serious illness. Attenuated vaccines are possibly the oldest vaccines. Ancient Chinese protected against small pox in the process of variolation. In 1796, Edward Jenner used cowpox virus to protect against small pox. In the 1880's, Louis Pasteur protected chickens from cholera and sheep from anthrax using attenuated forms of the pathogens and in 1921, protection was achieved against tuberculosis using BCG. To date, use of attenuated vaccines has been the most successful vaccination procedure, protecting against measles, mumps, rubella, varicella, polio etc.

Several studies have shown that attenuated malaria vaccines are feasible. Immunisation with irradiated sporozoites protects or partially protects rodents [5], monkeys [7] and humans [8-10] against sporozoite induced infection. These studies showed that attenuated sporozoites vaccination has the capacity to prevent induction of blood stage infection and induce sterile protection against liver stage infection. To immunise large numbers of people with attenuated sporozoites vaccines, they must be delivered either by bite of infected mosquitoes or by intravenous injection. Although this is likely to be clinically and logistically difficult, effectiveness of attenuated sporozoites vaccines indicates that a multi-valent pre-erythrocytic stage vaccine might induce sterile immunity. This type of vaccine would prevent all manifestations of disease and may have an important role in limiting malaria related morbidity and mortality.

Many scientists have focused on understanding the mechanisms of attenuated sporozoite induced immunity, their antigenic targets and on developing vaccine delivery systems that can mimic the protection. Much of this work has been done using *P. berghei* and *P. yoelii* and has led to a number of candidate vaccines [11-13].

Attenuated malaria parasite vaccines are expected to mimic natural protective responses induced in endemic areas by presenting a larger antigen repertoire to the host thereby evoking protective immune responses that will be boosted by a natural infection. Attenuated vaccines are administered without adjuvants and the natural infection boosts the immune responses. However, because sporozoites can not yet be cultured in mass, this does not yet form the basis of an effective vaccine.

Transfection of malaria parasites now permits the experimental evaluation of molecular techniques for developing live-attenuated blood stage parasite vaccines that could induce full

protection. Malaria blood stage infection is the cause of malaria associated morbidity and mortality, making it a major focus of malaria vaccine development.

1.2.2.2 Cytokine mediated immunomodulation of pathogens

Studies using viruses, and protozoan parasites have shown that pathogens expressing host cytokines *in vivo* can be immunopotentiated and/or attenuated, subsequently manipulating host-pathogen interaction and stimulating protective host responses. In addition, non pathogenic micro-organisms expressing host cytokine have been shown to immunomodulate unrelated diseases.

Vaccinia viruses expressing host IL-2 and IL-12 separately became attenuated and enhanced immune responses [14, 15]. In another experiment, recombinant vaccinia viruses expressing either IL-2 or IL-15 were attenuated in athymic nude mice leading to increased peripheral NK-cell mediated immune responses [16]. These studies indicated that localised expression of host immunomodulatory cytokines by intracellular pathogens could lead to immunopotentiation of the pathogens. Expression of host IFN-γ in another type of virus, simian immunodeficiency virus, was shown to be safe and immunogenic in rhesus monkeys [17].

A recent study by Steidler *et al*., [10] showed that cytokine expressing bacteria can be used to immunomodulate a chronic disease leading to cure. Murine colitis was effectively treated using the bacteria *Lactococcus lactis* expressing host interleukin-10. This study indicated that cytokine expressing micro-organisms also have the potential for therapeutic use.

The protozoan parasite *Leishmania major* expressing host IFN-γ was significantly attenuated in nude mice, indicating that localised expression of host immunomodulatory cytokines by an intracellular protozoan parasite enhances protective immune responses [19].

Thus far, expression of host cytokines by malaria parasites has not been examined. The development of transfection technology for malaria parasites (see section 2.3) now enables expression of recombinant host proteins such as cytokines in *Plasmodium*. It is tempting to speculate that genetically modified malaria parasites that express immunomodulatory cytokines can induce effective immune responses *in vivo*.

1.3 Transfection of malaria parasites

Transfection is a mechanism for mutating eukaryotic cells by introducing exogenous genetic material [20]. The introduced genetic material is expressed and retained either temporarily (transient transfection) or functionally maintained for longer periods (stable transfection) resulting in a genetically modified organism [21]. In transfection, a particular gene is modified followed by

assessing the phenotype of the mutant cell. Consequently, transfection is useful for determining the precise relationship between the structure of a gene and its expression and function.

Earlier attempts to develop transfection constructs and introduce DNA into malaria parasites were unsuccessful. Cloning *Plasmodium* DNA in *Escherichia coli* proved to be difficult as a result of high AT content of *Plasmodium* DNA [22]. Furthermore, successful transfection required DNA to cross four membranes to reach the nucleus of the predominantly intracellular *Plasmodium* parasite. Finally, in 1993, the extracellular gametocytes of *P. gallinaceum* were transfected by electroporation [23]. Since then *P. falciparum* [24], *P. berghei* [25], *P. knowlesi* [26], *P. cynomolgi* [27] and *P. yoelii* [28] have been successfully transfected.

1.3.1 Transient transfection of malaria parasites

Transient transfection is a procedure by which DNA is introduced into a eukaryotic recipient cell, retained and expressed temporarily. Developing the procedure has facilitated analysis of how gene expression is developmentally controlled in malaria parasites. Transient transfection provides the opportunity to identify genetic elements that control gene expression in malaria parasites [26, 27]. These studies showed that different regions of *Plasmodium* promoters were associated with controlling varying levels of expression, reminiscent of regulatory elements in other eukaryotes.

Transient transfection has been reported for *P. gallinaceum* [23], *P. berghei* [29] and *P. falciparum* [30]. In each of the systems, either luciferace or chloramphenical acetyl transferase (CAT) were used as reporter genes to study regulation of *Plasmodium* genes [31, 24]. Information resulting from these studies has been useful for further development of transfection technologies including identification of suitable promoters to drive transgene expression and in gaining insight into the basic processes that direct gene expression in malaria parasites.

1.3.2 Stable transfection of malaria parasites

During stable transfection, DNA is introduced into a eukaryotic recipient cell and maintained in a functional state for extended periods of time. This results into a heritable change of the transfected cell. Selectable markers are used to grow transfected parasites under drug pressure. This is required for selection and maintenance of foreign genes in transfected parasites.

Stable transfection technology is used when analysis of the transfected cell is required over several generations. Stable transfection can be categorised as episomally based when the introduced DNA is maintained as an extrachromosomal replicating plasmid. Alternatively, transfected DNA can integrate into the genome of the host cell. This is referred to as integration dependent transfection. Integration dependent transfection relies on the recombination between two copies of a

sequence, one present in the genome (target DNA sequence) and the other in the incoming DNA (targeting construct) [32].

Stable transfection of malaria parasites is therefore a versatile genetic tool for expressing foreign genes and for disrupting, modifying or replacing genes to analyse protein function. Currently, stable transfection is possible in *P. falciparum* [33], *P. berghei* [25], *P. knowlesi* [26], *P. cynomolgi* [27] and *P. yoelii* [28].

1.3.3 Parameters for transfection of malaria parasites

Transfection of malaria parasites requires a careful choice of the **target gene** for integration dependent transfection. The gene should be non-essential in the parasite developmental stage you are selecting in. Genetic alteration of an essential gene could be lethal to the mutant parasite consequently failing to generate transfected parasites.

Introduction and integration of DNA into *Plasmodium* genome is a **low frequency** combination of events, estimated to vary but could be as low as 10^{-7}. Thus drug selection is required for selecting the transformed events [34].

Integration of DNA into the *Plasmodium* genome occurs almost exclusively by site specific **homologous recombination** mechanisms. However, there are species specific differences in the mechanisms involved [34]. *P. falciparum* will only accept integration of circular DNA either by single [35] or double [33] crossover mechanisms. In the rodent malaria parasite *P. berghei*, integration occurs via linear DNA [36, 37]. The transfection construct for integration by double crossover mechanisms is usually linearised at two positions. This is usually at the ends defining the vector backbone DNA sequence. Integration by single crossover mechanisms in *P. berghei* only occurs when the transfection construct is linearised in the target DNA sequence region thereby defining the exact point of integration [37].

There are a number of **regulatory DNA sequences** available for controlling gene expression in *Plasmodium* (Table 1). Promoters currently in use, vary in strength and stage specificity, enabling gene expression at different levels and at distinct stages of the parasite [38-39]**.** Control of (trans)gene expression depends on promoter stage specificity. For example, expression of *P. falciparum* apical membrane-1 antigen (AMA-1) was restricted to mature schizont stages when expressed under the *ama-1* promoter but the stage specificity was lost when the gene was expressed under a constitutive promoter [40]. Certain promoters can also drive gene expression in more than one *Plasmodium* species (**heterologous promoter activity**). The 5' untranslated region (UTR) from the *P. chabaudi* dihydrofolate reductase thymidylate synthase *(dhfr-ts)* gene is transcriptionally active in *P. falciparum* [30], while the *P. falciparum* calmodulin (CAM) promoter region is active

in *P. berghei* [31]. This offers the possibility of developing shuttle vectors for transfection of *Plasmodium*. A detailed list of heterologous regulatory DNA sequences is shown in Table 1.

Four types of **transfection constructs** exist for transfection of *Plasmodium* [41]. First are constructs for transient transfection. In these constructs, the gene to be expressed is flanked by 5' and 3' UTR sequences. Together these three elements make up the expression cassette. Second, plasmids for stable episomal transfection. These constructs contain two expression cassettes, one for expressing the selectable marker (selection cassette) and the other for expressing the gene of interest (expression cassette). Plasmids are maintained in transfected parasites under conditions where the plasmid provides a selective advantage such as drug resistance. Removal of drug pressure results in plasmid loss over time due to incomplete daughter cell segregation. This leads to plasmid copy number variability and/or inefficient replication [42, 43]. Third, constructs for integration by single crossover mechanisms. These are referred to as insertion constructs. The constructs contain an expression cassette(s) and a single gene targeting sequence. A segment of the target gene is used as the gene targeting sequence. Finally, constructs for integration by double crossover mechanism. These are also known as replacement constructs. They contain two target gene sequences disrupted by a selection cassette and/or an expression cassette of the gene under study. Transfection of *P. falciparum* using insertion or replacement constructs requires circular constructs [41, 33]. However, in integrating DNA into *P. berghei* genome, the insertion construct is linearised within the target sequence, while the replacement construct is regularly linearised by removing bacterial plasmid sequences [41].

Because *Plasmodium* developmental stages are haploid (only the zygote is diploid), a single **selectable maker** is sufficient for knockout and gene expression by site directed integration. This has so far relied on using mutant forms of the *dhfr-ts* gene. Two types of selectable markers are commonly used for transfection of malaria parasites. **Positive selectable markers** donate a drug resistant trait to transfected parasites [34], allowing growth under drug pressure. Alternatively, applying drug pressure to parasites transfected with **negative selectable markers** [33, 44] confers survival disadvantage. Selectable markers currently used for transfection of *Plasmodium* are shown in Table 1.

Transfection and selection of mutants in malaria parasites is a slow process. In *P. berghei*, transfected parasites can be selected within a period of 3 weeks after transfection [45]. In contrast, it takes about 3 months to select for *P. falciparum* integrants [42].

Table 1. Parameters for transfection of malaria parasites

Event	Transfection system						References
	*P. gallinaceu*m	*P. falciparum*	*P. berghei*	*P. knowlesi*	*P. yoelii*	*P. cynomolgi*	
Electroporator settings	1.5, 2.5 and 3 kV, 25μF, 200Ω in 0.4 cm cuvettes	2.5 kV, 25μF, 200Ω in 0.4 cm cuvettes ; 0.31 kV, 960μF, in 0.2 cm cuvettes	800V, 25μF, in 0.4 cm cuvettes	1.5, 2 and 2.5 kV, 25μF, 200Ω in 0.4 cm cuvettes	800V, 25μF, in 0.4 cm cuvettes	1.5, 2 and 2.5 kV, 25μF, 200Ω in 0.4 cm cuvettes	[24,25, 46,47]
Transfected parasite stage	Extracellular gametocytes	Merozoites Intra-erthrocytic	Merozoites Intra-erythrocytic	Merozoites	Merozoites	Merozoites	[23,24, 26,28, 47]
Positive selectable markers	-	*T. gondii dhfr-ts* Human *dhfr-ts* Puromycin N -acetyl transferase, BSD, NEO	*T. gondii dhfr-ts* Human *dhfr-ts* *P. berghei dhfr-ts*	*T. gondii dhfr-ts* *P. berghei dhfr-ts*	*T. gondii dhfr-ts*	*T. gondii dhfr-ts*	[41,48, 38,49-50]
Negative selectable markers	-	Cytosine deaminase, Thymidine kinase	-	-	-	-	[33]
Integration transfection and selection	-	6 weeks to 3 months	Less than 3 weeks	-	Less than 3 weeks	-	[28,51, 45,46]
Promoters	pgs28	*P. falciparum hsp* 86, *cam*, *hrp3* and *dhfr-ts*; *P. chabaudi dhfr-ts*,	*P. berghei dhfr-ts* *ama-1*, *ef*-1α, *trap*, and *cs*; *P. chabaudi dhfr-ts*, *P. falciparum pfs*25	*P. berghei dhfr-ts* and *pbs21*; *P. falciparum hrp3*,	*P. berghei dhfr-ts*,	*P. berghei dhfr-ts*,	[25,28, 29,33, 38,39, 52,50]
Transfection culture	*In vitro*	*In vitro*	*In vivo*	*In vivo*	*In vivo*	*In vivo*	[23,24, 26,28, 47]
Current transfection mechanisms	Transient	Transient, stable episomal, integration by single and double cross-over	Transient, stable episomal, integration by single and double cross-over	Stable episomal,	Stable episomal, integration by double cross-over	Stable episomal	[23,24, 26,28, 47]
Transfection constructs	Circular	Circular	Circular, linear	Circular	Circular, linear	Circular	[23,24, 26,28, 47]

BSD, Blasticidin s deaminase; NEO, Neomycin phosphotransferase II; HSP 86, heat shock protein 86; CAM, calmodulin; TRAP, Thrombosponding related anonymous protein; CS, circumsporozoite protein; HRP3, histidine rich protein 3; *pfs25*, *P. falciparum* Pfs25; *pgs28*, *P. gallinaceum* antigen Pgs28; *pbs21*, *P. berghei* antigen Pbs21

1.3.4 Application of transfection

Transient expression of plasmids in *Plasmodium* has allowed for promoter mapping in order to define regions controlling gene expression. Functional analysis has been performed on the *P. falciparum hsp86* and *hrp3*, *cam*, *dhfr-ts* and *gp130* promoters and promoter regions and on the *P. chabaudi dhfr-ts* promoter (reviewed in [38]).

Transient transfection is also useful for rapid analysis of promoter activity. Stage specific promoter activity and heterologous promoter activity in *Plasmodium* was also shown using the procedure [29, 41, 26, 53].

Transfection techniques can be used to study the expression of genes that are uniquely processed by *Plasmodium* in a particular stage of the life cycle, for example expression of mosquito stage proteins in blood stage parasites [54]. This was clearly demonstrated for Pbs21. In this study, it was shown that stage specific expression is dependent on the promoter and not post-transcriptional modification. As deletions were made towards the transcriptional start site sites, stage specificity was lost and genes were expressed constitutively [55].

A major application of gene knockout studies is to analyse protein function *in vivo*. Gene knockout studies have shed useful information into the function of leading vaccine candidates. Menard *et al*., [45] reported that knocking out *P. berghei csp* gene resulted in the failure of sporozoites to infect hepatocytes, salivary glands and to undergo later stages of sporogony in the oocyst. Another knockout involving *P. berghei* TRAP, showed that the molecule was necessary for sporozoite gliding motility [37]. In a study involving knob associated histidine-rich protein [56], the knockout parasites bound weakly to CD36 and lost knob formation showing that the protein is important for knob assembly and for sequestration.

Through integration dependent transfection in *P. berghei*, it was shown that genetic complementation following gene knockout studies can confirm if a gene knockout is lethal. The knockout parasite can be complemented by a similar or modified version of the gene to restore gene function [52, 50]. This procedure is useful in confirming gene function.

The ability to transfect and express transgenes in *P. falciparum* has been an important technological breakthrough in determining the role of different malaria proteins in phenotypes such as drug resistance [57]. The direct role of particular mutations in the DHFR-TS enzyme was confirmed by transfection of the mutant *dhfr-ts* gene into *P. falciparum* to obtain parasite lines that were resistant to the expected levels of pyrimethamine [42]. Furthermore, transfection of the different mutant alleles of the gene for the bi-directional enzyme PPPK-DHPS has determined the mutations in *dhps* that confers sulphadoxine resistance [58]. Gene knockout and replacement with a modified version of the *cg2* gene has shown that it is not involved in chloroquine resistance and

instead a closely linked *pfcrt* gene is responsible [59, 60]. More recently, polymorphisms in the *pfmdr1* gene were determined through gene replacement strategies to affect resistance and sensitivity to mefloquine, halofantrine and quinine [61].

A number of genes from higher eukaryotes and other protozoan parasites have successfully been expressed in *Plasmodium* as transgenes. Reporter molecules from the jellyfish *Aequorea victoria* (GFP) [62, 63] and Luciferase [23, 24, 29] from the firefly were the first proteins from higher eukaryotes to be expressed in *Plasmodium.* A leading malaria vaccine candidate AMA-1 molecule from *P. falciparum* and *P. knowlesi* were successfully expressed in *P. berghei* [40, 47]. This provides wide possibilities to express *P. falciparum* proteins in animal malaria parasites and to study their function(s) using animal models. In another study, O'Donell *et al*., [64] successfully transfected *P. chabaudi* MSP-1_{19} in *P. falciparum* and concluded from the experiments that antibodies against MSP-1_{19} are a major component of the invasion-inhibitory response in individuals and mice immune to malaria.

Additional selectable markers are required for genetic complementation and for modification of essential genes. Current transfection of *P. berghei* and *P. knowlesi* has relied on *dhfr-ts* as the selectable marker of choice. Transfection has been used to develop new selectable markers based on blasticidin, geneticin and puromycin for use in *P. falciparum* [48, 49] and experiments are underway to adapt them to other *Plasmodium* systems. In the *P. berghei* transfection system, a new marker based on the drug WR92210 and the human dihydrofolate reductase gene is currently in use [52, 65].

Three exogenous reporters (GFP, luciferase and CAT) have been expressed in transfected *Plasmodium* [38]. These reporters are useful as molecular indicators of gene expression and in the study of intracellular molecular trafficking. In *P. falciparum*, expression of GFP was also used to determine signal peptide activity [66]. Fluorescence activated sorting of GFP expressing parasites was used to harvest pure parasite developmental stages for specific analysis, hence facilitating the characterisation of their phenotypes [62, 67]. Another reporter gene, the firefly luciferase has been used to develop the transfection systems for *P. gallinaceum* and *P. falciparum* and to determine promoter activity in *P. berghei* [23-25].

Transfection of malaria parasites can be applied to generate genetically engineered attenuated malaria parasites for possible inclusion into an attenuated malaria parasite vaccine. Nonhuman malaria parasites such as *P. knowlesi* offer the added advantage of testing host-parasite interaction of the attenuated parasites under natural host-parasite setting. Therefore, it is necessary to develop transfection systems for these parasites. The subsequent sections describe the *P. knowlesi* system.

1.4 The parasite *P. knowlesi*

The life cycle of *P. knowlesi* (Fig. 1) begins when an infected mosquito transmits sporozoites into a susceptible vertebrate host via blood sucking. The sporozoites migrate to the liver cells, where, in a process termed exo-erythrocytic schizogony (liver stage development), they undergo many rounds of replication and transform into liver schizonts. The infected hepatocytes rupture releasing merozoites which infect circulating erythrocytes immediately. In the cyclical pattern of development termed intra-erythrocytic schizogony (blood stage development) that follows, the parasites develop to rings, trophozoites and schizonts releasing more merozoites that continue to invade, colonise and replicate within erythrocytes. Schizont infected erythrocytes rupture releasing an average of 10 merozoites that continue the cycle of infection. The asexual cycle in blood is quotidian lasting 24 hours. During the blood stage development, *P. knowlesi* invades both mature erythrocytes and reticulocytes [68]. Ring forms appear in blood and appliqué forms are frequently seen. Band forms are common in the trophozoite stage. Mature schizonts have 10 merozoites on average, but the number can go as high as 16. Regular rings have one or more accessory chromatin dots. The factors which signal sexual development are poorly understood, but at some signal, a proportion of re-invading merozoites start to differentiate into male and female forms (micro- and macrogametocytes respectively). Mature gametocytes are seen in 3 days post-inoculation if the infection is heavy. Mature microgametocytes (8.5 μm) are smaller than the macrogametocytes (7 μm) and stain brick red with Giemsa, compared to the light blue colour of macrogametocytes. Gametocytes are taken up by a mosquito in a subsequent blood meal. In the mosquito midgut, gametocytes differentiate into mature micro- and macrogametes that mate to produce a zygote. The zygote differentiates into a motile ookinete that crosses the mosquito midgut and develops into a multi-nuclear oocyst. A mature oocyst, filled with sporozoites, ruptures releasing sporozoites into the heamocoel, from where they migrate to the salivary gland, ready to infect a vertebrate host during the next blood meal [69, 67, 71].

The mosquito hosts for *P. knowlesi* include *A. dirus*, *A. balabacensis* and *A. hackeri* [69, 70], all of which are found in Asia. Other possible vectors include *A. freeboni*, *A. maculatus*, and *A. quadrimaculatus* [70]. Development in the mosquito (sporogony) takes 10-12 days at a temperature of 26 to 28°C while the exo-erythrocytic phase lasts 5.5 days.

1.4.1 Animal models of *P. knowlesi*

The natural vertebrate hosts for *P. knowlesi* are *Macaca fascicularis* and *M. nemestrina*. Experimental infection can also be induced in a number of monkeys such as *M. mulatta*, *M. radiata*, *M. assamensis*, *Presbytis entellus*, *Callithrix jacchus*, *Aotus trivigatus*, *Saimiri sciureus* and

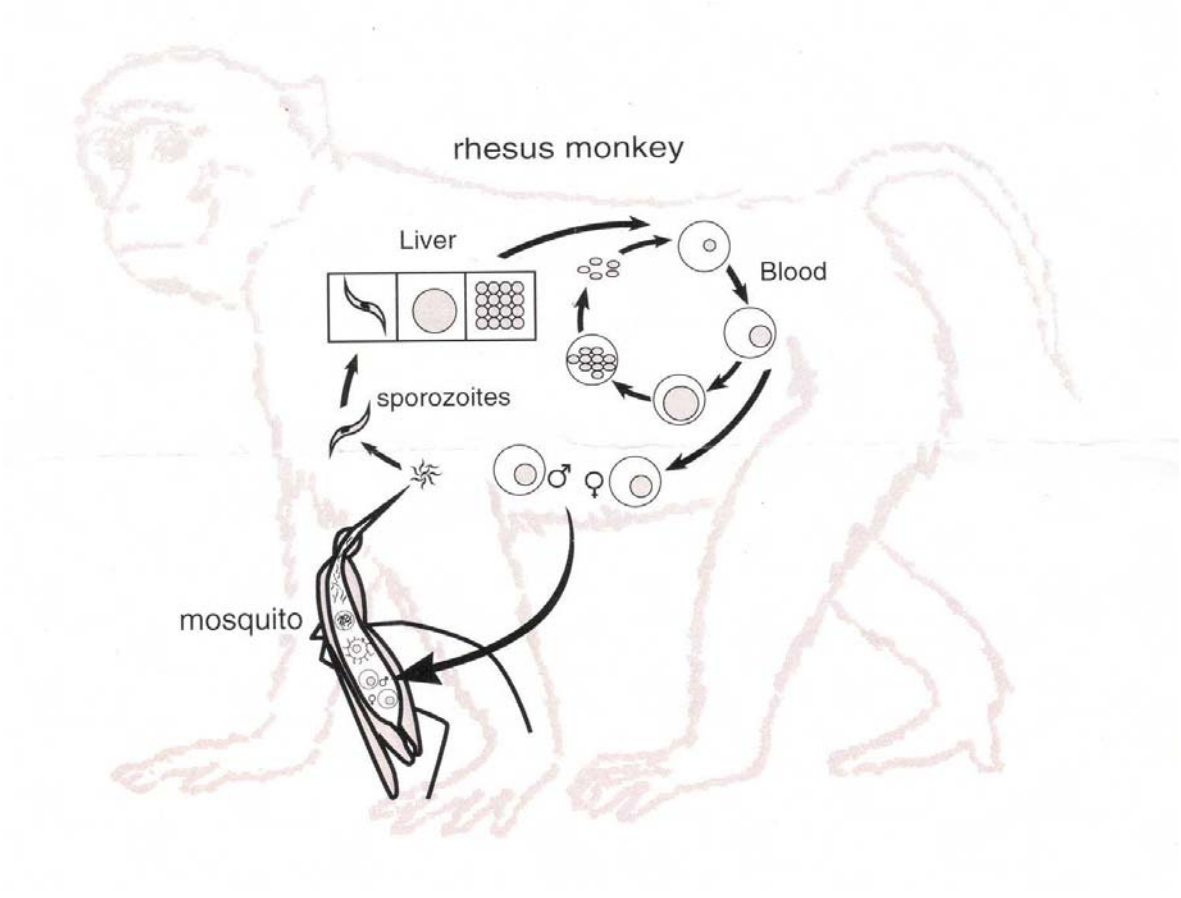

Figure 1. The life cycle of the simian malaria, *Plasmodium knowlesi*. Malaria infection of the monkey begins when an infected mosquito injects sporozoites into the blood circulation. The sporozoites circulate in the blood for less than 1 hour before invading liver cells, and develop into tissue schizonts in less than one week. The liver schizonts release thousands of merozoites into the blood stream, where they invade erythrocytes in less than 30 minutes. The newly invaded merozoites quickly develop into ring forms. The ring forms grow and differentiate into actively feeding forms known as trophozoites. A number of the ring forms differentiate into male and female sexual forms referred to as gametocytes. As the trophozoites feed and develop, the nucleus undergoes between 8 and 16 rounds of division (schizogony), without subsequent cell division. The multi-nucleate trophozoite (schizont) starts to divide its cytoplasm producing a similar number of haploid merozoites. The fully mature schizont (segmenter) ruptures the erythrocyte, releasing free merozoites that invade other erythrocytes, thereby completing the blood stage cycle. When a mosquito ingests blood with mature gametocytes, they emerge from the erythrocyte and differentiate into gametes. The male gametocyte (microgametocyte) undergoes three rounds of nuclear division, producing 8 flagellated microgametes (exflagellation), while the female gametocyte differentiates into a single gamete celled macrogamete. In the mosquito midgut, the microgamete immediately fertilises the macrogamete to form a diploid (2N) zygote. The diploid zygote undergoes one more round of genome division into a polyploid (4N) zygote. The zygote then undergoes meiotic division and transforms into a motile ookinete, which penetrates the midgut wall, and rounds up below the midgut epithelium as an oocyst. The oocyst enlarges and the nucleus divides frequently, followed by multiple cell fission to form thousands of midgut sporozoites. The sporozoites are released into the haemocoel where they migrate to the salivary glands, waiting for being injected into a new vertebrate host [69,70].

baboons [69, 70, 72-76]. In baboons, the infection has been induced in *P. cynocepahalus*, *P. doguera*, *P. jubileaus* and *P. papio* [70]. The parasite does also infect humans [77-80]. In humans, *P. knowlesi* causes mild infection seldom exceeding 1% parasitaemia. However, infection could get virulent on repeated passages in humans [81]. In the natural hosts, the infection is chronic with several relapsing peaks. The highest parasitaemia is usually in the first peak and rarely passes 5%. In the rhesus monkey (and most experimental models), the infection is acute and usually fatal, killing the animals in 7 to 14 days post-infection [69]. It requires several infections and cure in order for *M. mulatta* to develop immunity against *P. knowlesi* [82-84].

The availability of natural and experimental hosts for *P. knowlesi* offers the possibility to study the biology of malaria parasite and its antigens in a natural host-parasite combination and in hosts whose systems are predictable of the human situation [85]. In addition, the dichotomous disease profile of *P. knowlesi* (a chronic infection in natural hosts and an acute disease in artificial hosts) provides opportunities for understanding the mechanisms of immunity to malaria [86]. Experimental *P. knowlesi* infection has not been characterised in *P. anubis*, the most commonly used baboon for biomedical research.

1.4.2 *In vitro* culture of *P. knowlesi*

In 1912 Bass and Johns [87] were the first to report *in vitro* development of malaria parasites. Defibrinated patient blood containing *P. falciparum* and *P. vivax* was cultured for two cycles in glass vials to which small amount of glucose had been added. Nearly 25 years later [88], short-term cultures of *P. knowlesi* in rhesus monkey erythrocytes were achieved. The medium used in these cultures was known as Harvard growth medium. It was based on a chemical analysis of monkey plasma. Improved growth and multiplication was obtained with subsequent modifications of this medium, but only a few erythrocytic cycles were achieved *in vitro* [89]. In these early experiments, blood from infected rhesus was mixed with uninfected erythrocytes to give an initial parasitaemia of 1%. The blood was washed and suspended in Harvard medium or one of the modifications of it, in a culture vessel of considerable surface area such as a 50 ml conical flask. The cultures were gently agitated on a rocking platform to maintain the cells in suspension. The parasites would mature through the first *in vitro* cycle and often would invade into new erythrocytes, with little or no increase in parasite numbers. At best the parasites developed for a few days but with decreasing numbers, after an initial increase, until the cultures no longer contained viable parasites.

These earliest *in vitro* experiments revealed specific *in vitro* nutritional requirement by *P. knowlesi* for para-aminobenzoic acid. Other specific nutritional requirements demonstrated by

short-time cultures included biotin, methionine and purines [90]. In addition the cultures were used to study certain aspects of parasite metabolism [91] and action of anti-malaria drugs [92].

During later years, attempts for long term culture of *P. knowlesi* were made based on the assumption that cultured erythrocytes rapidly became unsuitable as host-cells for the parasites. To that effect, fresh erythrocytes were frequently added with increments of as high as 40% packed cell volume. However, the rate of parasite multiplication could not keep ahead of the rate of erythrocyte dilution and the cultures died out within 7 days [93]. Later experiments [94] further demonstrated that less multiplication was progressively achieved with successive cycles of development *in vitro*. These experiments not only achieved low parasitaemia, for example, in the range of 0.92% and 3.6% [95] but also generated morphologically abnormal parasites [96]. Culturing was done using the petri-dish candle jar method [95] and a sophisticated semi-automated tipping culture apparatus [96]. These culturing conditions were insufficient for generating large amounts of parasites for analysis. Furthermore, cloning was not achieved. These studies show that long term *in vitro* culture of *P. knowlesi* has remained elusive.

1.4.3 *P. knowlesi* as a model parasite for malaria research

Although human malaria parasites are seen as a major priority for research because of the mortality associated with this infection, they are not always the optimal system in which to study the biology and immunology of *Plasmodium*. The host specificity of human malaria parasites represents a major constraint on *in vivo* studies. The parasites can not be maintained in convenient small laboratory animals. As a result, numerous species of rodent (e.g. *P. berghei*) and avian (e.g. *P. gallinaceum*) malaria parasites have been widely used as laboratory models to study the biology of Plasmodia [97]. Although there is proximal phylogenetic relationship between *P. falciparum* and avian Plasmodia such as *P. gallinaceum* [98], differences in their life cycles, insect hosts and in the immune systems of their vertebrate hosts [4] limit their usefulness as models for human malaria. A good laboratory model should be relevant to human malaria and offer the ability to study the biology of the parasite at the cellular and molecular level. Biological, chemotherapeutic and immunological studies have made use of the fact that a number of *Plasmodium* species found naturally in monkeys are very similar biologically and antigenically and have similar host-parasite relationships to the human Plasmodia in man. This is in part due to the close phylogenetic relationships between the nonhuman primate and human malaria parasites [99].

P. knowlesi is an attractive experimental system for malaria research. Firstly, it is a parasite of monkeys that have immune and metabolic systems very similar to those of humans [85]. Secondly, *P. knowlesi* is phylogenetically close to *P. vivax*, sharing many vaccine candidate molecules [100].

Thirdly, the entire genome has recently been sequenced to 5-fold coverage [101], availing more gene sequences for analysing parasite biology and discovering vaccine candidate genes. Finally, tools for genetic manipulation in *P. knowlesi* are available. Because *P. knowlesi* has a 24 hour life cycle, this allows rapid generation and analysis of transfected parasites.

1.5 Outline of this thesis

Transfection of malaria parasites now permits the experimental evaluation of molecular techniques for developing live-attenuated blood stage parasite vaccines that could induce strong protection comparable to attenuated sporozoite vaccines. In the studies described in this thesis, an *in vitro* transfection system of the primate malaria parasite *P. knowlesi* has been developed and used to investigate molecular ways of immunopotentiating malaria parasites, as a potential component of an attenuated blood stage vaccine. This is because 1) there is no suitable animal model for the evaluation of attenuated *P. falciparum* (New World monkeys become rapidly immune to further infection or require splenectomy) 2) as a natural parasite of old world monkeys *P. knowlesi* will infect animals on multiple occasions before immunity develops, and interactions can be evaluated in an immunological and metabolic environment similar to humans.

The content of this thesis is grouped into three categories. Chapters 2, 3 and 5 focus on development of tools required for *in vitro* transfection of *P. knowlesi* and *in vivo* analysis of transfected parasites. In chapters 4 and 6, the *in vitro* transfection system of *P. knowlesi* is applied to analyse expression of a host cytokine, IFN-γ as an approach for immunopotentiating malaria parasites. Finally, chapter 7 is devoted to development of a new model for *in vivo* analysis of transfected parasites.

In order to transfect and select malaria parasites *in vitro*, long term *in vitro* culture techniques, similar to the *P. falciparum* system [102] are required. In **chapter 2**, a long term *in vitro* culture system was developed for *P. knowlesi* blood stages. Culture adapted parasites were continuously cultured *in vitro* for over 12 months in order to determine their long term culture characteristics. In addition, *in vitro* cloning by limiting dilution was tested. *In vivo* characteristics of culture adapted parasites were also characterised in rhesus monkeys (*Macaca mulatta*). *P. knowlesi* was successfully adapted to long term *in vitro* culture. *In vitro* adapted parasites regained wild type characteristics after a single passage through an intact rhesus monkey. Cloning by limiting dilution, and *in vitro* transfection were successfully achieved using culture adapted parasites. In addition, integration into the CSP locus was done. Microscopic evaluation of developing oocyst from mosquitoes that had fed on CSP knockout parasites showed impairment of sporozoite formation.

In studies described **in chapter 3**, a transient transfection system was developed for *P. knowlesi* using luciferase as a reporter molecule. Expression of luciferase was tested under the control of the stage specific *P. berghei* apical membrane antigen-1 (*pbama-1*), the constitutive *P. berghei* elongation factor-1 alpha (*pbef-1*α) and *pbdhfr-ts* promoter regions. In addition stable episomal transfection technology was used to analyse *in vitro* stage specific expression of GFP under the *pbama-1* promoter. The *P. berghei pbama-1*, *pbef-1*α and *pbdhfr-ts* promoter regions successfully drove expression of luciferase in *P. knowlesi* using transient transfection technology. This study identified the *pbef-1*α promoter as a strong heterologous promoter ideal for over-expression of transgenes in *P. knowlesi* blood stages. Stable episomal transfection was successfully applied to express GFP in *P. knowlesi*. These experiments showed that the *pbama-1* promoter maintained a tight stage-specific expression of GFP in *P. knowlesi*.

Expression of host cytokines in malaria parasites offers the opportunity to investigate the potential of an immunomodulatory approach by generating immunopotentiated parasites. **Chapter 4** of this study analysed the capacity of malaria parasites to express host IFN-γ. The *in vitro* transfection protocol for *P. knowlesi* was applied to explore the conditions for expressing host cytokines in malaria parasites. *In vitro* culture adapted *P. knowlesi* blood stage parasites were transfected with DNA constructs for expressing rhesus monkey IFN-γ under control of the *pbama-1* promoter. *P. knowlesi* blood stage parasites were shown to produce IFN-γ into the culture medium. *In vitro* analysis through inhibition of virus cytopathic effects and activation of *M. mulatta* peripheral blood cells showed that the parasite produced IFN-γ was bioactive.

The vast majority of antibodies available for characterisation of host responses in primates are anti-human. The molecules recognise the same epitopes in the primate under study [103]. These antibodies were selected through their optimal cross-reactivity with similar primate antigens. In order to generate cross-reactive antibodies for use in characterisation of host responses to malaria parasites, a large number monoclonal antibodies were characterised for their cross-reactivity with the chimpanzee, the squirrel monkey, and the rhesus monkey cell surface antigens. These monkeys are common nonhuman primate hosts of malaria. Several antibodies that reacted with T-cell antigens and cytokine receptors were identified for *in vivo* immunological studies. The list of cross-reactive antibodies and their levels of activity is tabulated in **chapter 5**. Suitable antibodies will be used to characterise host-parasite interaction between IFN-γ expressing *P. knowlesi* and the rhesus monkey.

In **chapter 6**, rhesus monkeys were infected with cytokine expressing parasites and clinical and immune response parameters measured to determine the efficacy and safety of IFN-γ expressing

parasites. This would also determine *in vivo* bioactivity of parasite produced IFN-γ and the vaccine potential of IFN-γ producing parasites. Animals infected with IFN-γ producing *P. knowlesi* developed a non-fulminating parasitaemia. Their liver and kidney functions were within normal ranges. There was a 2.5 to 5 fold increase in γδ T-cells compared to a 1 to 2 fold increase in the controls. After challenge infection, there was a 5 to 6 day longer pre-patent period compared to the controls. Control monkeys also developed a fulminating infection averaging 5 days earlier.

In order to determine the suitability of olive baboons (*Papio anubis*) as experimental hosts for *P. knowlesi*, 10 baboons were experimentally inoculated with *P. knowlesi* blood stages. The animals were observed for parasitaemia profiles, clinical and pathological parameters. Historical data was collected from rhesus monkeys infected with a similar *P. knowlesi* strain and used for comparative purposes. The results of this study are reported in **chapter 7**. All baboons infected with blood stage *P. knowlesi* developed either a severe moribund disease accompanied by multiple system organ dysfunction and cerebral malaria, or a mild infection characterised by low level parasitaemia and splenomegaly. These studies demonstrated for the first time that *P. anubis* is a suitable *P. knowlesi* host for studying clinical symptoms and pathology.

Chapter 8 is the general discussion. This chapter discusses the current status of *P. knowlesi* transfection. Long term *in vitro* culture protocol was established thus expanding experimental possibilities for transfection. *In vitro* and *in vivo* transfection protocols currently in place will minimise the use of resourceful monkeys for the simple process of generating parasites, while retaining the possibility of *in vivo* experimentation as need arises. Expression of host cytokines such as IFN-γ offers new approaches for developing attenuated and immunopotentiated malaria vaccines. The genome of *P. knowlesi* has been sequenced, indicating that genes for all possible drug and vaccine candidates are accessible. Transfection should play a role in deciphering the function of these molecules and by analogy, similar molecules could be isolated from human malaria parasites and expressed in *P. knowlesi*. Subsequently, the drug and/or vaccine potential of selected candidates can be determined using *in vitro* and *in vivo* systems. As a parasite of primates that also infects humans, *P. knowlesi* currently is the only malaria transfection system that combines the benefits of a simple long term *in vitro* culture system and analysis of host-parasite interaction in natural and experimental host systems similar to human.

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CHAPTER 2

Plasmodium knowlesi provides a rapid *in vitro* and *in vivo* transfection system that enables double-crossover gene knockout studies

Clemens H. M. Kocken, Hastings Ozwara, Annemarie van der Wel, Annette L. Beetsma, Jason M. Mwenda and Alan W. Thomas

Department of Parasitology, Biomedical Primate Research Centre, P.O. Box 3306, 2280 GH Rijswijk, The Netherlands, Departments of Infectious Diseases and Reproductive Biology, Institute of Primate Research, National Museums of Kenya, P. O. Box 24481, Karen, Nairobi, Kenya.

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ABSTRACT

Transfection technology for malaria parasites provides a valuable tool for analysing gene function and correlating genotype with phenotype. Transfection models are even more valuable when appropriate animal models are available in addition to complete *in vitro* systems to be able to fully analyse parasite-host interactions. Here we describe the development of such a model by using the nonhuman primate malaria *Plasmodium knowlesi*. Bloodstage parasites were adapted to long-term *in vitro* culture. *In vitro*-adapted parasites could readapt to *in vivo* growth and regain wild-type characteristics after a single passage through an intact rhesus monkey. *P. knowlesi* parasites, either *in vitro* adapted or *in vivo* derived, were successfully transfected to generate circumsporozoite protein (CSP) knockout parasites by double-crossover mechanisms. *In vitro*-transfected and cloned CSP knockout parasites were derived in a time span of only 18 days. Microscopic evaluation of developing oocysts from mosquitoes that had fed on CSP knockout parasites confirmed the impairment of sporozoite formation observed in *P. berghei* CSP knockout parasites. The *P. knowlesi* model currently is the only malaria system that combines rapid and precise double-crossover genetic manipulation procedures with complete *in vitro* as well as *in vivo* possibilities. This allows for full analysis of *P. knowlesi* genotype-phenotype relationships and host-parasite interactions in a system closely related to humans.

INTRODUCTION

The development of transfection technology for blood-stage malaria parasites [1-6] is of great importance in the postgenomic era. It provides a direct way in which to correlate genotype with phenotype, and this enhances the further understanding of parasite biology. This will facilitate rational design of new vaccines and drugs, which are urgently needed to fight the malaria epidemic that kills annually between 1.5 and 2.7 million people, mainly young children, in Sub-Saharan Africa alone [7].

Four species of *Plasmodium* are natural to humans [8], and two of them, *Plasmodium falciparum* and *P. vivax*, are the most prevalent and important in terms of disease. Phylogenetically, *P. falciparum*, the more deadly form of the two, forms a separate clade with *P. reichenowi*, which causes chimpanzee malaria, and *P. vivax* clusters with simian malarias [9]. The nonhuman primate malaria, caused by *P. knowlesi*, a natural parasite of *Macaca fascicularis*, has a relatively broad host range extending to humans, where it causes a mild disease [10]. The parasite is closely related to *P. vivax* [9], and many genes identified in *P. vivax* have homologues in *P. knowlesi*. To date, transfection techniques developed for malaria

parasite bloodstages [11] include episomal transfection and targeted integration with linear constructs for the rodent parasite *P. berghei* [3, 4], episomal transfection and targeted integration with circular DNA for the human parasite *P. falciparum* [6, 8], and episomal transfection for the nonhuman primate malaria parasites *P. knowlesi* [2] and *P. cynomolgi* [1]. Targeted integration experiments in *P. falciparum*, exploiting the results from the genome project [12, 13], are time-consuming, requiring several cycles of drug pressure to select for integration events based on a single crossover [5]. In addition, this system offers only restricted *in vivo* possibilities in scarce New World primate systems. The *P. berghei* system offers a rapid targeted integration regime through double crossover [3]. It has, however, only limited *in vitro* possibilities for bloodstage parasites due to reticulocyte restriction, precluding *in vitro* transfection and selection procedures and thus, for example, the use of selectable markers that are toxic to the host.

P. knowlesi offers an especially powerful experimental system, since both the natural host, *M. fascicularis*, and an experimental host, *Macaca mulatta*, are phylogenetically close to humans. Genetic manipulation of this parasite species [2] offers the unique possibility to study parasite-host interactions in a system that is highly predictive for the human situation [14, 15]. A complete *in vitro* transfection and selection system for this parasite would greatly enhance the experimental possibilities, since some analyses could be carried out without any requirement for primates. Furthermore, where host-parasite interactions are being studied, *in vitro* selection of the cloned parasite of the correct genotype can be assured before primate use is required. Publications on *in vitro* cultivation of *P. knowlesi* bloodstage parasites are scarce, and the reported technology is cumbersome [16-18]. Culture medium was refreshed at least twice daily [16], red blood cells were added five times per week leading to up to 40% erythrocyte concentrations, and multiplication rates of <2.5 per 24-h lifecycle were reported [16, 18]. These labour-intensive culture conditions do not allow the generation of enough parasites of good quality to perform transfection experiments.

We set out to develop a complete *in vitro* system for *P. knowlesi* to allow long-term *in vitro* culture and an extremely rapid procedure for *in vitro* transfection, selection, and cloning of transfected parasites. In addition, we further developed the *in vivo* and *in vitro* transfection technology for *P. knowlesi* to include targeted integration with linear constructs. This was demonstrated by targeting the circumsporozoite protein (CSP) locus and phenotypical analysis of the CSP knockout. The *P. knowlesi* system now is the only malaria system that combines rapid genetic manipulation procedures with complete *in vitro* as well as *in vivo* possibilities, allowing full analysis of genotype-phenotype and parasite-host relationships in a

host closely related to humans.

MATERIALS AND METHODS

Parasites and animals. *P. knowlesi* Nuri [19] and H strain [8] cryopreserved stocks were used to initiate blood-stage infection in rhesus monkeys by intravenous inoculation of 10^5 parasites. *In vitro* cultures were started with the same parasite strains.

Adult rhesus monkeys (*M. mulatta*) of either sex, each weighing over 4 kg, were used in these studies. They were infected with cryopreserved stocks of *P. knowlesi*, *in vitro*-adapted blood-stage *P. knowlesi* parasites, or blood from another rhesus monkey. All experimental animal work in these studies was carried out under protocols approved by the independent Institutional Animal Care and Use Committee and performed according to Dutch and European laws.

Long-term *in vitro* culture of *P. knowlesi*. *In vitro* cultures were initiated with either cryopreserved or fresh *P. knowlesi* parasites isolated from an infected rhesus monkey. Rhesus red blood cells were used to culture *P. knowlesi*. White blood cells were removed by Plasmodipur filtration (Eurodiagnostica, Apeldoorn, The Netherlands), and red cells were stored for up to 2 weeks in RPMI at 4°C. Parasites were grown in static cultures at 36.5°C under reduced oxygen conditions (5% CO_2, 5% O_2, 90% N_2) in RPMI 1640, supplemented with 20% pooled, heat-inactivated rhesus serum and rhesus erythrocytes at a 2.5% haematocrit. During the adaptation period medium was changed every 24 h, and fresh erythrocytes were added every 4 days to a maximum haematocrit of 5%. Once they had been established in culture, parasites were maintained under the same conditions except for medium changes every 48 h and subculturing when parasitaemias exceeded 5%. Parasitaemia was determined by microscopic examination of Giemsa-stained thick and thin films prepared from the cultured material at regular intervals. The culture-adapted parasites were subsequently adapted to growth in RPMI 1640 supplemented with 20% pooled, heat-inactivated human A or AB serum, by using a similar strategy. Parasites were cryopreserved at the young ring stage of development by standard protocols [20]. Cloning of culture adapted *P. knowlesi* was performed by limiting dilution.

Transfection constructs. All transfection constructs contained a heterologous selection cassette based on mutagenised *Toxoplasma gondii* dihydrofolate reductase/thymidine synthase gene (*dhfr/ts*) conferring pyrimethamine resistance, flanked by *P. berghei dhfr/ts* flanking sequences [21].

Sequences for *P. knowlesi* CSP locus were retrieved from GenBank entries (accession

numbers K00822 and M19749). To prepare a CSP knockout construct, 5' and 3' regions from *csp* were amplified by PCR. These regions were as follows: 5' nucleotides (nt) -1495 to -560 and 3' nt 1 to 1089 (for numbering, nt 1 is the start of open reading frame [ORF]). Through a series of cloning steps plasmid $pD_B.D_{Tm}.D_B$/CSPko was derived (Fig. 1A). After purification on Plasmid Maxi columns (Qiagen), the vector backbone was excised from the construct by *Eco*RI digestion, and the linear DNA was used for transfection of *P. knowlesi*. Recombinant DNA manipulations and analyses were performed according to standard procedures [22].

Transfection procedures and selection of transfected parasites. Transfection of mature blood-stage schizonts of *P. knowlesi* H-strain derived from a donor rhesus monkey and *in vivo* selection of transfected parasites by using oral dosing of pyrimethamine were performed as described previously [2], except that pyrimethamine was given at 1 mg/kg of body weight daily for five consecutive days and thereafter every other day until the end of the experiment. For transfection of culture-adapted parasites, up to 2 ml (packed cell volume) of red cells containing between 0.5×10^9 and 1×10^9 mature schizont-infected red cells was washed with incomplete Cytomix [6], mixed with 100 μl of transfection construct containing 50 μg of DNA, and electroporated (Bio-Rad GenePulser) in three 700 μl aliquots at a pulse of either 1.5, 2, or 2.5 kV, with 25-μF capacitance and 200 Ω resistance (the time constant ranged from 0.7 to 1.1 ms). Samples electroporated under the same conditions were pooled, incubated on ice for 5 min, and used to initiate 20 ml *in vitro* cultures at a 10% haematocrit. Culture medium was refreshed daily for 3 days to remove parasite material resulting from dead parasites and lysed erythrocytes. After that, the medium was replenished every other day. Pyrimethamine selection (25 ng/ml [final concentration] from a stock solution in 0.5% lactic acid in phosphate-buffered saline) was started 24 h after culture inoculation and maintained throughout the culture period.

DNA analysis. Total parasite DNA was isolated (Gentra Systems, Inc., Minneapolis, Minn.) directly from *in vitro* cultures or after Plasmodipur filtration of *P. knowlesi*-infected rhesus monkey blood according to the manufacturer's instructions. Parasite DNA was analysed through plasmid rescue by electroporation into *E. coli*, PCR, and Southern blotting according to standard procedures [19]. The PCR primers used for confirming integration into the CSP locus were A (5'-GTGTCTATATTACCAACTC-3') and B (5'-GTCAAAAAAGGGTCAGTCAAAAAGGG-3') (Fig. 1A).

Transmission studies. Rhesus monkeys were infected as described above, and *Anopheles stephensi* mosquitoes were allowed to feed for 10 min on the shaven chests of sedated monkeys 6 to 8 days after infection, when the parasitaemia was between 0.5 and 3%. Unfed

mosquitoes were removed, and engorged mosquitoes were maintained at 26°C and 80% relative humidity. From day 6 onward, midguts were dissected and examined by light microscopy (400× magnification) to monitor oocyst development and sporozoite formation within oocysts.

RESULTS

Long-term *in vitro* culture of *P. knowlesi*. To improve the versatility of the *P. knowlesi* transfection system [2], *in vitro* protocols are required to allow the generation of genetically transformed parasites without the need for primates. Transformed parasites of the desired genotype can then be used *in vivo* to study biological questions at the parasite-host interface. Development of an *in vitro* transfection and selection protocol required establishment of stable, long-term *in vitro* culture of parasites. *In vivo* we have observed different growth characteristics between the H strain and the Nuri strain. Nuri strain reproducibly gives fulminating parasitaemias with an average 15-fold multiplication rate that rises to >20% ca. 1 week after infection. Some rhesus monkeys infected with H strain showed similar parasite development but with a four- to fivefold multiplication rate. Others showed a rapid rise to ca. 5% parasitaemia that subsequently developed into a chronic infection. *P. knowlesi* H strain and Nuri strain, originating from cryopreserved stocks or directly from an infected rhesus monkey, were both successfully adapted to *in vitro* culture by using RPMI 1640 supplemented with 20% rhesus serum and an incubation temperature of 36 to 37°C. After an initial lag phase of at least 3 weeks in which parasites were hardly detectable in the cultures, adapted parasites emerged that now had been in continuous culture for >18 months (Fig. 2). Routinely, three- to fourfold multiplication rates per 24 h were obtained for the H strain and slightly higher (four- to fivefold) for the Nuri strain parasites in static cultures, and parasitaemias of up to 20% could easily be obtained. Culture adapted H-strain parasites were subsequently adapted to growth in medium containing 20% human serum, in which similar growth rates were observed. The use of temperatures of 39°C (the normal temperature of rhesus monkeys) during cultivation slowed down the parasite growth, and temperatures of 36 to 37°C appeared to be the optimal growth temperature. Culturing parasites under shaking conditions or in a roller bottle system did not dramatically improve growth rates, but roller bottles now routinely supplied 10^{10} parasites from a 200 ml culture in a 2-liter flask. Finally, culture-adapted parasites were cloned by limiting dilution (Table 1), demonstrating the feasibility of the procedure required to obtain clonal parasite populations after transfection.

The *P. knowlesi* H strain (either wild type or culture adapted) was used in subsequent

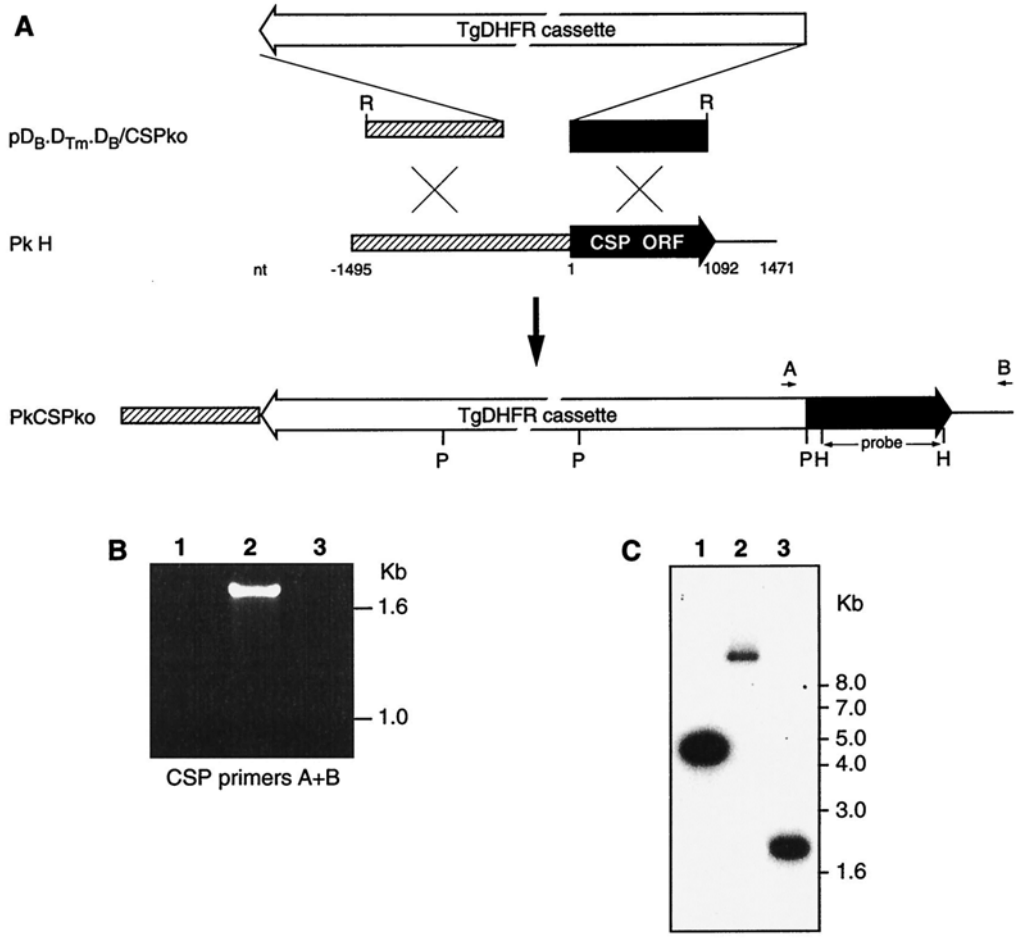

Figure 1. DNA constructs and analysis of integration into *P. knowlesi* CSP locus after transfection. (**A**) Linear DNA construct designed for integration into the *P. knowlesi* CSP locus. The 5-kb selectable marker cassette (TgDHFR cassette) contains *P. berghei dhfr/ts* flanking regions controlling expression of mutagenised *T. gondii dhfr/ts*. Relevant restriction sites are indicated: R, *Eco*RI; P, *Pst*I; H, *Hin*dIII; B, *Bam*HI. (**C**) The location of the PCR primers A and B and the specific probe used for Southern blotting are shown. ORF-containing sequences are marked with an open arrow, and sequences used for targeted integration are indicated. (**B**) PCR analysis of transfected parasites. Lanes 1 to 3 show PCR with CSP integration-specific primers A and B. Lanes: 1, *P. knowlesi* H-strain DNA; 2, PkCSPko clone DNA; 3, $pD_B.D_{Tm}.D_B$/CSPko vector DNA. PkCSPko and the transfection construct were all positive for PCR with the two *T. gondii dhfr/ts*-specific primers (not shown). (**C**) Southern blot analysis of transfected parasites. *Pst*I-digested DNA from *P. knowlesi* H strain (lane 2), PkCSPko clone (lane 3), and the transfection vector $pD_B.D_{Tm}.D_B$/CSPko (lane 1) was used to prepare a Southern blot. The blot was probed with a *P. knowlesi* CSP-specific probe (see panel A).

transfection experiments.

Culture-adapted parasites can be readily readapted to *in vivo* growth. To determine whether *P. knowlesi* H strain that had been maintained in culture for 11 months was still able to grow in rhesus monkeys, a rhesus monkey was infected with 10^5 parasites. Patent

parasitaemia was observed after 12 days, and a chronic low-level parasitaemia developed with a peak parasitaemia of 0.2%. On day 17, 1 ml of blood from this monkey was transferred to a second rhesus monkey, and this monkey developed a fulminating parasitaemia from day 6 onward, reaching 19% parasitaemia on day 10. Two further infections were initiated by using stocks from the first passage, resulting in peak parasitaemias of 5% on day 8 and 1.2% on day 10, respectively. After the peak, the parasitaemias declined in both rhesus. Two rhesus monkeys were infected with a stock from the second passage, and patency started on day 4, resulting in 4.2% parasitaemia on day 7 and 6.3% parasitaemia on day 9, respectively. At that time, the animals were bled to provide parasites for further transfection experiments. These results indicate that the *in vivo* H-strain phenotype of either a fulminant infection or a chronic infection after a ca. 5% peak parasitaemia is obtained after one passage through an immunologically intact rhesus monkey. When these parasites were cultured *in vitro* again they immediately developed like *in vitro*-adapted parasites, without any adaptation period.

Transfected *P. knowlesi* can be readily selected *in vitro*. By using previously determined transfection conditions [2], *P. knowlesi* schizonts, derived either from an infected rhesus monkey or from an *in vitro* culture, were transfected with plasmids containing the *T. gondii dhfr/ts* selection cassette. After transfection, parasites were maintained in 20 ml cultures, and pyrimethamine selection was applied from day 1 onward. The only transfection condition that consistently resulted in selection of transfected parasites was at 2.5 kV, 200 Ω, and 25 μF.

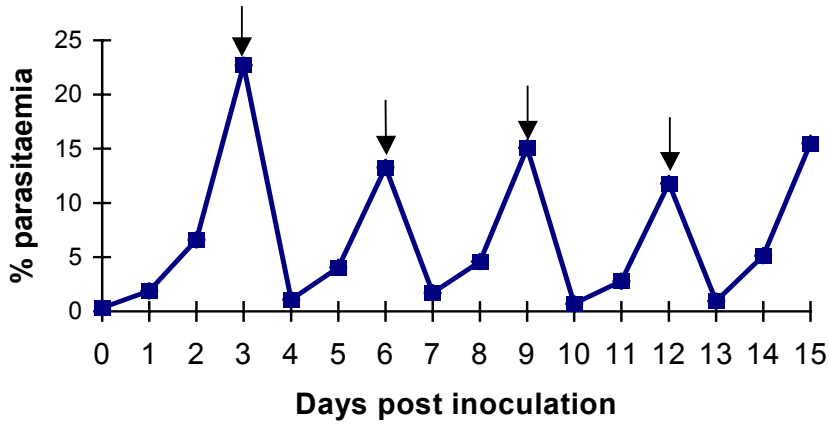

Figure 2. *In vitro* growth characteristics of *P. knowlesi* H strain. Parasitaemia development over a 15-day period. Parasites were cultured as described in Materials and Methods, and after 3 months the parasitaemia level was determined daily and expressed as a percentage of infected red blood cells. Arrows indicate the days on which the culture was diluted.

Pyrimethamine-resistant parasites emerged in culture after 21 days for parasites derived from the monkey and after 8 days for *in vitro*-adapted parasites. Plasmid rescue from the resistant pools of parasites demonstrated the presence of plasmid DNA in an unrearranged form (not shown).

Targeted integration through double crossover can be obtained *in vivo* as well as entirely *in vitro*. Previously, we reported transfection of *P. knowlesi* with plasmids that were maintained as episomes [2]. To determine whether targeted integration in this system is feasible with linear constructs, as has been reported for *P. berghei* [3], an integration construct for the *P. knowlesi* CSP locus was prepared. The construct had 0.93 kb of *P. knowlesi* CSP 5'-untranslated region (UTR) sequence located 5' of the selection cassette and 1.0 kb of *P. knowlesi* CSP ORF sequence located 3' of the selection cassette (Fig. 1A). Parasites derived from a rhesus monkey were transfected with linearised constructs and selected *in vivo* as described above. In parallel, the CSP knockout construct was used to transfect *in vitro* cultured schizonts, and selection was performed *in vitro*. As with the episomal transfections, 2.5 kV consistently resulted in selection of pyrimethamine-resistant parasites. *In vivo* and *in vitro*, pyrimethamine-resistant parasites were detected on day 8. Plasmid rescue experiments from these parasites consistently failed to produce ampicillin-resistant *E. coli*, suggesting the absence of (recircularised) episomes within the transfected parasite populations. Cloning by limiting dilution of *in vitro*-selected transfectants was started on day 8, and clones were observed in thin films by day 18. Multiplication rates for these transfected parasites were about four times per 24 h *in vivo* and about three times per 24 h *in vitro*. PCR on *in vitro*-selected and cloned PkCSPko genomic DNA with primers A and B (Fig. 1 A) (amplifying a 1.7 kb *P. berghei dhfr/ts* 5'-UTR–CSP ORF region only present when targeted integration has occurred) showed integration into the CSP locus (Fig. 1B, lanes 1 to 3). This was also confirmed by Southern blotting with a 0.89 kb *Hin*dIII fragment (Fig. 1A) of the CSP ORF as a probe. As shown in Fig. 1C, lanes 1 to 3, the 10 kb wild-type *Pst*I fragment from the CSP locus (lane 2) is converted to a 2.5 kb fragment in PkCSPko (lane 3) due to the introduction of *Pst*I sites contained in the transfection construct.

CSP knockout *P. knowlesi* does not produce sporozoites in oocysts. To examine the phenotype of CSP knockout *P. knowlesi*, rhesus monkeys were infected with either *P. knowlesi* H strain or the PkCSPko blood-stage parasites. The development of parasitaemia in both monkeys was similar, showing a rapid rise to ca. 10% parasitaemia on days 8 and 9 postinfection. *A. stephensi* mosquitoes were allowed to feed on day 8 on the wild-type infected monkey (0.9% parasitaemia) and on days 6 and 7 on the PkCSPko-infected monkey

(0.5 and 3% parasitaemias, respectively). Fed mosquitoes were isolated and kept at 26°C to allow oocyst and sporozoite development. From day 6 postfeeding onward, mosquitoes were dissected and midguts were examined for the presence of oocysts and sporozoites. Day 7 feeding of the PkCSPko resulted in the highest transmission rates (100%), with ca. 50 oocysts per midgut. The wild-type feeding resulted in 83% infection and ca. 26 oocysts per midgut. Wild-type oocysts produced sporozoites in the heamocoel from day 8 postfeeding onward, as determined by light microscopic examination of midguts (Fig. 3A and C). Spontaneously ruptured oocysts showed release of sporozoites, and rupture by applying gentle force to the coverslip also released sporozoites from the oocysts. As expected, due to rupture and release of sporozoites, the number of oocysts dramatically declined from days 8 to 11 postfeeding. Salivary gland sporozoites were not detected, since *P. knowlesi* sporozoites are not able to invade the salivary glands of *A. stephensi* [23]. In contrast, in the PkCSPko oocysts no sporozoites developed, and this remained so until the experiment was stopped at a point when the great majority of the wild-type oocysts had matured and ruptured (Fig. 3B and D). The number of oocysts did not decline from days 8 to 12 as observed with wild-type oocysts. PkCSPko oocyst development otherwise appeared to be similar to that of wild-type oocysts, as observed by light microscopy (Fig. 3A and B), resulting in similar-sized oocysts at day 11.

Table 1. Cloning of *in vitro* culture adapted *P. knowlesi*

Seeding density*	Percentage of positive wells‡	Probability of clones§	Number of clones obtained
100	100	0	0
20	100	0	0
4	100	0	0
0.8	75	46.2	0
0.16	21	91.7	5
0.032	4	98.4	1

*Average number of parasites used to start cultures in 24 wells of a 96 well plate.
‡ Cultures were maintained for 30 days before scoring as negative
§Probability that parasites are derived from a single infected erythrocyte is calculated according to Gritzmacher and Reese [24]

DISCUSSION

The postgenomic analysis of the sequences now being obtained for a range of malaria-causing species will require versatile tools for genetic manipulation. Transfection permits targeted genomic manipulation of malaria parasites to characterise structural and functional relationships. Analysis of transfected parasites in a natural host-parasite setting is not possible

with transfected human malaria parasites. In this respect *P. knowlesi*, a natural parasite of *M. fascicularis* [8], is a system with great potential because (i) the parasite is phylogenetically closely related to human malarial parasites, such as *P. vivax* [9] (in fact, the parasite can also infect humans [10]); (ii) animal hosts are available for experimentation [14, 25]; (iii) animal hosts are primates, enabling analysis of transfectant phenotypes in systems closely related to humans [15]; and (iv) we have developed *in vivo* transfection systems [2], allowing functional genome analysis.

We have now further improved and extended the *P. knowlesi* system in several ways to provide more widely available access to genetic manipulation of this parasite species. First, two *P. knowlesi* strains, Nuri and H, were adapted to long-term *in vitro* growth by using simple protocols. This is important since it obviates the need for primates as parasite donors to provide parasites for genetic manipulation. Second, by using protocols developed for *in vivo* selection of transfected parasites, transfected parasites were selected *in vitro* based on the *T. gondii dhfr/ts* gene providing resistance to pyrimethamine.

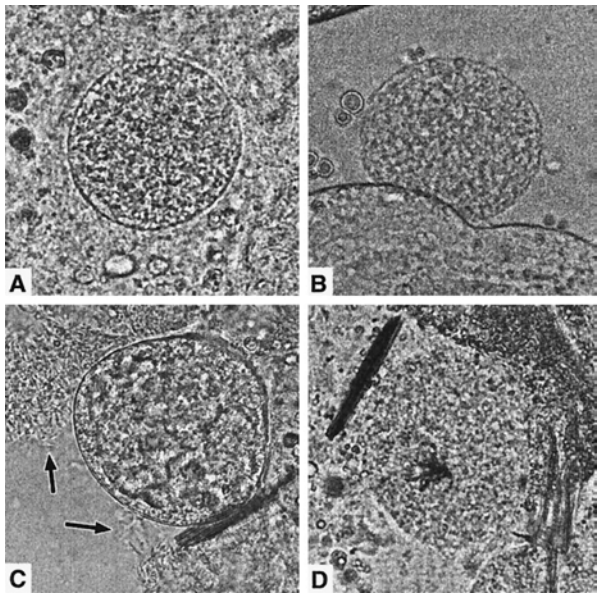

Figure 3. **Microscopic evaluation of wild-type and CSP knockout *P. knowlesi* oocyst development in *A. stephensi* midguts.** (**A**) Intact wild type oocyst 9 days postfeeding. (**B**) Intact CSP knockout oocyst 11 days postfeeding. (**C**) Wild-type oocyst releasing sporozoites (marked with arrows) 8 days postfeeding. (**D**) Force-induced rupture of CSP knockout oocyst 11 days postfeeding. Sporozoite release was never observed in CSP knockout oocysts.

This also obviates the need for primates as transfected parasite recipients during the initial selection for transfectants and allows development of new selectable markers, including those that would otherwise be toxic to the host, exploiting the benefits of complete *in vitro* selection. Third, to determine whether gene targeting through double crossover was possible in *P. knowlesi* or whether the lengthy *P. falciparum* procedures needed to be adopted, transfection experiments with linear DNA constructs designed to target the CSP locus were performed. These experiments, performed *in vivo* as well as *in vitro*, showed that targeted integration could be obtained with linear constructs and entirely *in vitro*. Recently, by using similarly designed constructs, the *P. knowlesi* thrombospondin-related adhesive protein gene was also efficiently disrupted (H. Ozwara *et al*., unpublished results). The *in vitro* procedure allows for deriving cloned parasites within 18 days of transfection, demonstrating the relative speed of this procedure compared to *in vitro P. falciparum* procedures, and allowing high-throughput analysis of gene functions.

Recently, the *P. knowlesi* genome sequence to a threefold coverage has become available (http://www.sanger.ac.uk/Projects/P_knowlesi/). This greatly expands the possibilities for transfection studies since homologues from *P. falciparum* genes and from genes available from the expanding *P. vivax* gene sequence tag database (http://www.ncbi.nlm.nih.gov/Malaria/plasmodiumblcus.html) can now easily be identified in *P. knowlesi*.

Finally, we have shown that parasites that have been growing *in vitro* for a long period of time can readily readapt to *in vivo* growth, displaying wild-type H-strain growth characteristics after single passage through an intact rhesus monkey. This observation is extremely important since *in vitro*-selected transfected parasites will be vital tools for studying host-parasite interactions. *In vitro* culture might lead to alteration of expression of SICAvar genes [26-28] and thus to loss of avoiding splenic clearance by sequestration, which can influence infectivity in nonsplenectomized rhesus monkeys. One passage through an intact rhesus is apparently sufficient to select for parasites that are readily able to infect rhesus monkeys and that can still be cultured *in vitro*.

In this study in a nonhuman primate malaria of the *P. vivax* type, we confirmed the earlier observations in a rodent malaria of the essential nature of CSP in sporozoite development [29]. In *P. knowlesi* CSP is also critical for sporozoite formation in developing oocysts in mosquito midguts, and the lack of CSP expression results in absence of sporozoite formation. Unlike the situation in *P. berghei*, oocysts of the CSP knockout have similar morphology to wild-type oocysts, and the phenotype only becomes clear at the time of sporozoite release.

This difference is mainly due to the fact that in *P. knowlesi* H-strain oocysts sporozoites are not visible "like rays from the sun" as described for *P. berghei* [18], and thus both wild-type and CSP knockout oocysts show an undefined morphology.

The *P. knowlesi* system now provides a unique malaria transfection system that allows for fast and simple *in vitro* genetic manipulations and combines this with the opportunity to perform *in vivo* studies in a nonhuman primate that is closely related to humans. It is the only malaria system described to date that is amenable to genetic manipulation that combines complete *in vitro* possibilities with studies *in vivo* in an animal model that is closely related to humans. This fact makes *P. knowlesi* highly suitable for the development of new antimalarial drugs and vaccines, as well as for studying basic biological questions in the postgenomic era at the parasite-host interface.

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Clemens Kocken and Hastings Ozwara contributed equally to this work.

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CHAPTER 3

Heterologous promoter activity in stable and transient *Plasmodium knowlesi* transgenes

Hastings Ozwara, Annemarie van der Wel, Clemens H.M. Kocken, Alan W. Thomas

Department of Parasitology, Biomedical Primate Research Centre, P.O. Box 3306, 2280 GH Rijswijk, The Netherlands, Institute of Primate Research, National Museums of Kenya, P. O. Box 24481, Karen, Nairobi, Kenya.

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A more rational approach to vaccine development and drug design requires a deeper understanding of the biology of malaria parasites. Transfection of malaria parasites [1–6] provides a broad range of tools to study malaria parasite biology. Transient transfection for malaria parasites provides the opportunity to precisely and rapidly examine control of gene expression in malaria parasites [7–9], an approach that is critical in understanding how gene expression is developmentally controlled in *Plasmodium* parasites. Luciferase and green fluorescent protein (GFP) are reporter molecules that have proven to be useful as markers for gene expression during transient and stable transfection in the avian parasite *Plasmodium gallinaceum* [1], the human parasite *Plasmodium falciparum* [10] and the rodent parasite *Plasmodium berghei* [11]. The use of heterologous promoters in transfection of *Plasmodium* [4, 6] is advantageous, especially in gene targeting experiments where it could reduce integration of the construct into loci different from that of interest. It is important to analyse regulation of expression under heterologous promoters to determine that they work in the same way as in the homologous system. Furthermore, promoters influence the timing of expression and eventual distribution of expressed transgenes [12]. The aim of this study was to characterise heterologous promoter activity upon transient and stable transfection in the simian malaria parasite *Plasmodium knowlesi*.

In order to rapidly characterise heterologous promoter activity in *P. knowlesi*, transient transfection was evaluated using luciferase as the reporter gene. The heterologous stage specific *P. berghei* apical membrane antigen-1 (pb*ama-1*) promoter [13], and the heterologous constitutive *P. berghei* elongation factor-1 alpha (pb*ef-1*α) promoter [13], were used to drive luciferase expression in *P. knowlesi*. To test these heterologous promoters, *P. berghei* luciferase constructs pE(A)$_b$.luc^D and pA$_b$.luc.^D$_b$ were used [13]. As a positive control, the construct pD$_b$.luc.^D$_b$ [13] for expressing luciferase under the heterologous pb*dhfr-ts* promoter was tested along side since the pb*dhfr-ts* promoter is known to be functional in *P. knowlesi* [4, 14]. The constructs were transfected into asynchronous *in vitro* culture adapted blood stage parasites using conditions described previously [14]. Luciferase assays were performed either as described [13] or using the luclite^{TM} kit (Perkin-Elmer Life Sciences) according to the manufacturer's instructions. Luciferase activity was measurable within 24 h post-electroporation using either of the two detection systems. The Top Count NXT Microplate Scintillation and Luminescence Counter (Perkin-Elmer, used with the luclite kit) has the capacity to rapidly measure luciferase activity either in a 96- or 384-well plate format. This is less labour intensive compared to the single tube procedure using the Lumat LB 9507 luminometer (EG & G Berthold), and has the potential for high throughput analysis.

The results (Fig. 1) show that luciferase activity was measurable using either of the three heterologous promoters and was consistently the highest using the construct with the pb*ef-1α* promoter. The *ef-1α* promoter drives expression in all parasite blood stages of *P. berghei* and *P. knowlesi* except mature schizonts [15], while the *dhfr-ts* and the *ama-1* promoters are active in late trophozoites/young schizonts [16] and mature schizont stages [17], respectively. Asynchronous parasite cultures were used for transfection and analysis of luciferase activity because they omit the time-consuming procedure of culture synchronisation.

Consequently, the pb*dhfr-ts* and pb*ama-1* promoters were active in only a proportion of the asynchronous parasites tested for luciferase activity.

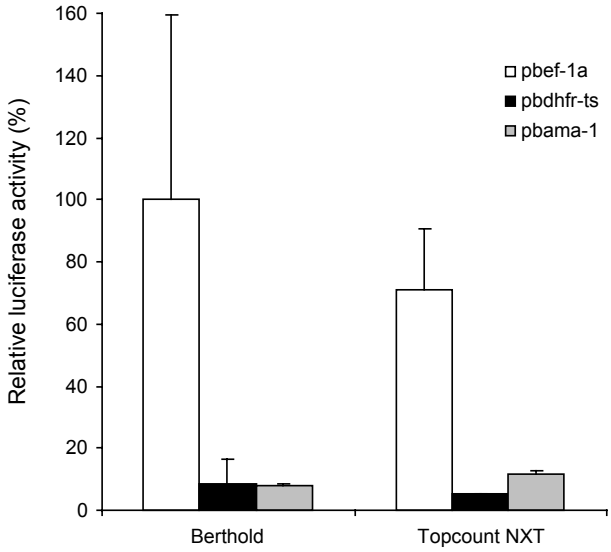


Figure 1. Transient transfection and heterologous promoter activity in *P. knowlesi* H strain. Asynchronous blood stage parasites were transfected with DNA constructs described in [13]. Parasites were electroporated in a gene pulser II (Biorad) as described previously [14]. Electroporations were done with 2 X 10^8 parasites and 50 μg of DNA resuspended in 800 μl of cytomix [19] in a 0.4-cm cuvette (Biorad). As controls for luciferase activity, parasites were electroporated without DNA. Electroporated parasites were cultured in 20 ml of complete medium as described previously [14]. Luciferase activity was measured 24 h later using a Lumat LB 9507 luminometer (EG & G Berthold) or a Top Count NXT Microplate Scintillation and Luminescence Counter (Perkin-Elmer). Each culture was split into two equal parts before harvesting and measuring. Luciferase activity values from control cultures were subtracted from all readings. To transform readings to percentage relative luciferase activity, the average luciferase light units measured under the pb*ef1α* promoter using a Lumat LB 9507 luminometer was scored as 100% [13]. The other readings were normalised against it. The luciferase activity shown is the average of two experiments per luminometer (±S.D.). pbama-1, parasites expressing luciferase under the pb*ama-1* promoter; pbdhfr-ts, parasites expressing luciferase under the pb*dhfr-ts* promoter; pbef-1α, parasites expressing luciferase under the pb*ef-1α* promoter; Berthold, Lumat LB 9507 luminometer; Topcount NXT, Topcount NXT Microplate Scintillation and Luminescence counter.

This, as well as actual differences in promoter strength, can explain the lower luciferase activity observed under the pb*dhfr-ts* and the pb*ama-1* promoters as compared to the pb*ef-1*α promoter if similar tight regulation is maintained in the heterologous parasite. Transient transfection protocols were used subsequently to determine the optimum electroporation conditions for mixed blood stage *P. knowlesi* transfection. We tested high voltage, low capacitance and low voltage high capacitance electroporation conditions, previously shown to transfect intracellular *P. falciparum* blood stages [3, 18] alongside previously published [14] *P. knowlesi* conditions, using plasmids for expression of luciferase under pb*dhfr-ts* and pb*ef-1*α promoters. Transfection using conditions of 2.5 kV, 25 μF, and 200 Ω consistently gave high and more reproducible luciferase activity (Table 1), indicating that these are the most optimal conditions for transfecting asynchronous and mature *P. knowlesi* blood stages [14]. Currently, both *P. knowlesi* episomal and integration types of transfection are routinely performed in our laboratory using electroporation conditions of 2.5 kV, 25 μF and 200 Ω, and 0.5 x10^9 to 1 x10^9 parasites resuspended with DNA in cytomix to 800 μl [19] in a 0.4 cm cuvette (Biorad). These conditions are suitable for transfecting as little as 20 μg plasmid DNA per electroporation (data not shown), but for uniformity of results, we use routinely at least 50 μg of DNA per electroporation.

Table 1. Electroporation conditions for asynchronous *P. knowlesi* parasites

Electroporation conditions	Luciferase activity (%) [a]			
	Expt 1	Expt 2	Expt 3	Expt 4
2 kV, 25 μF, 200 Ω	56.2[b]	100.0	21.0	29.9
2.5 kV, 25 μF, 200 Ω	92.5	97.3	100.0	100.0
0.37 kV, 975 μF	28.3	8.5	1.7	1.1
0.4 kV, 975 μF	62.7	47.6	3.6	3.2
0.45 kV, 975 μF	100.0	46.3	2.9	8.6

[a] For each experiment, luciferase activity was normalised on electroporation conditions that yielded the highest activity.
[b] Each culture was split into two equal parts prior to reading and measuring. Luciferase activity values are based on average of the readings.
Expt. experiment.

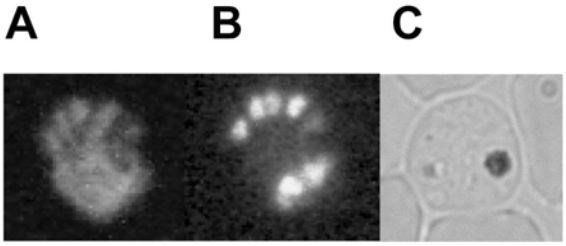
The asynchronous nature of *P. knowlesi* parasite cultures used in transient transfections precluded precise determination of stage specific control of expression under the pb*ama-1* promoter. Gene expression under the pb*ama-1* promoter in the homologous system is

restricted to mature schizonts [12] when six or more nuclei are present [17]. Using GFP as a reporter molecule [20], stable drug-selectable transfection experiments were done to analyse stage specific control of gene expression under the heterologous pb*ama-1* promoter in *P. knowlesi*. GFP is easily quantifiable [21, 22], non-toxic to cells [23] and allows analysis of stage specific expression in an asynchronous parasite culture by direct microscopic examination.

Plasmid $pD_B.D_{TM}.D_B/A_B.G_{AM3}.D_B$ was constructed for stable episomal expression of GFP in *P. knowlesi*. The *gfp* mutant 3 gene was isolated by *Bam*HI digestion of plasmid $pD_B.D_{TM}.D_B/D_B.GFP.D_B$ [21] and gel purification. The insert was cloned into the *Bam*HI digested $pD_B.D_{TM}.D_B/A_B.D_B$ [12] to generate the transfection plasmid $pD_B.D_{TM}.D_B/A_B.G_{AM3}.D_B$. *P. knowlesi* blood stage parasites (5×10^8) were transfected with this plasmid and selected *in vitro* using pyrimethamine. Drug resistant parasites were observed 8 days post-electroporation. Plasmid rescue experiments showed that the transfection plasmid was present in a stable form in *P. knowlesi*. Parasites expressing GFP were examined freshly for fluorescence after addition of 4,6-diamidino-2-phenylindole (DAPI) to stain parasite DNA, allowing precise determination of parasite developmental stage. Strong GFP fluorescence, diffused throughout the cytoplasm (Fig. 2), was observed in mature schizonts (Fig. 2D), indicating similar tight regulation of the heterologous pb*ama-1* promoter in *P. knowlesi* as observed in the homologous parasite *P. berghei* [12]. Occasionally, GFP fluorescence was observed in young ring stages, possibly due to high levels of GFP expressed in schizonts from multiple plasmid copies [16]. Due to the apparent relatively short half life of GFP in this system, this only results in minor carry over to subsequent ring stages. These experiments also indicated that GFP was successfully expressed by *P. knowlesi* blood stages, and that it can be used as a reporter.

Episomally transfected parasites maintain several copies of plasmids leading to over-expression of genes [16] and mutants of GFP may require up to 10,000 molecules for accurate detection inside a cell [24]. Whereas over-expression by episomal transfection readily achieves the threshold, we wanted to determine whether single copy gene expression was also sufficient. Experiments were performed to integrate a single copy of the *gfp* gene under control of the pb*ama-1* promoter into the non-essential *P. knowlesi* 140 kDa locus [25] by double cross-over mechanisms [14]. GFP fluorescence was readily detected in parasites that were confirmed by PCR and plasmid rescue to only contain a single copy of the *gfp* gene integrated in the 140 kDa locus (Fig. 2D). This demonstrates that single copy gene expression under control of the *pbama-1* promoter is sufficient for detectable GFP expression in *P.*

knowlesi. These experiments indicate that GFP is a suitable reporter in *P. knowlesi* under over-expression and single gene copy expression conditions. In all the experiments, there were no indications that expression of GFP compromised parasite viability.



D

Developmental stage	**GFP expression**[a]	
	Episomal	Integrated[b]
Rings	±[c]	-
Trophozoites	-	-
Immature schizonts[d] (N≤4)	±	±
Mature schizonts[d] (N≥6)	+++	+++

Figure 2. **Expression of green fluorescent protein (GFP) in *Plasmodium knowlesi* mature schizont stage.** GFP fluorescence was observed under fluorescein isothiocyanate (FITC) filter settings using a Nikon Microphot-FXA fluorescence microscope (**A**). DAPI was added to culture smears at a final concentration of 1.9 μM to stain parasite nuclei and the smears were directly observed as described above for GFP except that the microscope filter was under UV settings (**B**). A bright field image of the parasitized erythrocyte is depicted in (C). The pictures were derived from parasites expressing GFP after integration into the 140 kDa locus. Characteristics of GFP expression in different blood stages are shown in (D). [a]Cultures were followed through an entire blood stage cycle and 400 parasitised erythrocytes were evaluated for each developmental stage. [b]In order to design the GFP expression construct for integration into the *P. knowlesi* 140 kDa locus [25], the ORF was isolated by *Bam*HI digestion of plasmid $pD_B.D_{TM}.D_B/D_B.GFP.D_B$ [21], and gel purification. Through a series of cloning steps, plasmid $p140K/D_B.D_{TM}.D_B/A_B.G_{AM3}.D_B/140K$ was generated. The plasmid was linearised by restriction digestion with *Eco* RI and *Not* I prior to transfection. Targeted integration of the GFP expression construct into the 140 kDa locus was determined by PCR analysis.
[c]Fluorescence was observed in young ring stages from some of the parasite culture smears examined. [d]Schizonts were classified either as immature if the number of nuclei were not more than four ($N \leq 4$) or mature if the number of nuclei was six and above ($N \geq 6$).
(-) no fluorescence;
(±) sparse fluorescence occasionally seen in some parasites;
(+++) strong fluorescence diffused in the parasite cytoplasm.

GFP experiments reported here show that the heterologous pb*ama-1* promoter maintains tight stage-specific regulation of expression in *P. knowlesi*. Further studies will be required to show whether *P. knowlesi* uses the same transcription start site(s) as used in *P. berghei*, and whether other tightly regulated *P. berghei* promoters are also similarly active in *P. knowlesi*. Luciferase and GFP will be useful tools in addressing these questions. Expression of these reporters in *P. knowlesi* will now provide an effective means to study both gene expression and protein trafficking in tagging experiments [10, 11]. This study has further identified the pb*ef-1*α promoter as a strong heterologous promoter ideal for targeted integration and over-expression [13] of transgenes in *P. knowlesi* blood stages. The tools reported here will be used to further develop the *P. knowlesi* transfection system to enable full exploitation of the advantages offered by this versatile transfection system.

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CHAPTER 4

Transfected *Plasmodium knowlesi* produces bioactive host gamma interferon: A new perspective for modulating immune responses to malaria parasites

Hastings Ozwara, Jan A. M. Langermans, Clemens H. M. Kocken, Annemarie van der Wel, Peter H. van der Meide, Richard A. W. Vervenne, Jason M. Mwenda, and Alan W. Thomas

Biomedical Primate Research Centre, Department of Parasitology, 2280 GH Rijswijk, U-Cytech b.v., Utrecht University, 3584 CJ Utrecht, The Netherlands, and Institute of Primate Research, National Museums of Kenya, Karen, Nairobi, Kenya

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ABSTRACT

Transgenic pathogenic micro-organisms expressing host cytokines such as gamma interferon (IFN-γ) have been shown to manipulate host-pathogen interaction, leading to immunomodulation and enhanced protection. Expression of host cytokines in malaria parasites offers the opportunity to investigate the potential of an immunomodulatory approach by generating immunopotentiated parasites. Using the primate malaria parasite *Plasmodium knowlesi*, we explored the conditions for expressing host cytokines in malaria parasites. *P. knowlesi* parasites transfected with DNA constructs for expressing rhesus monkey (*Macaca mulatta*) IFN-γ under the control of the heterologous *P. berghei* apical membrane antigen 1 promoter, produced bioactive IFN-γ in a developmentally regulated manner. IFN-γ expression had no marked effect on *in vitro* parasite development. Bioactivity of the parasite-produced IFN-γ was shown through inhibition of virus cytopathic effect and confirmed by using *M. mulatta* peripheral blood cells *in vitro*. These data indicate for the first time that it is feasible to generate malaria parasites expressing bioactive host immunomodulatory cytokines. Furthermore, cytokine-expressing malaria parasites offer the opportunity to analyse cytokine-mediated modulation of malaria during the blood and liver stages of the infection.

INTRODUCTION

Recombinant pathogenic microorganisms expressing host cytokines such as gamma interferon (IFN-γ) have been shown to modulate immune responses, leading to enhanced protection [1–7]. Vaccinia virus and simian immunodeficiency viruses expressing a range of host cytokines were attenuated *in vivo*, leading to enhanced immune responses [1–3], and *Leishmania major* expressing host IFN-γ was significantly attenuated in nude mice [7]. These data indicate that *in vivo* expression of host cytokines by pathogens can manipulate the host-pathogen interaction and generate protective host responses. Thus far, expression of host cytokines by malaria parasites has not been examined. The development of transfection technology for malaria parasites [8–11] now enables expression of recombinant host proteins, such as cytokines in *Plasmodium.*

IFN-γ is one of the central effector cytokines in host response to malaria infection, especially during the liver stage [12 –17] and hence is attractive for expression in malaria parasites. *In vitro* and *in vivo* studies in rodent models of malaria have demonstrated that IFN-γ plays a central role in protection against malaria liver-stage infection, possibly by

inducing the infected hepatocyte to produce nitric oxide that kills parasites [14, 18]. In clinical vaccination studies with an attenuated sporozoite vaccine [reviewed in reference 19], vaccinated humans were protected from subsequent infection through IFN-γ-dependent responses. In separate studies in mice and monkeys, sterile protection was achieved through IFN-γ-dependent responses after exogenous treatment with interleukin-12 (IL-12) [13, 16]. Studies using rodent and human malaria models have demonstrated that IFN-γ also plays a role in protection against malaria blood stages when either endogenously produced [20 – 25] or exogenously administered [26 – 29].

Although cytokines have been shown to mediate protection against malaria infection after exogenous delivery [13, 16, 27, 29], systemically delivered cytokines are short-lived and require repetitive administration (often in large doses that could be toxic to the host) [28, 30, 31], and only a small portion reaches the site of infection [30, 32]. Alternatively, cytokine expression by the pathogen itself will ensure that the cytokine is released where its activity is required, as long as the infection persists and in proportion to the level of infection. In this report, *Plasmodium knowlesi*, a natural malaria parasite of macaque monkeys [33] and an experimental system for human malaria, was transfected *in vitro* to express *Macaca mulatta* IFN-γ. *In vitro* expression and bioactivity of *P. knowlesi*-expressed rhesus monkey IFN-γ (rhIFN-γ) was characterised, showing for the first time that malaria parasites can produce a bioactive recombinant host cytokine.

MATERIALS AND METHODS

Parasites. *In vitro* culture-adapted *P. knowlesi* H strain [34] wild type and transfected blood-stage parasites were maintained and cloned where necessary as described previously [35].

Transfection constructs and procedures. All transfection constructs contained a heterologous selection cassette based on a mutagenised *Toxoplasma gondii* dihydrofolate reductase/thymidine synthase gene (*dhfr/ts*) conferring pyrimethamine resistance, flanked by *P. berghei dhfr/ts* flanking sequences [36]. To construct the rhIFN-γ expression vector, the open reading frame (ORF) of *M. mulatta* IFN-γ was isolated by *Xba*I and *Spe*I restriction digestion of the *M. mulatta* IFN-γ cloning vector (a gift from F. Villinger) [37], Klenow polymerase treatment, and purification with the Qiagen gel extraction kit (Qiagen, Chartsworth, Calif.). The IFN-γ gene was cloned into the blunted *Bam*HI site of plasmid $pD_B.D_{TM}.D_B/A_B.\text{-}.D_B$ [36] to generate $pD_B.D_{TM}.D_B/A_B.\gamma_{MM}.D_B$ (Fig. 1A). The construct was

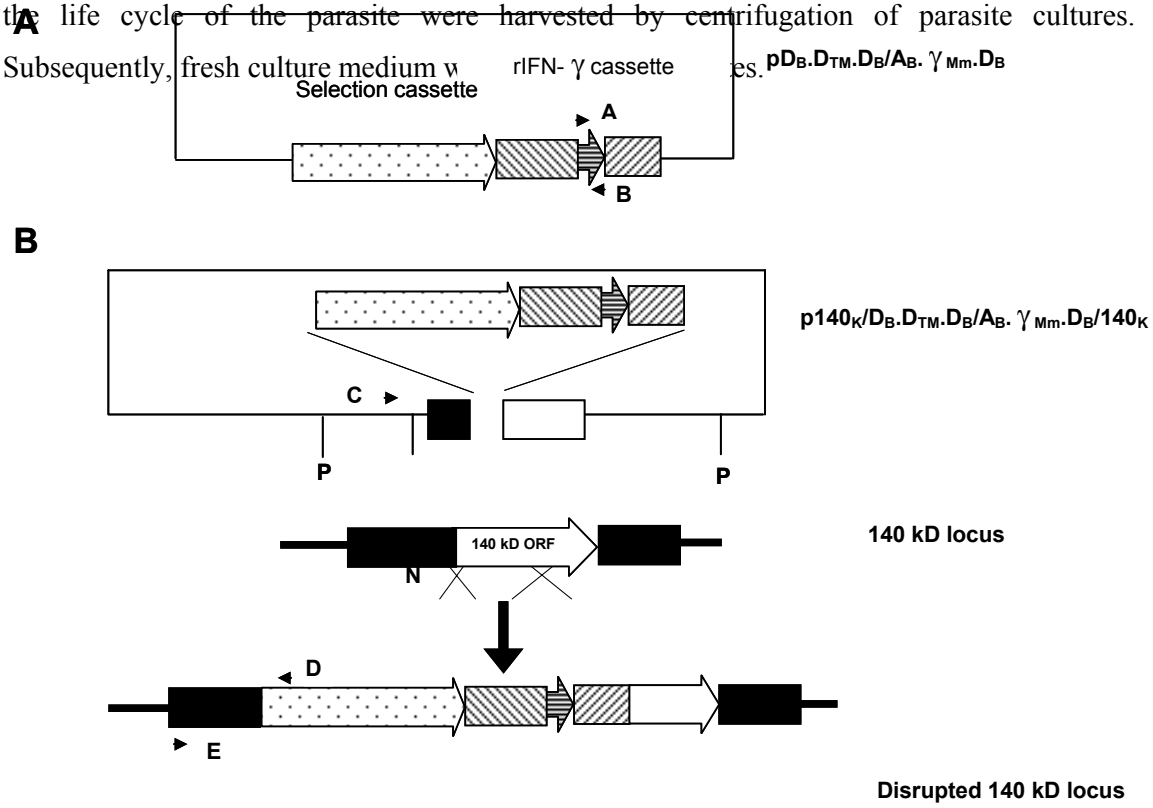
used for episomal transfection of *P. knowlesi* [35]. The 140-kDa merozoite surface antigen was shown to be nonessential during the bloodstage development of *P. knowlesi* [38]. Therefore, we designed the rhIFN-γ expression construct for integration into the *P. knowlesi* 140- kDa locus. The *M. mulatta* IFN-γ ORF was isolated as described above, and through a series of cloning steps, plasmid p140K/D_B.D_{TM}.D_B/A_B.γ_{MM}.D_B/140K was generated (Fig. 1B). Given that integration into the *P. knowlesi* genome by a double crossover mechanism requires linear constructs [35], the construct for integration into the 140 kDa locus was linearised by restriction digestion with *Pvu*I and *Not*I prior to transfection. As controls, parasites were transfected separately with pD_B.D_{TM}.D_B/A_B.-.D_B and p140K/D_B.D_{TM}.D_B/A_B.-.D_B/140K constructs. Parasite cultivation and *in vitro* transfection and selection procedures were performed as described elsewhere [35].

DNA analysis. Total parasite DNA was isolated (Gentra Systems, Inc., Minneapolis, Minn.) directly from *in vitro* cultures according to the manufacturer's instructions. The DNA from episomally transfected parasites was analysed through plasmid rescue by electroporation into *Escherichia coli* and PCR according to standard procedures [39]. PCR was performed on total parasite DNA with primers A (5'GGCTTTTCAGCTCTGCATTG-3') and B (5'-CCGCTCGAGGCTGGGATGCTCTTCGACC-3') to detect rhIFN-γ (Fig. 1B). Primers A and B amplify the ORF of rhIFN-γ from nucleotide positions - 24 to - 478. In order to discount the presence of episomes following integration-dependent transfection, primers C (5'-GTCATAGCTGTTTCCTG-3') and D (5'-GTGTCTATATTACCAACTC-3') were used to amplify the plasmid backbone of the integration construct. Primers E (5'-GAATTCCATTTATGAATATCC-3') and D (Fig. 1B) were subsequently used to confirm integration into the 140-kDa locus, by amplifying the downstream region of the disrupted locus.

In vitro analysis of rhIFN-γ expression. Transfected and control parasite cultures were expanded *in vitro* [35], and culture supernatants were harvested and frozen at -80°C. The culture supernatants were analysed for the presence of rhIFN-γ by enzyme-linked immunosorbent assay (ELISA, using a macaque IFN-γ ELISA kit (U-Cytech, Utrecht, The Netherlands) according to the manufacturer's instructions.

To monitor release of rhIFN-γ, control and rhIFN-γ-expressing *P. knowlesi* cultures were synchronised by alanine treatment [40]. Schizont-stage parasites (5×10^8) were inoculated into 20 ml of culture medium with 0.1 μM pyrimethamine and 5% haematocrit and cultured *in vitro* [35]. Culture supernatants for determining release of IFN-γ over 6 h time spans during

the life cycle of the parasite were harvested by centrifugation of parasite cultures. Subsequently, fresh culture medium w es.



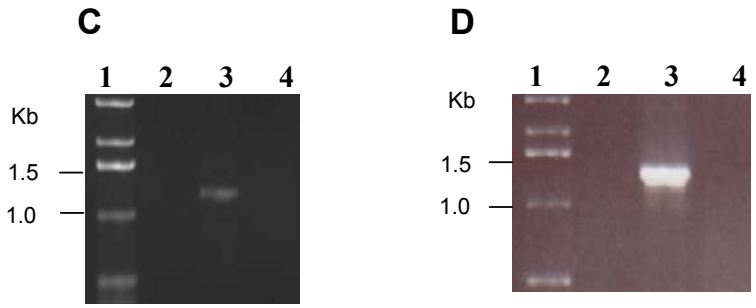


Figure 1. DNA constructs and analysis of integration into the *P. knowlesi* 140 kD locus. (A) Plasmid $pD_B.D_{TM}.D_B/A_B.\ \gamma_{Mm}.D_B$ for episomal expression of rIFN-γ. The selection cassette contains *P. berghei dhfr/ts* flanking regions controlling expression of mutagenized *T. gondi dhfr-ts*. Rhesus IFÑγ cassette contains the rIFÑγ gene under the expression control of the *P. berghei* apical membrane antigen-1 5' UTR and the *P. berghei dhfr/ts* 3' flanking sequence. **(B)** Disruption of the *P. knowlesi* 140 kD locus to express rIFN-γ. Restriction sites used for linearizing the 13.4 kb plasmid $p140_K/D_B.D_{TM}.D_B/A_B.\ \gamma_{Mm}.D_B/140_K$ to generate the integration construct are indicated: N, *Not* I; P, *Pvu* I. The location of PCR primers A, B, C, D and E are shown. **(C)** PCR analysis of transfected parasites with integration-specific primers D and E. Lanes: 1, 1 kb DNA marker; 2, *P. knowlesi* H strain DNA; 3, DNA from transfected parasites; 4, $p140_K/D_B.D_{TM}.D_B/A_B.\ \gamma_{Mm}.D_B/140_K$ vector DNA. **(D)** Analysis of transfected parasites for circular DNA using plasmid-specific primer C and selection cassette-specific primer D. Lanes: 1, 1 kb DNA marker, 2, *P. knowlesi* H strain DNA; 3, $p140_K/D_B.D_{TM}.D_B/A_B.\ \gamma_{Mm}.D_B/140_K$ vector DNA and 4, DNA from transfected parasites.

All harvested supernatants were stored at -80°C until assayed. In order to determine the *in vitro* stability of rhIFN-γ, 500 μl of parasite culture supernatant containing parasite-produced rhesus IFN-γ was incubated for 30 h with wild-type *P. knowlesi*-infected erythrocytes or in culture medium only. Parasite-containing cultures had a haematocrit of 2.5% and a starting parasitaemia of 3% (90% rings, 4% schizonts, and 6% trophozoites). Aliquots of the culture supernatant were harvested at 6 h intervals and stored at -80°C. To assay for rhIFN-γ present in the culture medium, supernatants were thawed on ice, and the rhIFN-γ concentration was determined by ELISA (UCytech).

Antiviral cytopathic effect assay. The bioactivity of rhIFN-γ was quantified by its ability to inhibit the cytopathic effect of vesicular stomatitis virus (VSV) in human HEp2 cell lines [41]. Human HEp2 cells [41] were plated in duplicate wells at a concentration of 2×10^4 per well and incubated at 37°C for 24 h in 1% foetal calf serum (FCS)–RPMI medium with culture supernatants of rhIFN-γ-expressing parasites. The cells were challenged with an appropriate dilution of VSV [41] and cultured for a further 24 h in 10% FCS–RPMI medium. As a control for IFN-γ specific activity, parasite culture supernatants containing rhIFN-γ were incubated with 15 μg of neutralising antibody per ml (U-Cytech) for 30 min at room temperature prior to incubation with the HEp2 cells. Supernatants from parasite-free culture medium and wild-type parasite cultures were also used as controls. Recombinant human IFN-γ (U-Cytech) was used as a standard for calibration of antiviral cytopathic effect. One antiviral unit of rhIFN-γ was defined as the inverse of the dilution that conferred 50% protection to the monolayer.

***In vitro* whole-blood-cell activation assay.** The bioactivity of rhIFN-γ was also determined by measuring release of tumour necrosis factor alpha (TNF-α) from whole-blood-cell cultures incubated with parasite culture supernatants. Blood was obtained by venipuncture from a naïve rhesus monkey and used immediately. The blood was washed three times in RPMI 1640, diluted 1:1 in 10% FCS–RPMI medium, and plated at 1 ml per well in 24-well culture plates containing parasite culture supernatants and controls. Whole-blood-cell culture supernatants were harvested 6 h later and stored at -80°C. Frozen culture supernatants were thawed on ice and assayed for TNF-α with a monkey TNF-α ELISA kit (U-Cytech) according to the manufacturer's instructions. Controls included parasite culture supernatants that were preincubated with 15 μg of IFN-γ neutralising antibody per ml (U-Cytech) for 30 min at room temperature prior to the activation assay, culture supernatants

from wild-type parasites, and bacterial lipopolysaccharide (LPS).

FACS analysis. IFN-γ stimulates monocytes to upregulate CD64 and major histocompatibility complex (MHC) class II DR expression in humans and rodents [42, 43, 44, 45]. To determine whether parasite culture supernatants from rhIFN-γ-producing parasites could produce a similar effect on rhesus monkey whole blood cells, rhesus monkey venous blood was obtained, processed, and cultured as described above, except that the cultures were incubated for 48 h. The blood cells were harvested, lysed with fluorescence-activated cell sorter (FACS) lysis solution (BD, Heidelberg, Germany), and stained with antibodies against human CD14, CD64, and MHC class II DR (BD). These antibodies cross-react with rhesus homologues. The number of CD64+ MHC class II DR+ cells within the CD14+ cell population was analysed by FACS as previously described [46].

RESULTS

Transfection of *P. knowlesi* and analysis of rhIFN-γ production. *P. knowlesi* blood-stage parasites were transfected with rhIFN-γ expression constructs $pD_B.D_{TM}.D_B/A_B.\gamma_{MM}.D_B$ and linearised p140K/ $D_B.D_{TM}.D_B/A_B.\gamma_{MM}.D_B$ /140K to determine the capacity of *P. knowlesi* to express host IFN-γ from episomal and integrated genes, respectively. Pyrimethamine-resistant parasites were observed in transfected cultures at 8 days postelectroporation. Plasmid rescue experiments and PCR analysis using primers A and B amplifying the IFN-γ ORF showed that plasmid $pD_B.D_{TM}.D_B/A_B.\gamma_{MM}.D_B$ was intact and therefore stable in *P. knowlesi* and that the gene was also present in parasites transfected with the integration construct. Integration into the 140-kDa locus was confirmed by PCR using primers D and E (Fig. 1C), and analysis for circular plasmids following integration-dependent transfection using primers C and D was negative (Fig. 1D). This showed that gene targeting into the 140-kDa locus in *P. knowlesi* by double-crossover mechanisms was feasible.

To determine rhIFN-γ production in transfected *P. knowlesi*, parasite cultures were expanded, and their supernatants were harvested and analysed for the presence of rhIFN-γ by ELISA with a macaque IFN-γ ELISA kit. rhIFN-γ was detected in culture supernatants from parasites transfected with plasmid $pD_B.D_{TM}.D_B/A_B.\gamma_{MM}.D_B$, and the integration construct but not from control cultures (Table 1). The results further suggested that both episomal and chromosomal expression of IFN-γ produced similar amounts (Table 1) and that empty vector-transfected parasites are equivalent to wild-type parasites. In all subsequent experiments, episomally transfected parasites were used.

The time course of rhIFN-γ production was evaluated in order to define the kinetics of rhIFN-γ release into culture medium. Cultures of rhIFN-γ-producing parasites were initiated at the schizont stage; supernatants were harvested at 6 h intervals and assayed for rhIFN-γ by ELISA. Culture medium was completely replaced at each harvest time point. rhIFN-γ was detected in the culture supernatants only after the rupture of schizonts (Fig. 2A).

N-glycosylation of IFN-γ protects the protein from rapid degradation by proteases [47]. However, malaria parasites have severely reduced capacity for N-glycosylation of proteins [48], prompting us to determine the *in vitro* stability of *P. knowlesi*-produced IFN-γ. It would be expected that highly unstable rhIFN-γ would be detected only immediately following schizont rupture. Evaluation of the stability of rhIFN-γ showed that, in the presence or absence of infected erythrocytes, the concentration of rhIFN-γ in the supernatant did not change significantly over a 30 h period (Fig. 2B).

Table 1. IFN-γ release from *P. knowlesi* following integration dependent transfection

Culture	Transfection construct	IFN-γ release (pg/ml)[a]
Wild type parasites	-	11.8
Red blood cells[b]	-	11.8
Transfected parasites: Integration	$p140_K/D_A.D_{TM}.D_B/A_B.\gamma_{Mm}.D_B/140_K$	1853.5
Integration control	$p140_K/D_A.D_{TM}.D_B/A_{\tilde{B}}..D_B/140_K$	10.0
Episomal	$pD_B.D_{TM}.D_B/A_B.\ \gamma_{Mm}.D_B$	1552.0
Episomal control	$pD_B.D_{TM}.D_B/A_B.\text{-}.D_B$	11.0

a Values show the IFÑγ concentration from 5×10^8 parasites cultured for 24 h and analyzed by ELISA.
b Complete culture medium with culture erythrocytes but lacking parasites.
This culture was incubated under the same conditions as the other cultures.

***P. knowlesi*-produced rhIFN-γ is bioactive.** The standard procedure for determining bioactivity of IFN-γ is to test the antiviral cytopathic effect [2, 41, 49, 50, 41]. To determine bioactivity of rhIFN-γ in *P. knowlesi* culture supernatants using an antiviral cytopathic effect assay, HEp2 cells [41] were incubated overnight with parasite culture supernatants. The cells were subsequently challenged with VSV and observed 24 h later for cytopathic effect. The HEp2 cells were protected from VSV cytopathic effect by culture supernatants from parasites transfected with the rhIFN-γ expression plasmid, but not by control supernatants (Fig. 3A). Protection from cytopathic effect was abrogated by preincubation with a neutralising anti-

IFN-γ antibody [41], demonstrating that the protection was mediated by rhIFN-γ.

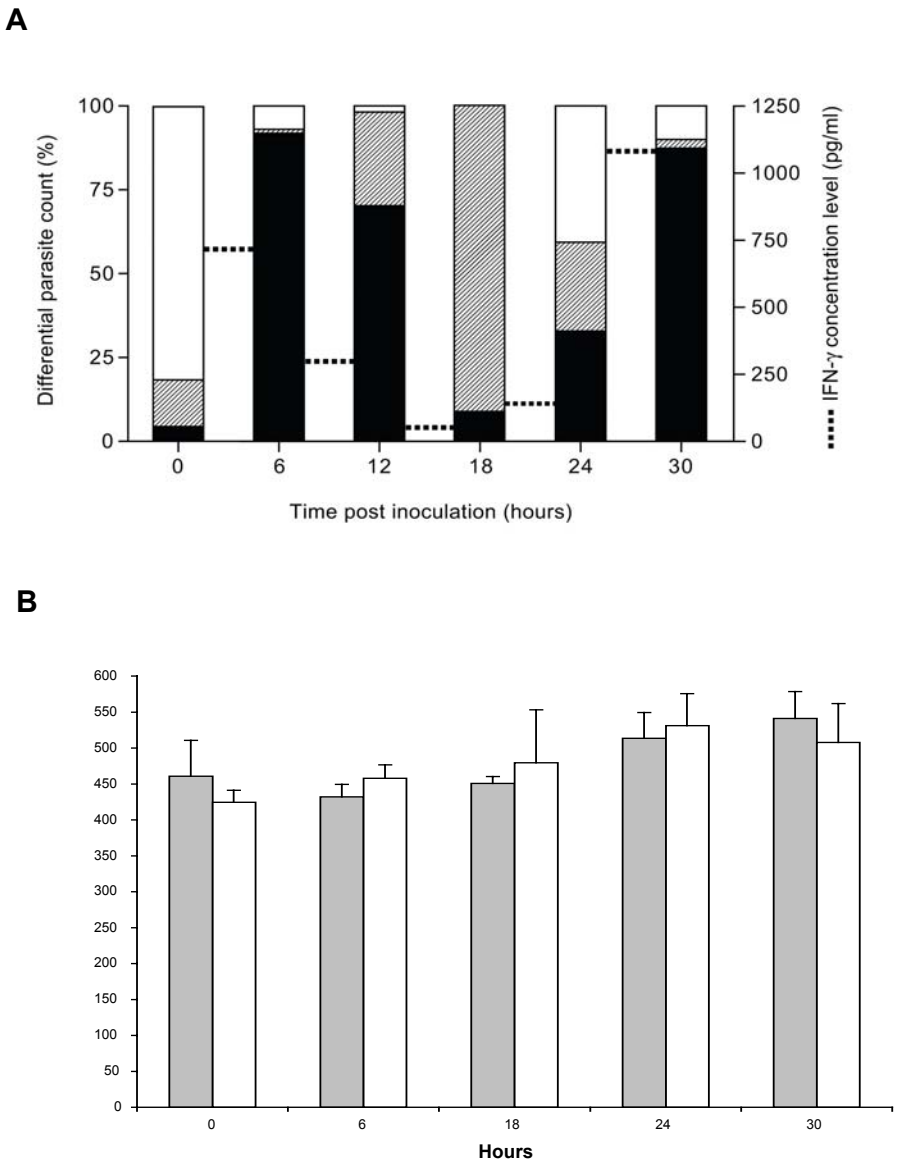


Figure 2. Characteristics of *P. knowlesi* produced rhIFN-γ. A., *In vitro*-transfected *P. knowlesi* H strain-produced rhIFN-γ coincides with schizont rupture. Schizont-stage *P. knowlesi* parasites were transfected *in vitro* to express rhIFN-γ under the stage-specific *P. berghei* apical membrane antigen 1 promoter. The release of rhIFN-γ was assayed by ELISA . The graph shows the relationship between parasite developmental stage and release of rhIFN-γ over a period of 30 h. The concentration of rhIFN-γ was analysed in culture supernatants harvested every 6 h. In order to assess rhIFN-γ production during each 6 h period, culture medium was completely replaced at each sampling time point. The developmental stage of the malaria parasites was determined at each time point and is expressed as a percentage of the total parasitaemia. The dotted lines show the accumulated concentration of rhIFN-γ in parasite culture supernatants during the 6 h sample windows. The bars represent the percentage of ring (■), trophozoite (▨), and schizont (□) stages at the time of harvest. B., *In vitro* stability of *P. knowlesi*-produced rhIFN-γ culture supernatants were harvested from rhIFN-γ-producing parasites, diluted 1:1 with fresh culture medium, and incubated with (gray bars) or without (open bars) wild-type *P. knowlesi*-infected erythrocytes over a 30-h period. At various timepoints, the rhIFN-γ concentration was determined by ELISA. The results represent an average of three

experiments ± standard deviation

The concentration of rhIFN-γ in parasite culture supernatants was calculated at 125 antiviral units per 5×10^8 schizonts.

Bioactive IFN-γ activates blood cells to release TNF-α [42]. To determine whether culture supernatants from rhIFN-γ-producing *P. knowlesi* activate rhesus whole blood cells to release TNF-α, blood was obtained from a rhesus monkey and incubated with culture supernatants from rhIFN-γ-producing parasites. Whole-blood-cell culture supernatants were harvested and assayed for TNF-α release. Significant TNF-α was released by whole blood cells incubated with culture supernatants from rhIFN-γ-producing parasites (Table 2). The release was blocked when culture supernatants from rhIFN-γ-producing parasites were mixed with neutralising antibody against human IFN-γ prior to incubation with whole blood cells (Table 2), confirming that activation of rhesus whole blood cells to release TNF-α was mediated via rhIFN-γ. TNF-α release from whole blood cells incubated with culture supernatants from control cultures was below detection levels (Table 2).

IFN-γ activates monocytes and macrophages to upregulate CD64 and MHC class II DR antigens [42– 45]. To determine the effect of culture supernatants from IFN-γ -producing *P. knowlesi* on the expression of CD64 and MHC class DR by monocytes from naïve rhesus monkeys, the whole blood was incubated with parasite culture supernatants, harvested, stained, and analysed by FACS for expression of CD14, CD64, and MHC class II DR antigens. Incubation of rhesus monkey blood cells with culture supernatant from rhIFN-γ-expressing *P.knowlesi* resulted in an increased number of CD14+ cells expressing CD64 and MHC class II DR (Fig. 3B). Neutralisation of IFN-γ inhibited this effect. Supernatants from control parasites and cell culture medium had no effect on the number of CD14+ cells expressing CD64 and MHC class II DR.

***P. knowlesi*-produced rhIFN-γ had no marked effect on *in vitro* parasite multiplication.** The *in vitro* growth rate of rhIFN-γ-producing parasites was comparable to that in wild type and control cultures (data not shown), indicating that the production of rhIFN-γ had no marked effect on parasite production.

DISCUSSION

In this paper, we show that *P. knowlesi* parasites, transfected with an rhIFN-γ gene under

control of the heterologous *P. berghei* apical membrane antigen 1 promoter, express bioactive IFN-γ. This is the first example of a host molecule being produced by a malaria parasite. It demonstrates that the malaria parasite transcription and translation machinery can effectively express a bioactive host cytokine.

Secreted malaria parasite proteins have to be transported across three bilayer membranes before getting out of the infected erythrocyte [51]. In order to determine the release characteristics of rhIFN-γ, time course experiments were done *in vitro*. The data shows that rhIFN-γ is released into culture medium after schizont rupture. Since the heterologous *pbama-1* promoter restricts expression to late-stage schizonts in *P. knowlesi* (Ozwara *et al*., unpublished data), it is not possible to determine from these experiments whether rhIFN-γ is actively secreted into the culture medium or whether it is released from parasitised cells during schizont rupture. However, rhIFN-γ minimally has to be secreted across one set of membranes to reach the parasitophorous vacuole, suggesting that the parasite effectively recognises higher eukaryotic secretion signals.

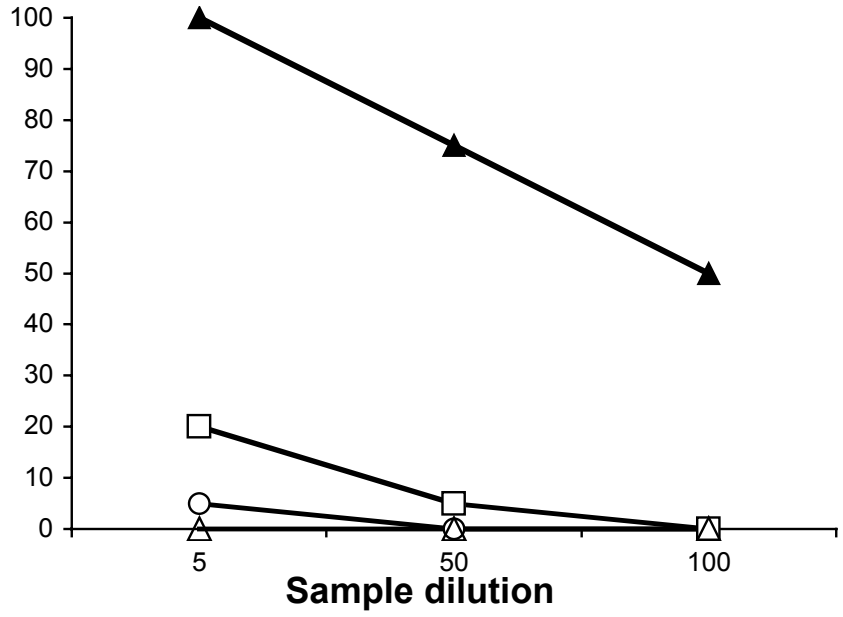


Figure 3A. **Antiviral cytopathic effects of *P. knowlesi* released rhIFN-γ.** The bioactivity of rhIFN-γ in parasite culture supernatants was determined by an antiviral cytopathic effect assay. Strong antiviral activity, as measured by the inhibition of the cytopathic effect, was observed in culture

supernatant from rhIFN-γ -expressing parasites (▲) but not from wild-type parasites (○) and parasite culture medium (△). The antiviral activity was abrogated when culture supernatants from rhIFN-γ-producing parasites were preincubated with neutralising antibodies **(□)**.

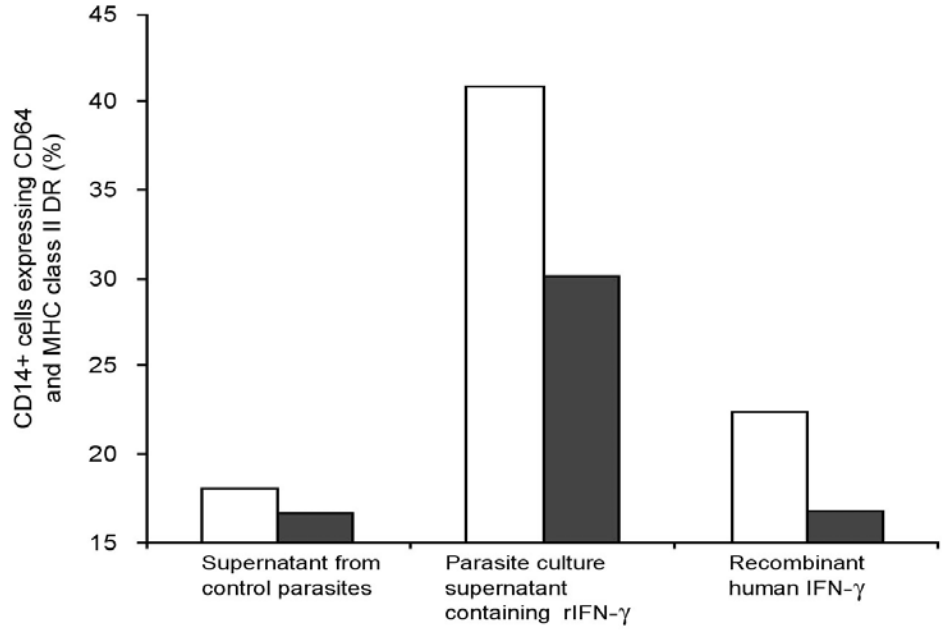


Figure 3B. **FACS analysis.** Rhesus monkey whole blood cells were incubated with culture supernatants from IFN-γ-producing *P. knowlesi* parasites and controls for 48 h and subsequently analysed for CD14+ cells. Within the CD14+ cell population, the expression of CD64 and MHC class DR was analysed without anti- IFN-γ antibody (□) or with anti- IFN-γ antibody (■).

Developmentally regulated promoters that restrict expression to the ring and trophozoite stages would be required to analyse the secretion process in more detail [52]. It is noteworthy that the rhIFN-γ produced by the parasites has no marked effect on parasite growth *in vitro*. This indicates that the cytokine itself, either present in the culture supernatant or accumulated in the infected cell, has no direct effect on parasite viability.

Arakawa et al. [53] showed that bioactivity of IFN-γ depends on protease processing of the carboxyl end of the protein. Furthermore, stable and bioactive IFN-γ exists as a noncovalent homodimer [54]. The fact that bioactive rhIFN-γis produced in P. knowlesi suggests that the molecule is properly processed and present in the homodimeric form. Interestingly, at in vitro culture concentrations, rhIFN-γwas stable for at least 30 h. This duration is likely to be sufficient for in vivo activity.

For example, despite clearance from rhesus monkey circulation within 6 h of exogenous administration [30], IFN-γ was protective in *Plasmodium cynomolgi*-infected rhesus monkeys

[22], suggesting that stability of IFN-γ for 6 h could be sufficient for mediating *in vivo* bioactivity in the monkeys.

We expressed IFN-γ because it is a key effector cytokine in protection against malaria, especially during the liver stages [13, 15–17, 19]. Studies in humans and animal models looking at endogenously produced and exogenously administered IFN-γ have shown that the cytokine is also required for protection against blood-stage infection [21, 22, 24, 25, 27, 29]. In order to determine the immunomodulatory effect of rhIFN-γ-expressing parasites during the malaria liver-stage infection, the host must be inoculated with sporozoites. Integration into the genome of the cytokine expression construct is required to express rhIFN-γ in the liver stages, since episomally transfected parasites undergo random segregation of plasmids [55], and in the absence of drug pressure during development in the mosquito, the plasmids could be lost. Therefore, we generated parasites expressing rhIFN-γ after integration-dependent transfection. The levels of rhIFN-γ produced were similar to those of episomally transfected parasites.

Table 2. TNF̃α release from rhesus monkey whole blood cells

Activation Sample	TNF-α release (pg/ml) in 6 hours
Culture supernatant from wild type parasites	<<[a]
Cell culture medium from control cells	<<
Commercial human IFN-γ (250 pg)[b]	168
Culture supernatant from IFN-γ producing parasites (400 pg/litre)[c]	265
Culture supernatant from IFN-γ producing parasites (400 pg/litre)[b] Pre-incubated with neutralizing antibody[d]	<<
Bacterial lipopolysaccharide (100 pg)	808

a <<, below detectable levels.
b Commercial human IFN-γ was purchased from U-Cytec.
c Concentration of IFN-γ in the culture supernatants as estimated by ELISA.
d Neutralizing antibody was antimacaque IFÑγ antibody from U-Cytech.

It is now feasible to generate malaria parasites expressing bioactive host cytokines, to characterise the capacity of the parasites to immunomodulate the infection, and to characterise the role of the expressed cytokine in host responses to malaria. It is important that the safety of cytokine-expressing parasites *in vivo* be determined prior to such experiments. A preliminary safety study with a small number of rhesus monkeys showed that

the levels of rhIFN-γ released by transfected malaria parasites were well tolerated by rhesus monkeys (data not shown). During this safety study, aspects of the immune responses of animals exposed to the rhIFN-γ parasites were compared with those of animals that had received parasites transfected with a control vector. All four animals exposed to rhIFN-γ parasites had a marked expansion (2.5- to 5-fold) of the γδT-cell compartment within 2 weeks of the start of infection compared with five controls (1- to 2-fold expansion). A more comprehensive analysis of host responses to rhIFN-γ-expressing *P. knowlesi* in *M. mulatta* is ongoing. However, this study already clearly demonstrates that the rhIFN-γ produced by the parasites is bioactive *in vivo*. In conclusion, the expression of host cytokines by malaria parasites, as demonstrated in our study, offers a new approach to explore the development of attenuated and immunopotentiated malaria vaccines.

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CHAPTER 5

Flow cytometric analysis on reactivity of human T lymphocyte-specific and cytokine-receptor-specific antibodies with peripheral blood mononuclear cells of chimpanzee (*Pan troglodytes*), rhesus macaque (*Macaca mulatta),* and squirrel monkey *(Saimiri sciureus)*

Hastings Ozwara, Henk Niphuis, Leo Buijs, Margreet Jonker,
Jon L. Heeney, Charanjit S. Bambra, Alan W. Thomas, and Jan A.M. Langermans.

Biomedical Primate Research Centre, P.O. Box 3306, 2280 GH Rijswijk, The Netherlands, and Institute of Primate Research, National Museums of Kenya, P. O. Box 24481, Karen, Nairobi, Kenya.

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ABSTRACT

There are relatively few monoclonal antibodies (mAb) that have been characterised for their applicability in studies on the immune system of various nonhuman primates. In the present study, we identified a large number of mAb that can be used in future immunological studies in three different nonhuman primates, i.e., chimpanzees, rhesus macaques, and squirrel monkeys. The reactivity of 161 anti-human mAb to T-cell antigens and cytokine receptors were tested on peripheral blood mononuclear cells (PBMC) from the three primate species by flow cytometric analysis. A total of 105 (65%), 73 (45%), and 68 (42%) antibodies reacted with PBMC from chimpanzees, rhesus macaques, and squirrel monkeys, respectively. Out of the 161 mAb, 38 reacted with all three species and 112 reacted with one or two of the species. No specific reaction was observed with mAb to receptors to GM-CSF, 4-1BB, FLT3, FLX2, common β-chain, IL-l (type I receptor), and IL-8.

INTRODUCTION

Nonhuman primates are frequently used as animal models for a broad range of disciplines, including immunological, drug, and vaccine development studies against human diseases [1, 2, 3, 4]. Optimal results from such studies require a thorough knowledge of the immune response in the nonhuman primate but the availability of characterised monoclonal antibodies (mAb) against various nonhuman primate immune systems is limited. However, due to the close phylogenetic relationship between nonhuman primates and man, their immune systems have many similarities [5, 6] and several studies have previously reported on the reactivity of mAb defined against human haematopoietic cells to cross-react with similar, if not identical, molecules expressed by cells from a variety of nonhuman primate species [5-12]. The ever-growing list of anti-human mAb, together with the identification of novel structures on human cells that play an important role in immune functions, e.g., co-stimulatory activity, prompted us to examine the reactivity of these new reagents to cross-react with cells from chimpanzees *(Pan troglodytes),* rhesus macaques *(Macaca mulatta),* and squirrel monkeys *(Saimiri sciureus)*. In the present study, a large panel of mAb against human T-cell markers and cytokine receptors were investigated for their reactivity with peripheral blood mononuclear cells (PBMC) from these three nonhuman primate species.

MATERIALS AND METHODS

Animals. From each of the three nonhuman primate species, three adult animals of either

sex were used. The animals were housed at the Biomedical Primate Research Centre (Rijswijk, The Netherlands) (BPRC) primate facilities. The squirrel monkeys were naturally infected with *Trypanosoma cruzi*, one chimpanzee was infected with HIV, and the rhesus macaque was infected with SlV.

Isolation and preservation of PBMC. Heparinised blood was drawn from each animal by either venipuncture (chimpanzees and rhesus macaques) or cardiac puncture (squirrel monkeys) and used as a source of PBMC. PBMC were isolated from peripheral blood by density gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC). Interface cells were collected, washed twice with RPMI 1640 (Gibco, Paisly, Scotland), adjusted to 1 x 10^7 cells/ml, and either used immediately or cryopreserved for later use. To cryopreserve cells, 1 x 10^7 cells were resuspended in 1 ml of RPMI 1640 containing 10% dimethyl sulfoxide (DMSO) and 25% heat-inactivated foetal calf serum (FCSi), placed in cryocell freezing containers filled with cold isopropanol, and kept at -80°C overnight. The cells were stored at -135°C in a cryostar ultralow freezer (New Brunswick Scientific, USA) until use.

Prior to use, cryopreserved cells were thawed rapidly in a water bath at 37°C and immediately diluted 1:1 with RPMI 1640 medium containing 20% FCSi, 2 mM L-glutamine, 100 μg/ml gentamycin followed by two washes in the same medium. Cells were adjusted again to 1 x 10^7/ml after the last wash.

Monoclonal antibodies. Antibodies against T-cell surface antigens and cytokine receptors were part of the panel presented during the VI International Workshop and Conference on Human Leukocyte Differentiation Antigens. The mAb names and specificities are included in the Results section. Further details on the mAb have been published elsewhere [13, 14].

Flow cytometric analysis. Single-label indirect immunofluorescence was used to screen the mAb for their reactivity against nonhuman primate PBMC. In short, 5 $\times 10^5$ cells were dispensed into each FACS tube on ice and washed in PBS pH 7.3 containing 2% FCSi and 0.1 % azide (FACS buffer). After centrifugation, the supernatant was aspirated, the cells mixed with 10 μl of antihuman mAb diluted 1:50 in FACS buffer, and incubated on ice for 30 min. After washing with cold PBS, the cells were resuspended in 15 μl of goat antimouse-PE conjugated antibody (Dako, Glostrup, Denmark) diluted 1:20 in FACS buffer, and incubated for 30 min on ice. Free antibody was washed off with cold PBS and cells were immediately fixed for 1-12 hr in 1 % formalin. Prior to FACS analysis, the cells were washed and resuspended in PBS. Fluorescence was measured on a FACSort (Becton Dickinson, Mountain View CA). Cells were first analysed by forward and right-angle scatter and the

lymphocyte population was gated to assess reactivity of the mAb with PBMC. In each sample, 5,000 events in the lymphocyte gate were measured. Data analysis was performed using Cellquest^{TM} software (Becton Dickinson) to determine frequencies and mean fluorescence intensities.

The reactivity of the mAb with PBMC was scored as type 0, 1, 2, or 3, depending on histogram patterns that emerged during FACS analysis (Fig. 1). In short, basal levels were set between fluorescence intensity 10^0 and 10^1. Type 1 reactivity was characterised by a slight shift of the basal peak toward a fluorescence intensity between 10^1 and 2-5 x 10^1.

Type 2 and type 3 reactivity were characterised by a peak at basal level with a second peak between fluorescence intensity 10^1 and 10^3 for type 2 and between 10^3 to 10^4 for type 3 reactivity.

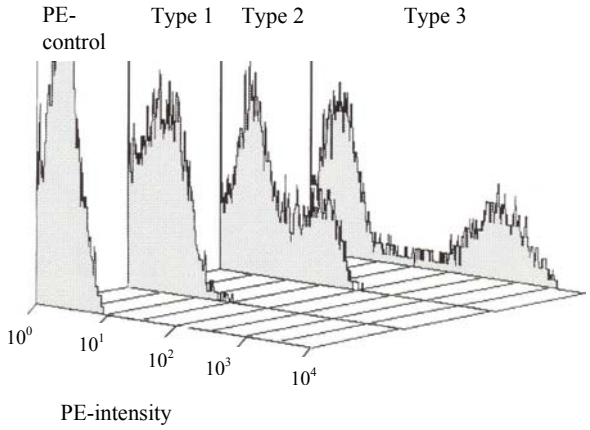

Figure 1. Histogram patterns observed during FACS analysis of PBMC from chimpanzees, rhesus macaques, and squirrel monkeys stained with antihuman mAb.

RESULTS

From the total of 161 anti-human mAb analysed, 58 antibodies were directed against T-cell surface antigens, 99 against various cytokine receptors, and 4 were directed against unknown antigens (Table 1). Of the antibodies against T-cell surface antigens, 81 %, 68%, and 63% reacted with PBMC from chimpanzees, rhesus macaques, and squirrel monkeys, respectively. For the cytokine receptors, 58%, 34%, and 32% of the mAb reacted with PBMC from chimpanzees, rhesus macaques, and squirrel monkeys, respectively.

There was no individual variation observed in the reactive mAb against T-cell antigens in

Table 1. Summary of antigens tested and number of reactive antibodies that emerged

		Number of positive antibodies		
Reactivity	Number tested	Chimpanzees	Rhesus macaques	Squirrel monkeys
C02	4	4	4	2
C03	4	4	1	3
C04	13	12	7	8
C05	5	5	1	5
C07	3	3	3	1
C08	12	11	10	8
C027	3	3	3	2
C028	4	4	4	2
COW109	3	0	1	1
C030L	2	0	2	2
C040L	5	0	3	2
MSPR	2	0	1	1
GM-CSFR	3	0	0	0
IL-4R	2	0	0	1
IL-7R	2	2	2	1
SCFR	11	1	1	2
4-1 BB	1	0	0	0
gp130	28	24	22	9
IL-2Ra	5	3	0	3
C071	1	1	1	1
FLT3	2	0	0	0
OX40	2	1	1	0
IL-6R	11	11	8	7
FLT3/FLX2	2	0	0	0
FAS	10	10	5	1
common	1	0	0	0
IL-1R type 1	2	0	0	0
IL-3Ra	1	1	0	0
IL-2R	2	2	1	1
IL-8R	1	0	0	0
IL-1R type II	1	0	1	1
TNFR/75kO	2	2	0	1
IL-2Ry	6	1	0	1
TNFR/55kO	1	0	0	1
UNKNOWN	4	0	0	1
TOTAL	161	105	73	68
%	100	65	45	42

Table 2. Reactivity of mAbs specific for human T cell surface antigens with PBMC from chimpanzees, rhesus macaques, and squirrel monkeys

CD number	mAb name	Chimpanzees	Rhesus macaques	Squirrel monkeys
2	LT2	3*	3	3
2	MEM-65	3	3	0
2	L303	3	3	0
2	RPA-2.10	3	3	3
3	LT3	3	3	3
3	IP30	3	0 or 1 **	1
3	Wu948	3	0 or 1	2 or 3
3	131F26	3	0	0
4	HIT4a	3	3	2 or 3
4	HIT4b	3	3	2 or 3

(continued)

Table 2. Reactivity of mAbs specific for human T cell surface antigens with PBMC from chimpanzees, rhesus macaques, and squirrel monkeys *(continued)*

CD number	mAb name	Chimpanzees	Rhesus macaques	Squirrel monkeys
4	LT4	3	0	0
4	MEM-16	0	3	0
4	IP34	3	0	0, 1, or 2
4	193-19	3	0	0
4	anti-C04	3	0 or 1	2
4	L3	3	3	3
4	RPA-T4	3	0 or 2	2
4	OKT4A	3	3	2 or 3
4	R2B7	3	0 or 2	0
4	8F4	3	3	3
4	LT4(UHKT)	3	3	2
5	HI211	3	0	1
5	HISM2	3	0	1, 2 or 3
5	SM3	3	1	1 or 3
5	MEM-128	3	0	1 or 2
5	7.8	3	0	1 or 2
7	LT7	3	3	3
7	MEM-186	3	3	0
7	BE57	3	3	0
8	HI212	3	3	0
8	HITBd	3	3	3
8	IP48	3	3	3
8	L2	3	2or3	3
8	BU88	3	3	3
8	ITI-5C2	3	3	3
8	5F2	0	0	0
8	RPA-T8	3	3	3
8	OKTBf	3	3	3
8	7B12/1G11	3	3	3
8	VIT8b	3	3	0
8	MC08	3	0 or 3	0
27	203-6	3	1 or 3	3
27	L128	3	1 or 3	3
27	1M	3	1 or 3	0
28	204-12	3	3	2 or 3
28	B-T3	3	3	3
28	B-L8	3	3	0
28	4B10	3	3	0
w109	701	0	0	0
w109	8A3	0	0	2 or 3
w109	02	0	0	0
30L	M81	0	1	2 or 3
30L	M82	0	1	0 or 2
40L	TRAP-1	0	1	0
40L	M79	0	1	0
40L	M90	0	0 or 2	0 or 2
40L	M92	0	2	2 or 3
40L	39-106	0	0 or 2	0

*0, 1, 2, and 3 are reactivity patterns that emerged during FACS analysis of PBMC from chimpanzees, rhesus macaques, and squirrel monkeys stained with anti-human mAbs. For details see Figure 1.
**The presence of more than one type of reactivity reflects intraspecies variation of binding.

the chimpanzees. However, there was intraspecies variation with some mAb against T-cell antigens in rhesus macaques (20%) and squirrel monkeys (26%). Intraspecies variation in reactivity of mAb against cytokine receptors was observed in all three species. The most variation was observed in rhesus macaques (31%); chimpanzees and squirrel monkeys showed 13% and 11% intraspecies variation, respectively.

T-cell surface antigens. Antibodies against 11 T-cell surface antigens were tested for reactivity with PBMC from chimpanzees, rhesus macaques, and squirrel monkeys. Most of the tested mAb reacted with chimpanzee cells and showed type 3 reactivity (Table 2). Of the mAb reactive with rhesus PBMC, most were type 3 while there was more variety in the type of reaction against markers of PBMC derived from squirrel monkeys (Table 2). In total, 22 mAb directed against T cell surface antigens reacted with PBMC from all three species (Table 2).

Cytokine receptors. A panel of mAb against 23 different cytokine receptors on PBMC was tested. Four antibodies whose reactivities in the human had not been fully characterised were also included (Table 3). Of the antibodies tested, several reacted with antigens on cells from all three species, i.e., with IL-7R, gp130, CD71, FAS, IL-2R/3, and IL6R (Table 3). Some antibodies only reacted with PBMC from one or two of the three species, whereas no mAb reactive with GM-CSFR, 4-lBB, FLT3, FLX2, common β, IL-IR type I, and IL-8R were found (Table 1).

DISCUSSION

In this study, we describe the reactivity of 161 antihuman mAb with PBMC from three different non human primate species, i.e., chimpanzees, rhesus macaques, and squirrel monkeys. We show that of this panel, 38 mAb reacted with PBMC from all three species tested and 112 mAb reacted with PBMC of one or two of the three species tested. These reactive antibodies most likely recognise antigens similar to those on human PBMC, as has been demonstrated in earlier studies [5, 6, 8, 10, 11]. Since these mAb have not been tested before in these three species, this increases the number of mAb that can be used in biomedical studies with these primate species.

Our results show that 65% of the tested mAb reacted with chimpanzee PBMC, whereas only 45% reacted with rhesus macaques and 42% with squirrel monkeys. This agrees with the assumption that reactivity between anti-human mAb and nonhuman primate systems increases with a decrease in phylogenetic distance [6, 8, 15]. The data from the present study also show that within the same species, several mAb reacted with one individual but not with

Table 3. Reactivity of mAbs specific for human cytokine receptors with PBMC from chimpanzees, rhesus macaques, and squirrel monkeys*

Reactivity	mAb name	Chimpanzees	Rhesus macaques	Squirrel monkeys
MSP-R	101	0*	0 or 2*	1
MSP-R	102	0	0	0
GM-CSF-R	SCO6	0	0	0
GM-CSF-R	SCO4	0	0	0
GM-CSFR	hGMCSFR-M1	0	0	0
SCFR	A3C6E2	0	0	0
SCFR	57A5	0	0	0
SCFR	10402	0	0	0
SCFR	NU-C-KIT	0	0	0
SCFR	L15	0	0	0
SCFR	NU-SCF2	0	0	0
SCFR	NU-SCF1	0	0	0
SCFR	MTK1	0	0	1
SCFR	MTK2	3	0,2 or 3	1
SCFR	17F11	0	0	0
SCFR	95C3	0	0	0
4-1 BB	ANTI-4-1 BB/4B4	0 or 2	0	0
gp130	B-K5	2	1 or 2	0
gp130	B-K11	0	0	0
gp130	B-L9	2	1 or 2	0
gp130	B-N4	2	1 or 2	0
gp130	B-N9	2	1 or 2	0
gp130	B-R9	2	1 or 2	0
gp130	B-S1	2	1 or 2	0
gp130	B-T6	2	1 or 2	1
gp130	B-T12	2	0 or 2	0
gp130	B-T2	0 or 2	1 or 2	1
gp130	B-T9	0	0	0
gp130	B-P8	2	1 or 2	0
gp130	B-P4	2	0 or 1	0
gp130	B-S12	2	1 or 2	0
gp130	B-R3	2	1 or 2	1
gp130	B-S8	2	1 or 2	1
gp130	A2	2	1 or 2	1
gp130	B1	2	1 or 2	1
gp130	C2	1	1 or 2	0
gp130	04	0	0	0
gp130	E1	2	0 or 1	0
gp130	F2	2	1 or 2	1
gp130	G1	0	0	0
gp130	H1	2	0,1 or 2	0
gp130	11	2	0,1 or 2	0
gp130	J1	2	1 or 2	1
gp130	GPX7	2	1 or 2	1
gp130	AM64	2	1 or 2	1
C071	BU56	1	3	1
OX40	BER-ACT35	2	0	0
OX40	L106	0	2	0
IL-1 R type 1	6B5	0	0	0
IL-1Rtype1	h1L-1 R-M1	0	0	0 or 2
IL-1R type 2	h1L-1 R2-M22	0	2	3
IL-2Rα	7G7B6	2	0	1 or 3
IL-2Rα	MEM-145	0	0	0

(continued)

Table 3. Reactivity of mAbs specific for human cytokine receptors with PBMC from chimpanzees, rhesus macaques, and squirrel monkeys* *(continued)*

IL-2Rα	MEM-140	0	0	1
IL-2Rα	Wu949	2	0	2
IL-2Rα	H-31	2	0	0 or 1
IL-2Rα	H-31	2	0	0 or 1
IL-2Rβ	2R-B	0 or 2	0**	0
IL-2Rβ	CF1	2	1**	1
IL-2Rγ	TUGh4	0	0	1
IL-2Rγ	3B5	0	0	0
IL-2Rγ	3G11	0	0	0
IL-2Rγ	AG14C	0	0 or 1	0 or 1
IL-2Rγ	AG43C	0 or 1	0 or 1	0
IL-2Rγ	AG184	1	0 or 1	0
IL-3Rα	9F5	2	0	0
IL-4R	hIL-4R-M57	0	0**	0, 1, or 2
IL-4R	S456C9	0 or 1	0	0
IL-6R	M11	0 or 1	0	3
IL-6R	M91	2	1 or 2***	3
IL-6R	M164	2	1***	0
IL-6R	M182	1 or 2	0 or 1 **	3
IL-6R	M195	0 or 2	1 or 2***	1
IL-6R	PM1	2	1***	3
IL-6R	M5	2	2	0 or 2
IL-7R	hIL-7R-M20	3	2 or 3	0
IL-7R	R34.34	3	3	3
FLT-3	4G8	0	0	0
FLT-3	BV10	0	0	0
FLT3/FLK2	SF1.340	0	0	0
FLT3/FLK2	SF1.394	0	0	0
FAS	7C11	2	0	0
FAS	OX2	3	3	3
FAS	Anti-Fas-(CH11)	2	0	0 or 2
FAS	B.029	3	3	0
FAS	B.E28	2 or 3	2	0
FAS	B.G27	3	0	0 or 1
FAS	B.G30	1 or 2	0	0
FAS	B.G34	1	2	0
FAS	B.K14	1 or 2	1	0
FAS	B.L25	1	0	0
IL-3R common	307	0	0	0
TNFR/55kO	htr9	0 or 2	0	1
TNFR/75kO	hTNFR-M1	2	0	3
TNFR/75kO	utr1	2	0	0 or 2
Unknown	G42	0	0	0
Unknown	HIM62	0	0	1
Unknown	M6-12	0 or 1	0	0
Unknown	WH2	0	0	0

*0, 1, 2, and 3 are reactivity patterns that emerged during FACS analysis of PBMC from chimpanzees, rhesus macaques, and squirrel monkeys stained with anti-human mAbs. For details see Figure 1.
**Reactivity of mAb with rhesus PBMC also described in reference 5.
***The presence of more than one type of reactivity reflects intraspecies variation of binding. Abbreviations: IL, Interleukin; MSP-R, macrophage stimulating protein receptor; GM-CSFR, granulocyte/monocyte colony stimulating factor receptor; SCFR, stem cell factor receptor, TNFR, tumour necrosis factor receptor.

another. This polymorphic reaction pattern was found for all three species with the cytokine receptor mAb panel, and for the rhesus macaques and squirrel monkeys also for the T cell surface marker panel. The observed intraspecies variation might reflect differences in activation levels between individual animals, possibly due to health status or polymorphism in the epitopes. Our findings agree with earlier studies in which polymorphic epitopes were demonstrated in T lymphocytes from other New World species, i.e., the spider monkey *(Atelus fusciceps)* and the owl monkey *(Aotus trivirgatus)* [5, 10]. Also, the finding that certain epitopes may be polymorphic in one species but monomorphic in another agrees with earlier studies [10].

In this study, we did not activate the cells prior to determination of mAb reactivity. Therefore, the lack of reactivity of the mAb with the known activation markers Cdw109, CD30L, and CD40L in chimpanzees should be treated with caution. The reactivity of these mAb with PBMC from rhesus macaques and squirrel monkeys might be due to aspecific binding or reflect the presence of circulating activated cells due to infections, although the reactivity of these antibodies was similar in the SIV-infected and non-infected rhesus macaques. Although it was not the objective of our study, we noticed that in the SlV-infected rhesus macaque the reactivity with all gpl30-specific mAb was less than in the uninfected monkeys. For all other mAb tested, we have no indication that there are major differences between infected and non-infected animals (data not shown).

Some of the mAb against the IL-2Rα and β and the IL-6R have been described before with respect to their reactivity with PBMC from rhesus macaques [16]. The data from the present study in general agrees with the earlier data, with the exception that some IL-6R-specific mAb that reacted weakly with rhesus PBMC in our study (M91, M164, M182, B-N12, BFI9, and PMI) were not reactive in the earlier study. One of the possible explanations for this discrepancy might be the use of PE-conjugated secondary antibodies in the present study, as opposed to FITC-conjugated antibodies in the earlier study since, in general, PE-conjugated antibodies give a stronger signal than FITC-conjugated antibodies.

Antibodies that reacted with PBMC from one species but not with PBMC from the other species were detected in this study. These antibodies could be used to study species-specific variation in the expression of epitopes they determine. In the present study, a large number of mAb that may be useful in immunological studies that use nonhuman primates have been identified. Presently, we are further characterising the reactivity of these mAb to determine

their value in the analysis of specific immunological processes in experiments using various nonhuman primate models.

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CHAPTER 6

IFN-γ expressing *P. knowlesi* is safe in rhesus monkeys and partially modulates host responses

Hastings Ozwara, Clemens Kocken, Jan Langermans, Richard Vervenne,
Jason Mwenda and Alan Thomas.

Biomedical Primate Research Centre, P.O. Box 3306, 2280 GH Rijswijk, The Netherlands and Institute of Primate Research, National Museums of Kenya, P.O. Box 24481, Karen, Nairobi, Kenya.

ABSTRACT

Our studies have previously shown that *in vitro* transfected *P. knowlesi* express bioactive rhesus IFN-γ. Preliminary safety and efficacy studies for these parasites were undertaken by infecting ("vaccinating") rhesus monkeys *in vivo* and measuring safety parameters and immune responses. In addition, immune response parameters were also measured in the monkeys after challenge with virulent *P. knowlesi* parasites. In total, four monkeys received IFN-γ expressing *P. knowlesi* and another five were inoculated with control parasites. All the animals developed patent parasitaemia; this peaked significantly earlier in vaccinated monkeys compared to the controls. Clinical chemistry and haematology data from these monkeys during the vaccination phase showed no abnormalities. This indicated that IFN-γ expressing parasites were safe and well tolerated in rhesus monkeys. Analysis of cellular changes revealed a significant increase in γδT cells in vaccinated animals compared to the controls, indirectly showing that IFN-γ released by parasites was bioactive *in vivo*. Subsequently, all animals were challenged with virulent *P. knowlesi* parasites. This resulted in a longer pre-patent infection period in vaccinated animals compared to the controls. This indicated that IFN-γ expressing *P. knowlesi* partially protected rhesus monkeys against virulent infection after a single vaccination. Analysis of cellular immune responses showed that peripheral blood cells from vaccinated monkeys showed higher levels of proliferation against crude parasite antigen than the controls. However, as the parasitaemia increased in these animals, the cellular responses waned.

INTRODUCTION

More than one quarter of the world population is at risk from malaria and between 1 and 2.7 million of them die annually [1]. Rapid spreading of parasite resistance to drugs of choice in the absence of a vaccine has made it difficult to combat the disease. New strategies, especially vaccines (being the most cost-effective way to prevent a disease) are urgently needed. Two observations suggest that a live malaria vaccine may be achievable. First, immunisation with attenuated sporozoites induces sterile protection in mice and humans[2, 3], mediated via IFN-γ dependent mechanisms and we have recently published data [4] showing that chimpanzees can be protected. Hence a vaccine based upon attenuated parasites is feasible. Second, adults in areas endemic for malaria develop variable levels of clinical immunity [5].

Studies have shown that pathogens expressing host cytokines *in vivo* become immunopotentiated, subsequently manipulating host-pathogen interaction and stimulating protective host responses [6-8]. We reasoned that parasites expressing immunomodulatory cytokines such as IFN-γ may become immunopotentiated and induce protective host responses better than normal parasites. *P. knowlesi*, a natural parasite of macaques [9], is an attractive experimental system to use in testing this assumption because it has potential use in humans (it is known to infect humans and recently an outbreak of infections has been reported in Asia) and it is closely related to human malaria parasites [10]. Recently *P. knowlesi* blood stage parasites were adapted to long term *in vitro* culture [11]. In contrasts to *in vivo* passaged parasites, the long term *in vitro* cultured *P. knowlesi* parasites tended to induce chronic parasitaemia in rhesus monkeys, characterised by low-level peak parasitaemia (0.2 to 1%). These parasites were used to generate *P. knowlesi* that expresses rhesus IFN-γ. Analysis of these parasites *in vitro* has shown that they release bioactive rhesus IFN-γ [12].

Repeated exposure to *P. knowlesi* infection is required to generate protective immune responses in monkeys [13], therefore under normal circumstances, animals are not protected after a single infection. Cytokine releasing parasites could potentially prime the host to destroy the parasites and develop protective responses after a single infection. The development of immunopotentiated malaria parasites that protect the host against a virulent infection could have far-reaching implications in terms of developing a live malaria vaccine.

In rodent and human malaria models, IFN-γ has been shown to play a role in protection against malaria blood stages when either endogenously produced [14-16] or exogenously administered [17-19]. In addition, administration of exogenous IFN-γ is safe and immunogenic in rhesus monkeys when administered in short term [20-23] and when expressed by simian immunodeficiency virus [7]. However, exogenously produced cytokines could be toxic to the host [24, 25]. Therefore, it is critical to evaluate the *in vivo* safety of IFN-γ expressing parasites.

In this report, we evaluated the safety and efficacy of IFN-γ expressing *P. knowlesi* blood stage parasites *in vivo* by vaccinating rhesus monkeys and measuring parasite development and host responses during vaccination and after challenge with virulent parasites.

MATERIALS AND METHODS

Animals. Ten adult intact malaria-free *Macaca mulatta* of either sex and weighing six kilos and above were used in the study. Each animal was housed individually in single cages

to avoid blood contact that could lead to cross infection. All experimental animal work in these studies was carried out under protocols approved by the independent Institutional Animal Care and Use Committee and performed according to Dutch and European laws.

Parasites. *In vitro* episomal transfected *P. knowlesi* H strain parasites used during vaccination were generated as described in Ozwara *et al*., [12]. Parasites from on going cultures were used to inoculate the monkeys. Wild type *P. knowlesi* H strain [26] parasites clone Pk1(A+) [27] for challenge infection were retrieved from liquid nitrogen followed by overnight *in vitro* culture.

Vaccination. Vaccinations were done in two independent experiments. Experiment 1 comprised 2 test and 2 control monkeys. The second experiment had 6 animals, 3 vaccinated and 3 control monkeys. Each vaccinated monkey was i.v. injected with 5×10^8 genetically modified blood stage *P. knowlesi* parasites in 1 ml of RPMI 1640 medium. Control monkeys were injected with *P. knowlesi* genetically modified with a similar plasmid but without the rhesus IFN-γ gene. Parasites used in inoculating both vaccinated and control animals were cultured under identical conditions. The time between parasite inoculation of the first and last animal (randomly divided over the two groups) was 1 h. Peripheral blood was obtained from each animal prior to parasite inoculation and used to determine pre-infection values for cytokine and cell surface maker levels, peripheral blood mononuclear cell (PBMC) isolation, antibody responses and clinical chemistry and haematology analysis. Thereafter, blood was obtained from each animal on a weekly basis to determine responses during infection. The general health status of the animals was monitored by regularly determining body temperature, weight, appetite and general behaviour. Body temperature and weight of animals was measured on a sedated animal at the time points of bleeding. Appetite was evaluated by closely monitoring the feeding habits of the animals. Finger prick blood was regularly taken from all animals for analysis of parasitaemia. Parasitaemia was determined in thin smears by separate enumeration of infected and total erythrocytes. Pyrimethamine (1 mg/kg body weight) was orally administered on a daily basis to maintain episomes in the parasites. Animals were radically cured when the level of parasitaemia reached 5% or higher, by injection of chloroquine sulphate (Rhone-Poulenc Rorer, Paris, France) at a dosage of 5 mg/kg daily for three days. At three weeks post-vaccination all untreated animals were radically cured with chloroquine sulphate.

Challenge infection. At five weeks post vaccination, all vaccinated and control monkeys in both vaccination experiments were challenged by i.v. injection of 1×10^5 virulent blood stage *P. knowlesi* parasites (in 1 ml of RPMI 1640 medium). Challenged animals were bled

immediately prior to parasite inoculation to determine pre-challenge immune, clinical chemistry and haematology values. Subsequently, blood was obtained from each animal on a weekly basis to determine responses during challenge infection. Finger prick blood was obtained from challenged animals on a daily basis starting five days post challenge infection and continued until the end-point. Animals were radically cured when the level of parasitaemia reached over 5%, by injection of chloroquine sulphate. Otherwise, experiments were stopped at two weeks post-challenge by treating the animals with chloroquine sulphate.

Clinical Chemistry and Haematology. Peripheral EDTA blood was obtained from all monkeys as described above, and analysed in a Sysmex R500 (Sysmex, Kobe, Japan) to determine reticulocyte changes. A Sysmex SF 3000 (Sysmex, Kobe, Japan) was used to analyse for changes in haematocrit and red blood cells, haemoglobin, and leukocytes. To determine kidney and liver functions, serum was processed from part of peripheral blood and analysed in a COBAS INTEGRA 400 (Roche, Schweitz, Germany) to measure albumin, liver enzymes AST, ALT and lactate dehydrogenase, creatinine, total bilirubin, and urea. The data were compared with normal values obtained from over 500 naïve rhesus monkeys of different ages and sexes.

Lymphocyte proliferation assays. Lymphocyte proliferative responses were determined in both vaccinated and control monkeys. Previously isolated PBMC were cultured in triplicate in 96-well microtitre plates. The cells were stimulated with crude *P. knowlesi* H strain whole parasite antigen for 72h. Cultures were then pulsed with [^{3}H]thymidine and cultured for 18h at 37°C prior to harvest and counting in a Top Count NXT Microplate Scintillation and Luminescence Counter (Perkin Elmer).

FACS analysis. Rhesus monkey peripheral blood was obtained as described above and 100 µl was lysed with FACS lysis solution (BD, Heidelberg, Germany). Lysed blood was stained with cross-reactive antibodies against human CD3, CD20, CD64, MHC class II DR, CD14 and pan γδT cell receptor surface markers (BD). These markers stained for T and B cells, γδT cells and activated monocytes. The stained cells were analysed for cell surface marker expression by fluorescence activated cell sorting (FACS) as previously described [28], except that 1×10^5 cells were scanned and analysed.

Statistical analysis. Parasitaemia curves for several days were compared by two-way repeated-measures analysis of variance. Comparison between two groups was made by using a two-tailed Student's *t* test. All statistical calculations were performed with SPSS, version 7.5 for windows (SPSS, Inc., Chicago, and Ill).

RESULTS

Vaccinated and control monkeys develop different parasitaemia profiles during the vaccination phase. Two studies, each comprising experimental and control animals were conducted six months apart under identical conditions. In one study one animal had to be removed from the experimental group for technical reasons unrelated to the experiment. The results from these experiments are pooled in what follows to yield data from a total of 4 experimental and 5 control animals (Table 1).

Table 1. Selected parameters of parasitaemia in monkeys infected with *P. knowlesi*

Animal	Group	Peak parasitaemia[a] vaccination		Pre-patent period[b] challenge
		% Parasitaemia	Day	Day
C117	Experimental[c]	1.4	9	11
C019	Experimental	0.4	6	9
C097	Experimental	0.7	9	11
C017	Experimental	0.6	6	9
C149	Control	0.3	14	NO[d]
C143	Control	0.1	16	5
C151	Control	0.1	16	5
C039	Control	0.8	14	6
C096	Control	0.6	14	6

[a] The highest level of parasitaemia detected in the 21 days that vaccinated monkeys were observed for peripheral parasitaemia.
[b] Period from day of challenge to when the day parasites were first detected in peripheral circulation by thin smear method.
[c] Experimental monkeys were infected (vaccinated) with IFN-γ expressing *P. knowlesi.*
[d] Peripheral parasitaemia was not detected in the animal during the entire challenge period of 14 days.

Monkeys in both experiments were vaccinated with 5×10^8 transfected parasites containing either empty expression plasmids (controls) or plasmids allowing the expression of rhesus IFN-γ in late stage schizogony. Both IFN-γ expressing and control parasites had similar growth rates *in vitro* prior to vaccination (data not shown). All plasmids contained the pyrimethamine resistance cassette and were maintained in the parasite population by treating the monkeys with pyrimethamine. After exposure for 3 weeks these genetically modified parasites were removed by chloroquine treatment. A short period of rest was then allowed for washout of chloroquin before vaccinated and control animals were challenged with 1 x 10^5 wild-type H strain *P. knowlesi* parasites (Fig. 1).

All monkeys during the vaccination phase developed patent parasitaemia by day 6 post-inoculation that developed into a low-level self-regulating parasitaemia (Fig. 2A). When compared with controls, vaccinated animals had significantly higher parasitaemias between days 6 to 10 ($P<0.007$), (Fig. 2A). The peak parasitaemia was achieved significantly earlier (day 8 against day 15, $P<0.001$) (Table 1). The parasitemia in control monkeys developed in similar fashion to that seen in rhesus monkeys inoculated with wild-type culture-adapted *P. knowlesi* H strain, a profile distinct from *in vivo* passaged parasites ([11] and our unpublished data). Animals vaccinated with IFN-γ parasites had a higher cumulative exposure to parasites than control animals (Fig 2B).

Changes in cellular immune responses were also monitored by FACS analysis of peripheral blood. Changes in B-cell, monocyte and most T-cell populations were comparable in experimental and control animals (not shown). However there was a significant (2-5 fold) increase in γδ T cells in experimental animals as compared to a 1-2 fold increase in controls (Fig. 3).

In blood taken at days 0, 7, 14 and 21 PBMC proliferative responses to crude *P. knowlesi* H strain antigen remained within background levels. Serum IgG levels were comparable between the two groups, suggesting that exposure to IFN-γ had not significantly enhanced humoral responses.

To determine whether animals had been exposed to parasites of the expected phenotype, blood obtained from each monkey immediately prior to chloroquine cure was cultured and supernatants analysed by ELISA for IFN-γ expression. Rhesus IFN-γ was detected in cultures of parasites derived from each of the experimental animals but was not detected in blood from control animals (data not shown).

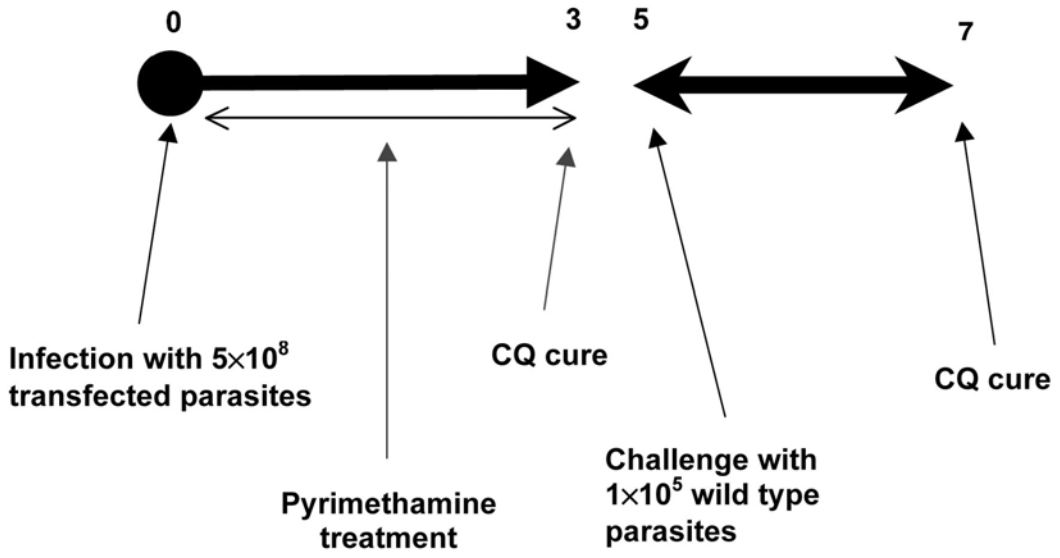

Figure 1. Vaccination and challenge schedule for rhesus monkeys infected with IFN-γ expressing and control *P. knowlesi* parasites. "Vaccination phase lasted 21 days and challenge infection followed 2 weeks later. Pyrimethamine treatment (1 mg/kg body weight) during vaccination was started less than 24 h post-inoculation. Monkeys were cured with chloroquine at the end of each phase. CQ, Chloroquine.

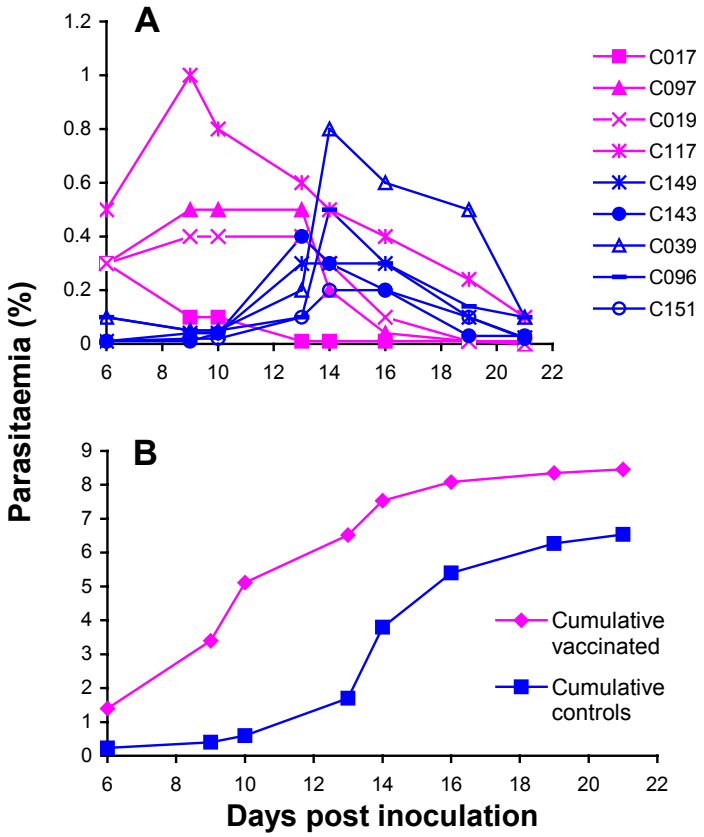

Figure 2. Individual monkey (A) and cumulative (B) parasitaemia profile in vaccinated and control monkeys

Host responses during vaccination. To determine whether exposure to IFN-γ expressing parasites was well-tolerated, blood samples from vaccinated animals collected on days 0, 7, 14 and 21 were assessed for haematological and clinical chemistry criteria. Haematological values, and clinical chemistry indicators of liver and kidney function remained within normal ranges in all the animals (not shown). In addition, the animals were observed for appetite, body weight and temperature changes. There were no significant changes in body temperature, weight and appetite during the course of vaccination (not shown).

Vaccinated rhesus monkeys show a modified course of challenge infection. The parasite challenge dose administered at week 5 had previously been infective to all naïve animals tested, producing patent infection within 7 days of inoculation ([11] and unpublished data). A parasitaemia of over 5% was radically drug cured because above this level, a fulminating infection normally develops in the monkeys.

As can be seen in Table 1 and Fig. 4A, three of the five control animals developed a fulminating parasitemia requiring treatment. Unexpectedly, two of the five control animals controled the parasitemia to undetectable levels (C149) or to low chronic levels (C143). This could be the result of extended exposure to the control *in vitro* adapted parasites during the vaccination phase of the study. By contrast, four of the four vaccinated animals had a significant delay in the onset of parasite development of 4 days ($P<0.001$). One animal (C017) had a peak parasitaemia at day 11, and subsequently controlled the parasitaemia. All other experimental animals required drug treatment but at time points significantly later than the controls. Pooled cumulative parasitaemia graphs for challenge infection (Fig. 4B) further demonstrate the difference between the two groups.

Host responses during challenge infection. Analysis was performed on blood samples collected from all monkeys prior to challenge infection, and on a weekly basis thereafter. Haematological values remained comparable in both experimental and control groups. Clinical chemistry values for liver and kidney functions were increased in monkeys with acute infection (data not shown). Cellular immune responses as determined by FACS analysis of peripheral blood changes in T cells, B cells and monocytes were similar in experimental animals compared to the controls (data not shown).

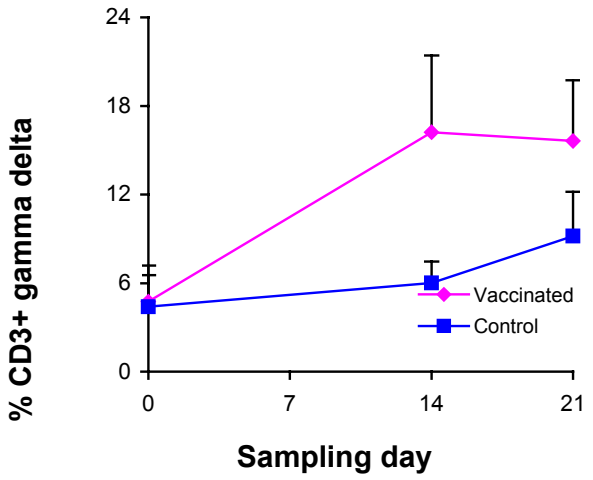

Figure 3. Average percentage of γ δT cells during the vaccination phase. Dark line graphs represent percentage of cells in monkeys inoculated with parasites transfected with control plasmids and grey lines in monkeys infected with IFN-γ expressing parasites. Procedure for cell staining and FACS analysis is described in Materials and Methods. Error bars are standard deviations

At one week post-challenge, *in vitro* PBMC proliferative responses to crude *P. knowlesi* H strain antigen were generally higher in vaccinated monkeys than in controls (Table 2). The proliferation indices were significant for three out of four vaccinated monkeys and for one out of four measured control monkeys. However, these responses had partially waned at the end of the experiment following development of acute parasitaemia (Fig. 4A and Table 2). Serum IgG levels were comparable between the two groups throughout, suggesting that vaccination had not primed for enhanced antibody responses.

Table 2. Stimulation of rhesus monkey PBMC with *P. knowlesi* total parasite antigen

Group	Pre-challenge[θ] (Day 0)	Challenge (Day 7)	End Pont challenge (Day 14)
Vaccinated			
C019	1.18[⋆]	**5.36**[‡]	**2.13**
C097	1.25	**2.53**	1.23
C117	1.35	**8.49**	**2.01**
C017	1.10	0.70	**2.60**
Control			
C143	1.29	1.5	1.62
C149	0.79	**3.76**	**5.37**
C151	1.40	1.47	1.42
C096	0.90	ND	1.20
C039	0.50	0.9	1.20

[θ] Pre-challenge day was 35 days post-vaccination. Samples were obtained immediately prior to inoculation of challenge parasites.
[⋆] Values are stimulation indices, calculated as readings from stimulated samples divided by background level values. ND = not done
[‡] Boldface figures are samples scored as having proliferated upon stimulation

DISCUSSION

These preliminary experiments were designed to establish the safety of the genetically modified parasites and to assess whether there was any indication that exposure to parasites expressing IFN-γ would modulate the immune response and modify the course of subsequent infections. We have previously shown that malaria parasites are able to express host cytokine genes, and because of the ease of manipulation and the ready availability of suitable assays, IFN-γ was selected as the first cytokine with which to show proof of principle.

We demonstrate that IFN-γ expressing *P. knowlesi* is well tolerated in rhesus monkeys. The *in vitro* adapted parasites induced as expected [12] a low level self-regulating infection that did not disturb important kidney and liver functions and did not induce significant changes in body temperature, weight and appetite during the course of vaccination. IFN-γ expressing *P. knowlesi* parasites induced an increase in γδT cells, and evoked responses

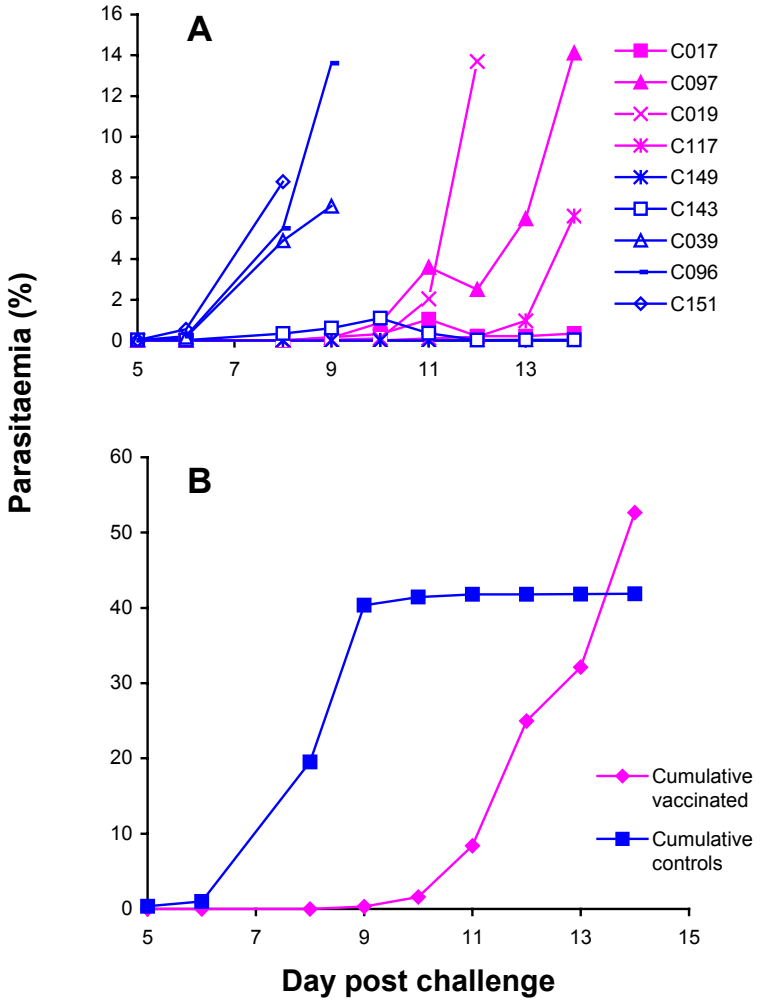

Figure 4. Individual monkey (A) and cumulative (B) parasitaemia profile in vaccinated and control monkeys during the challenge phase.

(presumably immune) that caused a delay in the development of infection in four out of four rhesus monkeys challenged with bloodstage parasites. Two out of five control monkeys also managed to control parasitemia, possibly caused by extended exposure to control *in vitro* adapted parasites during the vaccination phase. These data suggest that parasite-produced IFN-γ possibly enhanced anti-*P. knowlesi* immune responses evoked by exposure to *in vitro* adapted parasites.

The main measurable difference in immune response between the experimental and control animals during vaccination was at the cellular level. Expansion of γδT cells during the vaccination period was significantly greater in the experimental group. This may simply

be due to the higher parasite load experienced in the experimental group (Fig 2B). However, there were no differences in the expansion of γδT cells during asexual blood stage challenge, despite the control animals having been exposed to a much higher parasite load between 8 and 12 days post challenge infection. It seems therefore more likely that the increase in γδT cells observed during the vaccination phase was largely due to IFN-γ release by *P. knowlesi*. Gamma delta T cells are known to proliferate and expand early during malaria infection [29, 30] and can secrete IFN-γ within a day of exposure to parasitised erythrocytes *in vitro* [31, 32]. Parasite produced IFN-γ is likely to have stimulated the γδT cells to proliferate and to release IFN-γ, leading to a positive feedback in IFN-γ production.

The role of γδT cells in malaria infection is not yet clear. Some studies have suggested that an increase in γδT cells could lead to an increase in parasitaemia (reviewed in [33]). Other studies have shown that γδT cells may also protect against malaria infection [34, 35]. It is tempting to attribute the observed increase in parasitaemia during vaccination with IFN-γ producing parasites to the effect of γδ T cells. However, there are no sufficient sampling data points to confirm this. In addition, care should be taken when interpreting the data because the findings are a single observation using only four animals.

Total proliferative cellular responses were generally higher in vaccinated animals than in controls. Total antibody responses were similar in both groups during vaccination and after challenge. The delay in parasitaemia observed after challenge in vaccinated animals may therefore have been mediated by cellular responses. This is surprising because protection against malaria blood stage infection is generally thought to be mediated by antibody dependent mechanisms [36]. Recent studies have, however, demonstrated that cell mediated mechanisms can mediate protection independent of antibody [37], especially at low parasite densities. A combination of exposure to low parasite density along with IFN-γ production may have stimulated a cellular response that is indeed able to control parasite development, albeit only for a short period.

Proliferative responses in experimental monkeys partially waned in the second week of challenge infection at the same time as high levels of parasitaemia emerged. The reasons for this are not clear, but may be related to the fact that excessive antigenic stimulation decreases T cell function and survival [38]. Malaria infected erythrocytes have also been reported to inhibit protective T cell responses [39, 40].

A more comprehensive analysis of host responses to IFN-γ expressing *P. knowlesi* and protection after challenge is now warranted. Nevertheless, this study has already

demonstrated that the parasites are well tolerated *in vivo* and that the IFN-γ produced by the parasites is bioactive *in vivo*. It is noteworthy that IFN-γ expressing *P. knowlesi* can mediate partial protection (a significant delay in peak parasitaemia) against challenge infection and this after only one vaccination episode. Studies in humans and animal models have shown that IFN-γ is intimately involved in protection during liver stage development of malaria parasites [41-43]. As far as we are aware, this is the first study to suggest that it may play an important role in the emergence of immune responses that control the development of asexual blood stage parasites.

Protection against asexual blood stage infection in monkeys can be induced by repeated exposure to parasites [13], a situation that is similar to the emergence of protection in human populations **(**reviewed in [44]**).** Our study suggests that by genetic manipulation with host immune response modifying genes, malaria parasites may be able to induce such protective responses in an accelerated manner. Nevertheless, although IFN-γ expressing parasites were immunopotentiated, they did not provide complete protection against challenge infection in rhesus monkeys, suggesting either that the expression of IFN-γ alone is insufficient to generate fully immunopotentiated parasites, or that multiple exposures to IFN-γ expressing parasites will be required to induce complete protection.

A vaccine for malaria is urgently required. A variety of approaches based on presentation of one or a few selected parasite components to the immune system are currently being developed. While these approaches have great promise, there are concerns as to whether the immune response can be stimulated for a sufficiently long period of time and whether the polymorphism displayed by most of the parasite target antigens represents an insurmountable problem. The induction of immune responses to whole parasites may have some advantage, in that responses to a broad range of host targets are induced, lessening the impact of polymorphism at any one locus. It is known that even individuals immune to the clinical effects of malaria lose this immunity after a relatively short period living outside the endemic area (and therefore no longer exposed to repeated sub-clinical boosting of immunity). It may be possible, with judicious use of host immune modulator genes, to induce more long lived responses against the parasite.

This approach of immunopotentiation of parasites, when combined with strategies to attenuate parasites (such as genetically programmed decreases metabolic capabilities) may provide an attractive and cheap alternative to sub-unit vaccines. Attenuated vaccines have been very successful against other diseases, and the possibility of such developments for malaria should not be ruled out. It will be important to continue this work and expand the

range of immune modulator genes being evaluated. A further study is warranted to confirm these results, and to allow a deeper analysis of the immune responses evoked by vaccination with immunopotentiated malaria parasites.

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CHAPTER 7

Experimental infection of the olive baboon *(Papio anubis)* with *Plasmodium knowlesi:* severe disease accompanied by cerebral involvement

Hastings Ozwara, Jan A. M. Langermans, Jenneby Maamun, Idle O. Farah, Dorcas S. Yole, Jason M. Mwenda, Horst Weiler, and Alan W. Thomas

Institute of Primate Research, National Museums of Kenya, Nairobi, Kenya; Departments of Parasitology and Animal Science, Biomedical Primate Research Centre, Rijswijk, The Netherlands

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ABSTRACT

Experimental systems that model some of the complex interactions between parasite and host can be extremely valuable in identifying and developing new prophylactics and therapeutics against human diseases. Because primates have similar immune systems to humans, we have characterised a baboon model for understanding host response to *Plasmodium knowlesi.* Ten intact olive baboons *(Papio anubis)* of either sex were experimentally infected with *P. knowlesi* H strain erythrocytic parasites. The infection in these baboons was either acute or chronic. Animals with acute infection developed multiple system organ dysfunction and cerebral involvement. In chronically infected animals, only the spleen was moderately enlarged. The *P. knowlesi* parasitaemia profile in baboons and rhesus monkeys was comparable. However, some clinical symptoms of the baboons and *P. falciparum*-infected humans were similar. These studies demonstrate for the first time that *P. anubis* is a suitable host for *P. knowlesi* for studying clinical symptoms and pathology.

INTRODUCTION

The evaluation of new therapeutics and prophylactics for use in humans often requires testing in animal models that develop a disease comparable to that in humans. In certain basic and applied studies, primates are the only animal models susceptible to the human disease under study [1, 2]. Similarities in biologic mechanisms between humans and nonhuman primates underlie the value of nonhuman primates as the final system for evaluating the safety and efficacy of drugs and vaccines developed in studies with other laboratory animals and systems [3-5]. Nonhuman primates are widely used in malaria drug and vaccine development [5-7].

The two major human malaria parasites *(Plasmodium falciparum* and *P. vivax)* have a very restricted host range,[8] which limits research on parasite biology especially at the host-parasite interface. However, experimental systems have been used to model some of the complex interactions between parasite and host. There are three *Plasmodium* groups that are mainly used in experimental studies on host-parasite interactions, i.e., rodent, avian, and primate. Rodent malaria parasites are used to study parasite biology [9]. However, these parasites are phylogenetically distant from human *Plasmodium* [10] and do not easily allow investigations of natural host-parasite interactions. Although *P. gallinaceum* and *P. lophurae,* the most widely used avian malaria parasites are closely related to *P. falciparum* [11] their development in nucleated cells and the wide phylogenetic distance between birds and humans limits their applicability to study important questions on host-parasite interaction relevant to

human malarias. Simian *Plasmodium* such as *P. knowlesi* have a comparable phylogeny and host-parasite relationships to human malaria parasites [l0, 12]. The parasites are used to identify, develop, and evaluate vaccine and drug candidates [7, 13, 14] and to characterize host responses [15-17].

Plasmodium knowlesi malaria infection has been described in *Macaca fascicularis* (natural host), as well as experimentally induced in a number of other nonhuman primates such as *M. mulatta, M. radiata, M. assamensis, Presbytis entellus, Callithrix jacchus, Aotus trivigatus, Saimiri sciureus,* and baboons [8, 12, 18, 22]. In baboons, the infection has been induced in *Papio cynocepahalus, P. doguera, P. jubileaus,* and *P. papio* [12]. The parasite also infects humans [12, 23] and clusters phylogenetically with *P. vivax* [24]. In most experimental models, *P. knowlesi* infection is acute, whereas in their natural host *M. fascicularis,* it generally induces a chronic infection [6, 12]. The availability of both natural and artificial hosts combined with the close relationship between primates and man make *P. knowlesi* infection in nonhuman primates attractive to study host-parasite interaction in detail.

We have recently developed protocols for long term *in vitro* culture and genetic modification of *P. knowlesi* [25, 26]. These are powerful tools for understanding parasite biology, especially gene function. This has been facilitated further by the recent sequencing of its genome (http://www.sanger.ac.uk/Projects/ Protozoa/) and that of *P. falciparum* [27]. The function(s) of attractive drug and vaccine candidates can be determined using homologous *P. knowlesi* genes. However, versatile *in vivo* systems are required to determine host-parasite interaction of the genetically modified parasites. The baboon is attractive because it is a well characterised and frequently used primate in biomedical research [4]. At the moment, the patterns and mechanisms of *P. knowlesi* infection in baboons are relatively unknown.

In this study, we determined the disease profile and pathology of the *P. knowlesi* H strain infection in the olive baboon as an experimental host system for understanding parasite biology of *P. knowlesi,* especially host-parasite interaction.

MATERIALS AND METHODS

Parasites. *Plasmodium knowlesi* H strain [23] parasites for inducing baboon infection were retrieved from liquid nitrogen and cultured overnight. The original parasite inoculum was clone Pkl(A+), previously cloned by micromanipulation and passaged in rhesus monkeys [28].

Animals. Adult *Papio anubis* (weight range = 11-21 kg) of either sex and originating from the Kajiado district of Kenya were used. Prior to the experiment, all animals were screened and determined to be free of infection with *Plasmodium* by a Giemsa-stained thick blood smear film. Ten baboons were inoculated intravenously with 1 x 10^4 to 1 x 10^6 *P. knowlesi* blood stage parasites. As controls for clinical symptoms, four uninfected animals were housed under similar conditions. All the animals were fed on a standard diet for baboons and water was provided *ad libitum.* The Institutional Animal Care and Use and the Scientific Review Committees of the Institute of Primate Research approved the baboon experiments. All experiments were performed in a biocontainment facility.

For comparative purposes, historical data were collected from six rhesus monkeys that had been infected with 1 x 10^5 *P. knowlesi* H strain parasites of the same stock. These experiments were done at the Biomedical Primate Research Center after approval by the Institutional Animal Care and Use Committee (the DEC).

Animal observation and sampling. Baboon health was monitored on a daily basis. Animals were assessed for 1) general agility, 2) playing habit, and 3) appetite (by weighing leftover food). Agility and playing habits of the monkeys were assessed by an attendant familiar with normal behaviour of the particular animals. Animals were weighed on a weekly basis. Parasitaemia was determined microscopically on finger prick thin blood smears stained with Giemsa and plotted as the number of infected cells in 1 x 10^4 erythrocytes.

Clinical chemistry and haematology. Venous EDTA blood was obtained from infected baboons after sedation with ketamine. Blood was analysed in a Coulter counter (Beckman Coulter, Mijdrecht, The Netherlands) to determine haematocrit and haemoglobin changes. Serum was collected before and during peak infection, and used to analyse blood creatinine, bilirubin and urea. Commercial kits for Creatinine (Biotech Laboratories, Ipswich, United Kingdom), total bilirubin (Biotech Laboratories), and urea (Randox Laboratories, Antrim, United Kingdom) were used according to manufacture's instructions.

Pathology. After the duty veterinarian had certified baboons with severe malaria as lethargic or comatose, they were humanely killed and autopsies performed immediately. Two animals died unexpectedly and were immediately presented for necropsy. Animals with chronic infection were killed at four weeks post-infection. Gross organ and pathophysiologic derangements were observed during necropsy. Brain, liver, lung, kidney, spleen, and lymph node specimens were collected and immediately fixed in formalin. Sections of 5-μm thickness were prepared from paraffin-embedded specimens, stained in haematoxylin and eosin and analysed for pathologic changes. Erythrocyte aggregation in the brain was

quantified by examining 100 small blood vessels and expressed as percentages, as described by Pongponratn and others [29].

RESULTS

Parasitaemia. All baboons inoculated with *P. knowlesi* H strain developed patent parasitaemia by day five post-inoculation (Figure lA). Seven animals had acute parasitaemia that systematically increased to greater than 500 parasites per 1 x 10^4 erythrocytes, reaching as high as 4,950 parasites per 1 x 10^4 erythrocytes at the time of killing. All baboons with acute infection had become lethargic by day 12 post-infection (Table 1). The remaining three animals developed chronic parasitaemia (Table 1) with peak levels of less than 300 parasites per 1 x 10^4 erythrocytes by day 16, which thereafter decreased to less than 50 parasites per 1 x 10^4 erythrocytes (Figure lA). At low parasitaemia, i.e., less than 300 parasites per 1 x 10^4 erythrocytes schizont parasite stages were rarely observed in the peripheral circulation, suggesting sequestration. At higher parasitaemia, as observed in animals with severe malaria, the number of schizont stages in the peripheral circulation increased slightly, indicating reduced sequestration. The inoculum size and *in vivo* passage of parasites used to inoculate all the baboons did not determine the parasitaemia and disease profile (Table 1).

Historical data on patterns of parasitaemia were collected from rhesus monkeys previously infected with the same parasites as the baboons. All rhesus monkeys had developed patent parasitaemia by day six post-inoculation (Figure 1B). The parasitaemia profile in three of the monkeys (Rh2T, Rh 4086, and Rh9154) was characterised as chronic (Figure 1B), with peak parasitaemia less than 700 parasites per 1 x 10^4 erythrocytes and thereafter decreasing (Figure 1B). The other three animals (Rh C029, Rh 3015, and Rh 3337) developed acute parasitaemia, i.e., levels greater than 700 parasites per 1 x 10^4 erythrocytes (Figure 1B).

Clinical symptoms. Onset of patent parasitaemia was followed by loss of appetite in all animals as measured by decreased food intake (Figure 2A) and marginal weight losses. Baboons with acute parasitaemia developed severe clinical symptoms and were characterised as having severe malaria. These symptoms included a significant increase in body temperature as the infection progressed (Figure 2C) and remaining in a sitting position in the cage (apathy) with raised fur. In addition, they progressively became lethargic, developed dyspnoea, and produced dark coloured urine suggesting cholestasis. There was reduced ocular tension and skin turgor indicating dehydration. Haematocrit and haemoglobin levels showed moderate to low decreases (Figures 2B and 2D).

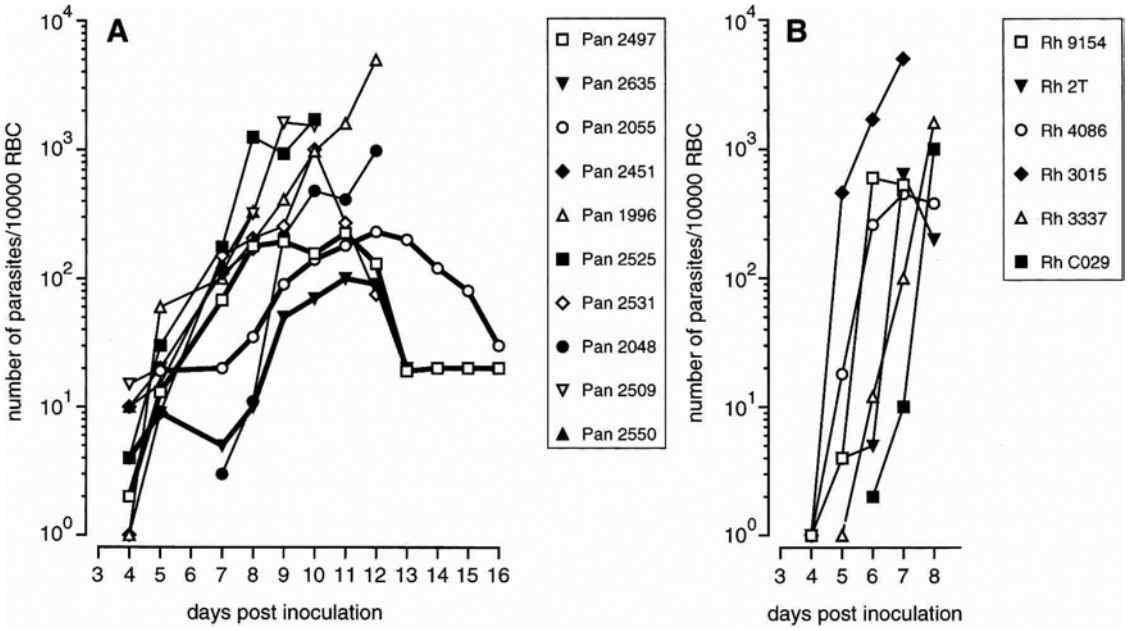

Figure 1. Parasitaemia profile of A. baboons and B. rhesus monkeys infected with *Plasmodium knowlesi* H strain. RBC - red blood cells.

Analysis of serum for bilirubin, creatinine, and urea to determine liver and kidney functions showed that in animals with severe malaria, there was a four-fold increase in total bilirubin (Table 2) and an increase in creatinine and blood urea, suggestive of severe haemolysis and dysfunctional kidneys (Table 2). By day 12 post-inoculation, all the baboons with severe malaria were either lethargic or comatose. Animals with chronic parasitaemia were classified as having mild malaria; they showed moderate to low level of the clinical symptoms observed in severely infected animals (Figure 2).

Table 1. Parameters of baboons infected with the *Plasmodium knowlesi* H strain

Baboon number	Inoculum	Infection profile		Inoculum passage in baboons**
		Profile	Day post-infection*	
Pan 1996	2×10^5	Acute	12	1
Pan 2525	2×10^5	Acute	10	1
Pan 2531	1×10^6	Acute	12	1
Pan 2497	1×10^6	Chronic	16	1
Pan 2048	1×10^6	Acute	12	2
Pan 2055	2×10^4	Chronic	16	3
Pan 2509	1×10^4	Acute	11	4
Pan 2635	1×10^4	Chronic	12	4
Pan 2550	1×10^4	Acute	6	4
Pan 2451	1×10^4	Acute	6	4

*Day post-infection when the animal profile was scored as acute or chronic.

**Parasite inoculum for the first passage was derived from rhesus monkeys and used to infect baboons after overnight *in vitro* culture.

However, they had a significant decrease in haematocrit and haemoglobin at peak parasitaemia (Figure 2). There was a marginal increase in total bilirubin, creatinine and blood urea (Table 2). After day 16 post-inoculation, the behaviour of these monkeys was similar to the uninfected and healthy control group.

Table 2. Analysis of liver and kidney functions in baboons experimentally infected with *Plasmodium knowlesi* H strain*

Group	*Creatinine (mg/dl)*		*Total bilirubin (mg/dl)*		**Blood urea (mg/dl)**	
	Pre-infected	Infected	Pre-infected	Infected	Pre-infected	Infected
Chronic (n=2)	1.66±0.19	2.80±0.36	0.78±0.16	1.07±0.20	30.25±7.35	45.53±8.61
Acute (n=7)	1.86±0.27	4,05±1.25	0.58±0.15	2.35±0.40	34.29±13.07	120.0±38.37

Samples from infected animals were collected at peak infection. Values are the mean ± SD.

Gross pathology. At necropsy, baboons with severe malaria were remarkably similar in the quality of gross appearance, varying only in the degree of manifestation of pathologic changes. As a general feature, all tissues, particularly the mesentery, were of yellow-tan appearance. Intramural vascular deposition of malaria pigment was a widespread finding. These animals presented with severe acute haemolytic anaemia, severe diffuse pre-hepatic jaundice, hydropericardium, hydroperitoneum and hydrothorax.

Baboons with mild malaria had no comparable alterations indicating severe acute haemolytic crisis. However, mucous membranes were pale with yellowish tinge but not extended to the mesentery.

In all infected baboons, the spleen was highly friable, hemorrhagic, and pronouncedly enlarged with cut surfaces bulging out. Lungs from animals with severe malaria were distended with patchy consolidations and oedematous. In animals with mild malaria, lungs had patchy consolidation and diffuse hyperaemia. The liver of animals with severe malaria was enlarged, firm, and hyperaemic. The lobes were distinct with rounded edges and the gall bladder was distended. Animals with mild malaria had a slightly enlarged liver with whitish streaks. Kidneys showed diffuse hyperaemia and adherence of capsule in animals with severe malaria while kidneys of animals with mild malaria were without alterations. The brain of animals with severe malaria was oedematous, congested on the external surfaces, and blood vessels were prominent. No pathologic changes were observed in the brain of animals with mild infection.

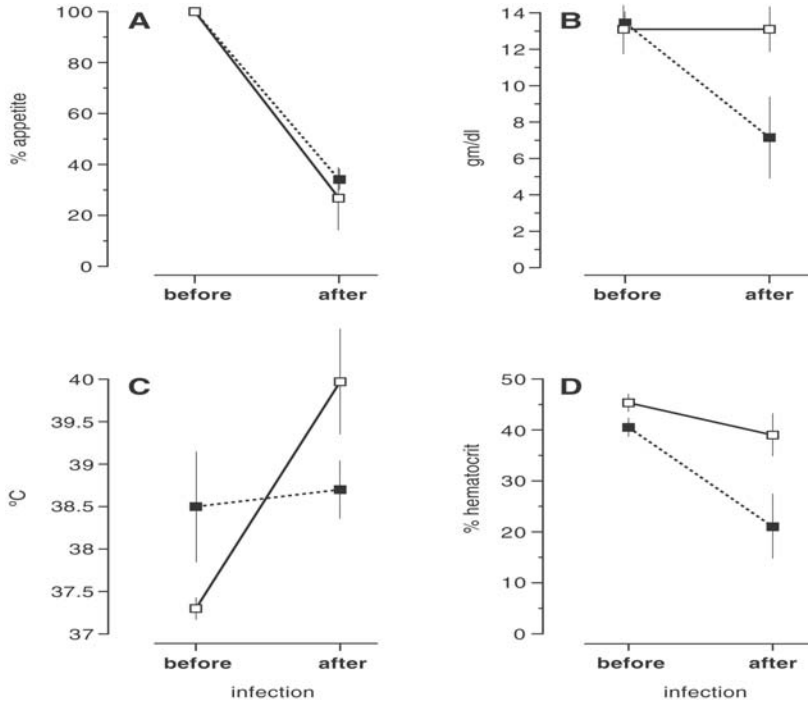

Figure 2. Changes in A, appetite, B, haemoglobin, C, body temperature, and D, haematocrit in *Plasmodium knowlesi*-infected olive baboons with severe (□—□) and less severe (■—■) clinical symptoms. before = before infection; after = during peak infection.

Histopathology. Histopathology showed that in animals with severe malaria, as expected from the high peripheral parasitaemia, blood vessels of all diameters down to the capillaries of all tissues studied were interspersed with parasitised erythrocytes.

The brains of animals with severe malaria showed pronounced oedema, multifocal neuronal degenerations, and mild gliosis (Figures 3A and C), in addition to widespread intravascular schizont-infected erythrocytes in microvessels (Figure 3C). More than 70% of brain microvessels of these animals were filled with aggregates of infected and non-infected erythrocytes which might represent sequestration (Table 3). Infiltrations of lymphocytes and phagocytic cells between endothelial cells of brain blood vessels, as observed in murine cerebral malaria [30] were not encountered in baboons. The brain of baboons with mild malaria was normal (Figure 3B).

In baboons with severe malaria, alveolar septa were increased in diameter due to multifocal to diffuse mononuclear cell infiltration, segmental neutrophil infiltration and interstitial oedema (Figure 4A). Pigment laden desquamated alveolar macrophages were commonly observed. In addition, multifocal neutrophilic granulocytes were found within alveolar lumina. Animals with mild infection showed low congestion of the lung.

The liver of animals with severe malaria showed cloudy to hydropic swelling of hepatocytes (Figure 4B), centrilobular dissociation, and necrosis of hepatocytes. Spaces of Disse were increased in diameter. Presence of hypertrophy pigment laden kupffer cells and pigment laden sinus lining endothelial cells was a common feature in animals with severe malaria but less pronounced in animals mild malaria.

Kidneys from animals with severe malaria were characterised by multifocal interstitial nephritis with infiltration of mononuclear cells (Figure 4C). Glomeruli often showed segmental or diffuse increase in volume of mesangial matrix. Atrophic glomeruli were an occasional finding. No lesions were observed in animals with mild malaria. In all baboons, spleens were characterised by the presence of reactive germinal centres with mantle zones and showed pronounced pigment depositions. Compared with animals with mild malaria, thickening of lienal capsules and trabeculae appeared more pronounced in animals with severe malaria.

Table 3. Erythrocyte aggregation in blood microvessels of *Plasmodium knowlesi*-infected baboon brain

Animal	PAN 1996	PAN 2525	PAN 2497
Parasitaemia profile	Acute	Acute	Chronic
Peripheral parasitaemia*	4,950	1,710	20
Blood microvessels containing**			
Parasitised erythrocytes	86	87	1
Aggregated erythrocytes	80	70	2

*Peripheral parasitaemia was determined as the animal was presented for necropsy. Values are the number of parasitised erythrocytes in 1 x 10^4 peripheral circulation erythrocytes.

**For each baboon, 100 blood micro vessels in the brain were randomly evaluated. Values are percentages of brain micro vessels.

DISCUSSION

In this report, the clinical spectrum and pathology of experimental *P. knowlesi* infection in *P. anubis* is presented for the first time. The disease profile was either severe or mild. Baboons with severe malaria developed multiple system organ dysfunction with cerebral involvement.

In baboons with severe *P. knowlesi* infection, the brain showed considerable pathology including congestion, oedema, neuronal degeneration, prominence of blood vessels, mild gliosis and aggregation of infected and uninfected erythrocytes in cerebral microvessels.

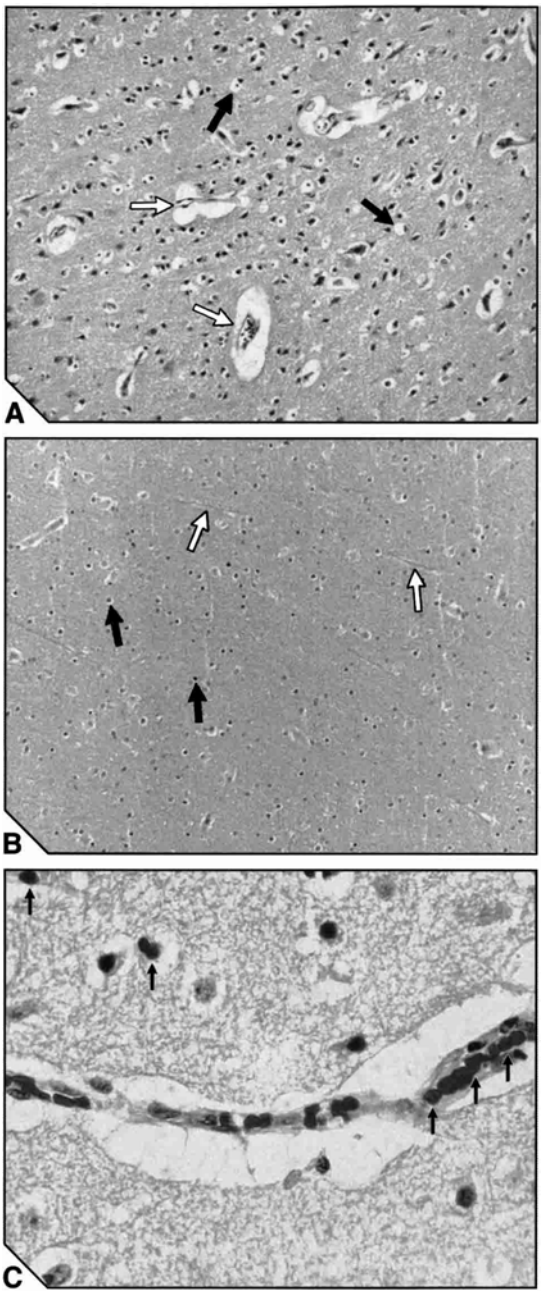

Figure 3. **A**, Overview of the brain of a *Plasmodium knowlesi*-infected olive *baboon* with severe malaria showing cerebral oedema, micro-vacuolisation around nuclei (**black arrows**), and vacuolisation around blood vessels (**white arrows**) (magnification x 200). **B**, Overview of the brain of *P. knowlesi*-infected olive *baboon* with mild malaria. **Black arrows** = micro-vacuolisation around nuclei: **white arrows** = vacuolisation around blood vessels (magnification x 200). **C**. Parasitised (**black arrows**) and non-parasitised erythrocytes in a blood microvessel from the brain of *P. knowlesi* infected olive baboon with severe malaria (magnification x 600). (Haematoxylin and eosin stained.)

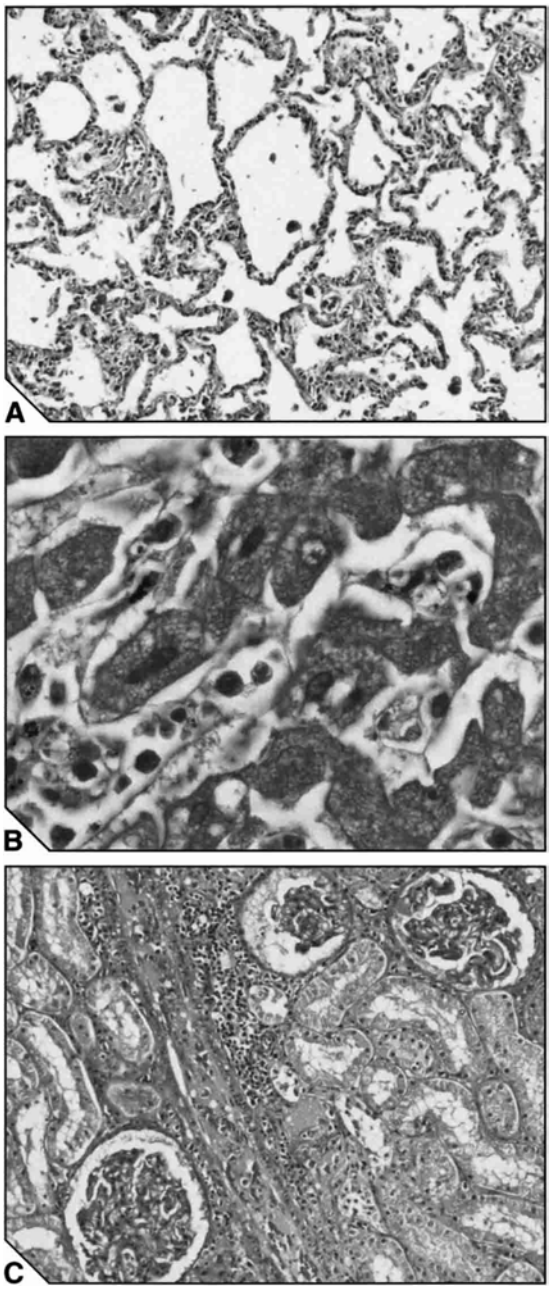

Figure 4. **A**, Lungs of a *Plasmodium knowlesi*-infected olive baboon with severe malaria showing increased cellular infiltration resulting in thickened width of alveolar septa. **B**, Liver of a *P. knowlesi*-infected baboon with severe malaria showing hydropic swelling of hepatocytes, widespread pigment deposition (dark spots) and oedema of the space of Disse. **C**. Kidney of *P. knowlesi*-infected baboon with severe malaria showing multifocal interstitial nephritis with infiltration of mononuclear cells. (Haematoxylin and eosin stained, magnification x 400)

The presence of aggregated erythrocytes in the brain of baboons with severe malaria suggests blockade of cerebral capillaries, which is associated with cerebral malaria in humans [31, 32].

Cerebral malaria is a serious neurologic condition that can lead to coma and death. It is defined as an altered consciousness in a patient who has malaria parasites in the blood and in whom no other cause of altered consciousness can be found [33]. Blockade of brain blood microvessels in *P. falciparum*-infected humans and *P. coatneyi*- or *P. fragile*-infected macaques is mediated through sequestration of knob-forming, mature, parasite-infected erythrocytes [31, 32, 34, 35]. Although knob-formation has not been defined in *P. knowlesi* [36, 37] sequestration of *P. knowlesi* infected erythrocytes might be mediated by schizont-infected cell agglutination variant antigens [38-40]. Further studies to elucidate cerebral phenomena in the brain of *P. knowlesi* infected baboons are required.

In the brain microvessels of baboons with severe malaria, many parasitised erythrocytes were present, but no lymphocytes and phagocytic cells were observed in contact with parasite-infected erythrocytes. This is similar to human cerebral malaria [29, 31, 32]. In contrast, numerous phagocytic cells are encountered in brain microvessels of rhesus monkeys infected with *P. knowlesi* and in rodent cerebral malaria [30, 36]. Together, our data suggest that the cerebral involvement in *P. knowlesi*-infected baboons resembles several aspects of human cerebral malaria.

Papio anubis developed either severe malaria or controlled the parasitaemia, resulting in a mild infection. This is also seen in rhesus monkeys [36, 37, 41-44]. The mechanisms that predispose *P. knowlesi*-infected monkeys to developing either severe or mild infection are unknown. In general, *P. knowlesi* produces a chronic self-regulating infection in the natural host *M. fascicularis.* However, Schmidt and others [45] showed that the course of *P. knowlesi* infection can differ in *M. fascicularis* from different geographic origins. In our study, animals originated from the same area, excluding monkey origin as a factor involved in the different infection outcomes. Our study also shows that inoculum size, age, and sex were not indicative of infection outcome. In humans, basal cytokine levels at the time of infection and host genetic factors are most likely involved in determining *P. falciparum* infection outcome [46, 47]. The precise mechanism that predisposes a dual outcome during malaria warrants further investigation and *P. knowlesi*-infected baboons might be helpful in this.

Our report shows that *P. anubis* is fully susceptible to experimental *P. knowlesi* H strain infection since all infected animals developed patent parasitaemia. The parasitaemia profile observed in the baboons was comparable to those in rhesus monkeys following infection with the same parasites [26] indicating that the virulence of this strain is similar in both monkeys,

although studies were not done in parallel. In contrast to rhesus monkeys, *P. knowlesi*-infected baboons develop clinical symptoms at onset of patent parasitaemia. *Plasmodium knowlesi*-infected rhesus monkeys frequently show minor clinical symptoms until they suddenly collapse due to massive parasitaemia [6, 12, 37, 4l-43] (Langermans JAM and others, unpublished data). One possible explanation is that rhesus monkeys are relatively resistant to endotoxin-like characteristics mediated by malaria parasites [6, 48, 49]. Humans infected with *P. falciparum* also frequently develop clinical symptoms at onset of parasitaemia [8, 33, 46].

Papio anubis was successfully infected with an inoculum size of 1 x 10^4 *P. knowlesi* parasites. This suggests that the infection can be initiated by mosquito bite since a single hepatocyte infected with *Plasmodium* could contain 1 x 10^4 merozoites. Sporozoite induced infection is necessary if the olive baboon is to be used to study *P. knowlesi* liver stage analyses. Moreover, we observed that *P. knowlesi* continued to produce gametocytes after four passages in baboons. The viability of the gametocytes has not yet been characterised.

Infection of olive baboons with *P. knowlesi* provides an additional malaria model that allows for *in vivo* analysis of mechanisms of host response during severe and mild malaria. The use of baboons to study *P. knowlesi* will specifically find relevance in facilities that are not home to other hosts of *P. knowlesi.* These include baboon source countries and primate research facilities with access to baboons. Baboons can also be used to analyse host-parasite interaction of transfected *P. knowlesi* (Ozwara H and others, unpublished data), which is an important tool for converting genome sequence information to medical use. Overall, our findings show that *P. anubis* infected with *P. knowlesi* show various clinical characteristics that are also seen in human malaria including cerebral involvement.

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CHAPTER 8

General discussion

General discussion

Transfection technology for blood stage malaria parasites offers new tools for direct correlation of genotype to phenotype, thus enhancing further understanding of parasite biology. Most of the existing malaria transfection systems rely upon artificial host-parasite combinations with limited capacity for investigating natural host-parasite interaction. Because both natural and experimental hosts for *P. knowlesi* are available, van der Wel and co-workers [1] developed an *in vivo* transfection system for the parasite (Fig. 1). The work described in this thesis was aimed at further development and application of the *P. knowlesi* transfection system. A long term *in vitro* culture protocol for *P. knowlesi* blood stages was developed to facilitate *in vitro* transfection and expand experimental possibilities of the system; an *in vitro* transfection protocol was developed; integration, transient and stable episomal transfection of the parasite was achieved; *in vitro* transfection technology was successfully applied to generate bioactive rhesus IFN-γ expressing *P. knowlesi*, a new approach to generating immunopotentiated malaria parasites and finally, reagents for characterising host responses and a new experimental host were identified, to facilitate *in vivo* phenotypic analysis of transfected parasites. These studies have shown that the *P. knowlesi* transfection system is unique in providing fast and simple *in vitro* genetic manipulations with the opportunity to perform *in vivo* studies in systems highly predictable of the human situation.

In section 1 of this chapter, the impact of long term *in vitro* culture on *P. knowlesi* research is discussed. The second section deliberates on the status of *P. knowlesi* transfection system and section 3 is devoted to technical aspects of the system. Sections 4 and 5 consider transgene expression in *P. knowlesi* and some perspectives on the studies reported in this thesis.

1. Long term *in vitro* culture of *P. knowlesi*.

Continuous and mass *in vitro* culture of *P. knowlesi* is required to reduce the need for resourceful monkeys for simple propagation of parasites for transfection and analysis. In chapter 2, blood stage parasites of two *P. knowlesi* strains, nuri and H, were adapted to long term *in vitro* growth using a simplified protocol. This was done in standard closed-cap culture flasks, under gas conditions of 5% CO_2, 5% O_2 and 90% N_2 and haematocrit of 2.5%. Culture medium was completely changed every other day and fresh erythrocytes were added at a minimum of once a week. These cultures were continuously maintained for over 18 months

without observable changes in parasite morphology and multiplication rates. Culture adapted parasites were originally maintained in medium with rhesus serum, but conditions were improved further to allow the use of human serum and 5% haematocrit. A 200 ml roller bottle culture yielded as high as 1×10^{10} parasites and a 50% parasitaemia was feasible. For the first time, cloning of *P. knowlesi* by limiting dilution was achieved in a 96 well plate. *In vitro* culture adapted parasites were regularly cryopreserved and retrieved for further culturing using a standard protocol [2].

The simplified long term *in vitro* culture system for *P. knowlesi* is in some aspects similar to the *P. falciparum* system [3] because (i) cloning by limiting dilution is feasible in a 96 well plate; (ii) parasite morphology and multiplication rates do not deteriorate after prolonged culturing; (iii) small scale culturing does not require specialised apparatus; (iv) adding fresh culture medium and erythrocytes depends on culture haematocrit, parasitaemia and volume; (v) cultures are readily expanded to provide enough material for transfection (chapter 2) and analysis; (vi) culture medium is supplemented with human serum. Previous protocols for long term *in vitro* culture of *P. knowlesi* blood stage parasites either achieved low parasitaemia in the range of 0.92% and 3.6% [4] or the parasites became morphologically abnormal [5]. Culturing was done using the petri-dish candle jar method [4] and a sophisticated semi-automated tipping culture apparatus [5]. In addition, cloning by limiting dilution was not done. Compared to our procedure, these culturing conditions are insufficient for generating large amounts of parasites for analysis.

Long term *in vitro* cultured *P. knowlesi* parasites were transferred into rhesus monkeys in order to determine their *in vivo* characteristics. Normal parasitaemia profiles were regained after a single passage through an intact rhesus monkey (chapter 2). In addition, parasites from first to third passage *in vivo* retained *in vitro* growth characteristics upon re-introduction into culture, allowing the possibility of generating shuttle parasites for *in vitro* to *in vivo* work.
These features offer an opportunity to analyse adaptations that the parasites may have undergone following adaptation to *in vitro* growth conditions. This is important because one of the potential risks in using parasites maintained *in vitro* is that they may change from the original isolate in both phenotype and genotype. It can be difficult to compensate for the possible impact of these changes on individual host-parasite interaction studies. Prolonged *in vitro* culturing of *P. knowlesi* might lead to alteration of *sicavar* genes [6] leading to loss of sequestration capacity and consequently to reduced virulence in intact monkeys [7].

Adaptation of *P. knowlesi* to long term *in vitro* culture greatly enhances the experimental possibilities of the system. Culture adapted parasites are readily transfected, drug selected and

cloned *in vitro* to provide a homogenous parasite population for molecular analysis (chapters 2 and 3). This has obviated the need for monkeys as parasite donors and recipients of parasites for genetic modification (Fig. 1). Furthermore, *in vivo* cloning of parasites is ethically complex because a large number of monkeys would be required (Fig. 1). Finally, long term *in vitro* drug screening is now possible using culture adapted *P. knowlesi* unlike in the rodent parasite *P. berghei* where long term *in vitro* drug screening is not readily achieved [8]; this approach is currently used for characterising new selectable markers for the *P. knowlesi* transfection system [9].

The current status of the *P. knowlesi* system shows that it is now feasible to culture parasites, use them for transfection and selection, clone the heterogeneously transfected parasites, determine the genotype of the resultant clone and characterise host-parasite interaction in monkeys. As further optimisation of the system takes place, it will be possible to infect mosquitoes (*Anopheles dirus/An. stephensi*) using cultured parasites and sporozoites used to infect hepatocyte cell cultures, which mature to yield invasive merozoites [10]. This requires generation of high gametocyte producing *in vitro* culture adapted parasite lines which is still ongoing. However, the entire life cycle of *P. knowlesi* can be maintained in principle in the laboratory without the need for primates (Fig. 2).

2. Transfection of *P. knowlesi*.

Transfection technology was originally developed in *P. knowlesi* through stable episomal expression of selectable marker genes in a rhesus monkey [1]. Constructs for transfection of *P. berghei* and *P. falciparum* were transfected into *P. knowlesi* to express dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) genes from *Toxoplasma gondii* and *P. berghei*. Studies described in this thesis (chapters 2-4) expanded the system by developing protocols for stable episomal, transient, and site-specific DNA targeting forms of transfection.

2.1. Stable episomal transfection of *P. knowlesi*.

Stable episomal transfection (chapter 1) was achieved in *P. knowlesi* using *P. berghei* and *P. falciparum* transfection plasmids [1]. These plasmids were designed for expression of the *T. gondii* and *P. berghei dhfr-ts* selectable markers under control of *dhfr-ts* and histidine rich proteins 2 and 3 (*hrp2*/*hrp3*) promoter regions from *P. berghei* and *P. falciparum* respectively [11, 12]. In chapter 3, plasmids for selection and expression of green fluorescent protein (GFP) were used to develop an *in vitro* stable episomal transfection protocol in *P. knowlesi* [8, 13]. In these experiments, GFP was expressed under control of the heterologous

pbama-1 and *pbdhfr-ts* UTRs. In order to maintain the plasmids in transfected parasites, drug pressure was continuously applied. These and earlier experiments [1] showed that plasmids for stable episomal transfection can be shuttled between *P. knowlesi* and other malaria parasites. Shuttle plasmids are suitable for comparing the biology of identical genetic components in different parasite/host backgrounds. Thus, the biology of genetic components from *P. berghei* and/or *P. falciparum* can be analysed in *P. knowlesi*. Because stable episomal transfection allows for long-term overexpression of genes [14], *P. falciparum* vaccine candidates can be overexpressed in *P. knowlesi* using shuttle plasmids and the vaccine potential of the candidates tested in monkeys. This would enhance the use of *P. knowlesi* as an adjunct system for testing vaccine candidates.

Recently (Beetsma A., unpublished), constructs were engineered for site-specific gene targeting of *P. falciparumcsp* in *P. knowlesi* under the control of *P. knowlesi* regulatory DNA sequences. The constructs were transfected in *P. knowlesi* as circular plasmids, and expression of the transgenes successfully characterised. Unlike in the *P. falciparum* transfection system [12], the circular plasmids did not integrate into the genome after prolonged culture under drug pressure. Therefore, based on analogy, the mechanisms for plasmid replication and segregation in *P. knowlesi* is similar to *P. berghei* [15], i.e., plasmids are maintained in monomeric form (chapter 3) and do not integrate into the genome.

2.2. Transient transfection of *P. knowlesi*.

In chapter 3, transient transfection technology was developed for blood stages of *P. knowlesi* using episomes designed for the *P. berghei* system [16]. The technology was used to characterise heterologous promoter activity of the *pbama-1* and *pbef-1*α 5' regulatory DNA sequences and to optimise transfection conditions for *P. knowlesi*. Using the promoters, expression of luciferase (an exogenous reporter) was achieved within 24h. This shows that it is now possible to ascertain rapidly expression of transgenes and activity of new heterologous regulatory DNA sequences in *P. knowlesi*. Although there are some differences in transient transfection methodologies between the various *Plasmodium* systems, the DNA constructs used have the same configuration (chapter 3; [14]).

Very little is known about how gene expression is developmentally controlled in malaria parasites. Transient transfection provides the opportunity to identify genetic elements that control gene expression in *P. knowlesi*, as was done for *P. falciparum* and *P. chabaudi* [17, 18].

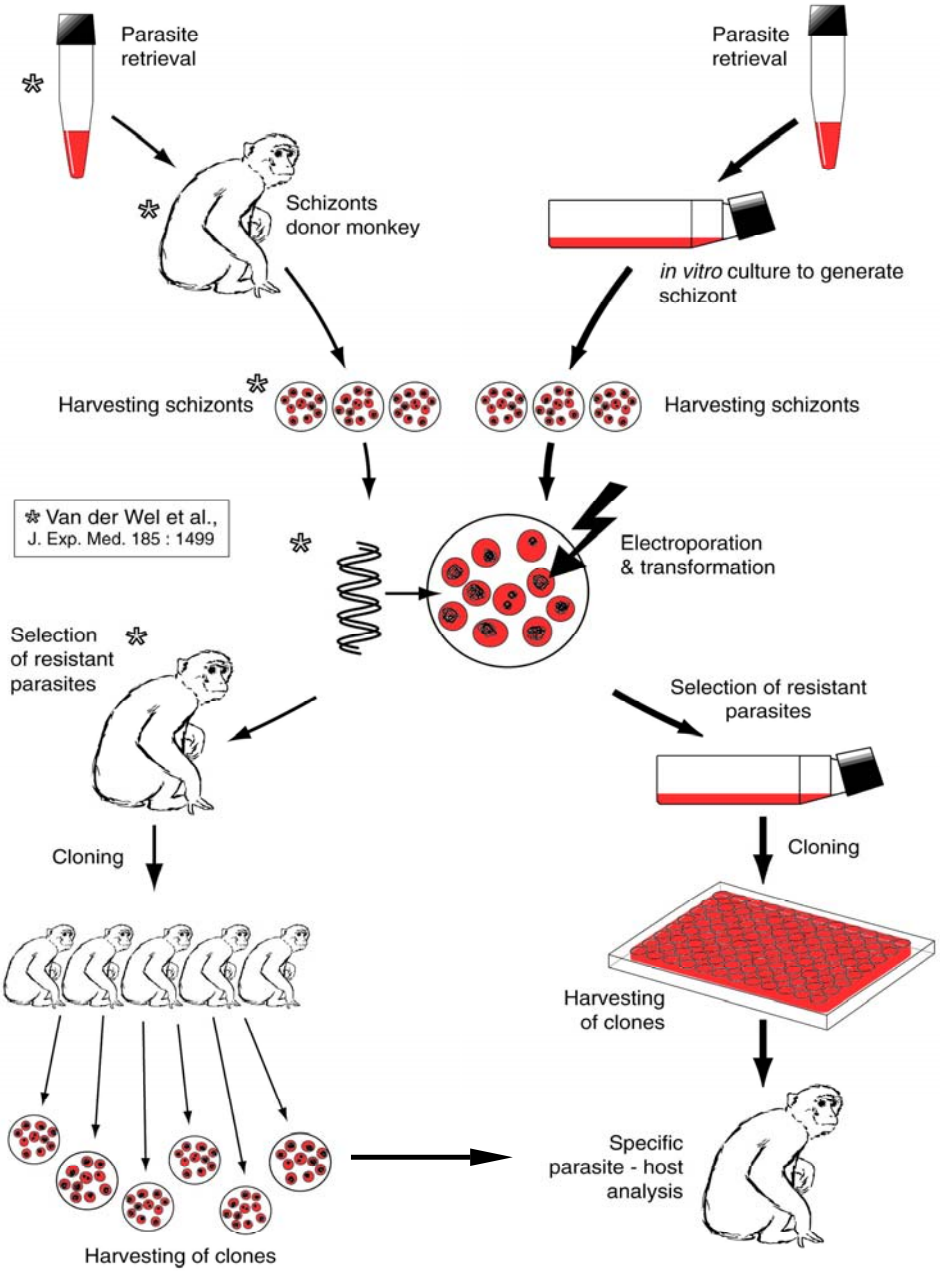

FIGURE 1. *In vitro* and *in vivo* transfection of *P. knowlesi*. In order to develop an *in vivo* transfection protocol for *P. knowlesi* blood stages [1], cryopreserved parasites were retrieved and used to infect a donor rhesus monkey. Blood was collected from the monkey when erythrocytes were infected with mature schizonts. The schizonts were mixed with transfection DNA and electroporated. The electroporated mixture was injected into a recipient monkey. Pyrimethamine was orally applied to the monkey for selecting drug resistant (transfected) parasites. *In vivo* cloning of transfected parasites would require an unacceptably high number of monkeys and was not persued. In order to develop an *in vitro* transfection protocol (the right half of the diagram), cryo-preserved blood stage parasites were retrieved and adapted to long term *in vitro* culture as described in chapter 2. Mature schizonts were harvested, mixed with transfection DNA and electroporated. These were transferred into *in vitro* culture system for overnight culture. Pyrimethamine was added to the culture for selection of drug resistant parasites. Where necessary, transfected parasites were cloned in a 96 well plate by limiting dilution to obtain parasites with the correct genotype. This enables further analysis of selected clones in the monkey. ☆, stages that were successfully done during *in vivo* transfection.

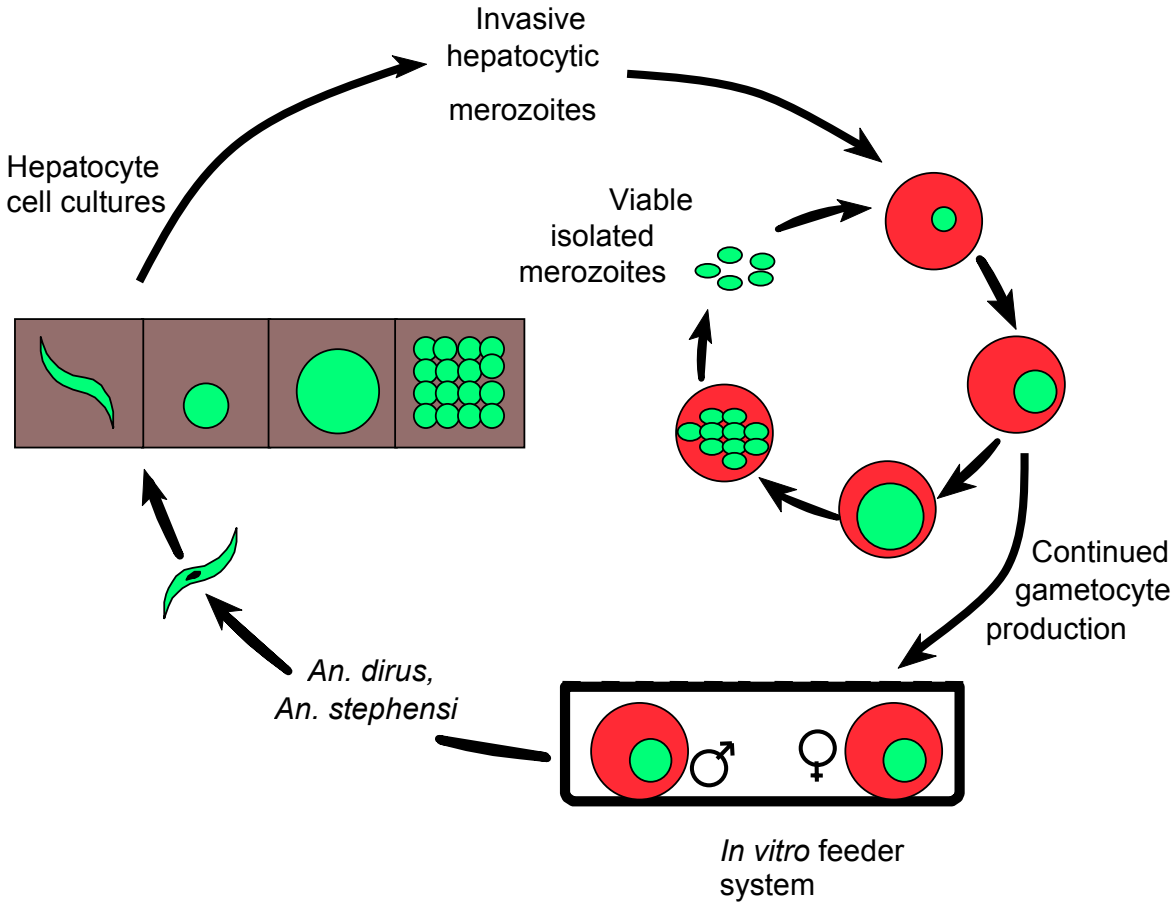

Figure 2. Complete primate free life cycle of *P. knowlesi*. In principle, the entire life cycle of *P. knowlesi* can be maintained in the laboratory without the need for primates. It is now feasible to culture, transfect and select drug resistant parasites *in vitro*. Once high gametocyte producing parasite lines have been established, feeder systems can be used to infect mosquitoes (*Anopheles dirus/An. stephensi*) with transfected parasites. Mature sporozoites would then be isolated and used to infect hepatocyte cultures *in vitro*. Exo-erythrocytic stages would develop in the hepatocytes, ultimately yielding merozoites to continue the cycle. Apart from transfection, this system can also be used to generate viable free merozoites for studying basic parasite cell biology mechanisms such as cell-cell interactions.

These studies showed that different regions of *Plasmodium* promoters were associated with controlling varying levels of expression, reminiscent of regulatory elements in other eukaryotes. Transient transfections can also be used to temporarily (over)express genes that are otherwise toxic to *Plasmodium* [14].

2.3. Site-specific DNA targeting in *P. knowlesi*.

Site-specific DNA targeting (gene targeting) relies on the homologous recombination between two copies of a sequence, one present in the genome (target DNA sequence) and the other in the incoming DNA (targeting construct) [19]. Gene targeting was originally achieved

in *P. knowlesi* (chapter 2) using a replacement type of construct (see section 3 below). The construct comprised 1 kb of the 5' and 3' homologous *csp* gene sequences disrupted by *tgdhfr-ts* selectable marker expression cassette, to allow for integration by double crossover mechanisms (chapter 2). *In vitro* transfection using the linearised construct generated CSP knockout parasites (chapter 2). In these experiments and similar ones that followed (Table 1), non homologous DNA recombination was not detected in *P. knowlesi*, suggesting that gene targeting in this parasite occurs only by homologous recombination (chapters 3 and 5). Evidently, non homologous recombination is uncommon in *Plasmodium* [14, 20].

Recently, integration into the *P. knowlesi* genome was achieved by single crossover mechanisms, using an insertion type plasmid ([9]; section 3 below). The plasmid was restriction digested in the target sequence prior to transfection, similar to the *P. berghei* transfection system [14]. Gene targeting experiments in *P. knowlesi* show that the transfection construct and selection procedures are identical to the *P. berghei* system (chapter 2;[14]). In *P. falciparum* both replacement and insertional constructs are transfected as circular plasmids [17, 21]. Furthermore, the procedure for integrating replacement constructs into *P. falciparum* requires both negative and positive selectable markers [21]. These studies show that the mechanisms for DNA integration into the *P. knowlesi* and *P. berghei* genomes are similar, but different from *P. falciparum*.

3. Parameters for *P. knowlesi* transfection.

The application of a transfection procedure is significantly influenced by the type of transfection construct, the regulatory DNA sequences driving gene expression and the nature of selectable marker(s) used. New construct designs, regulatory DNA sequences and selectable markers are continuously being identified and incorporated into malaria transfection systems. The status of these parameters in the *P. knowlesi* transfection system is elaborated in this section.

Transfection Constructs

Vectors for the transfection of *P. knowlesi* are based on a bacterial plasmid backbone for propagation in *Escherichia coli*. They are assembled in a cassette-like structure so that DNA sequences can be exchanged easily (chapters 3-5; [8]).

Different DNA constructs are used in genetically modifying *P. knowlesi*, depending on the type of transfection. Expression cassettes for transient transfection constructs (chapter 3), consist of the gene to be expressed flanked by 5' and 3' UTRs of *Plasmodium* origin. The

cassette is cloned into a high copy number plasmid for cloning in *E. coli*. Transient transfection constructs are electroporated into *P. knowlesi* as circular plasmids. The design of constructs for transient transfection is remarkably similar in all *Plasmodium* systems [14].

DNA constructs for stable episomal transfection of *P. knowlesi* contain a selection cassette and an adjacent expression cassette cloned either in a head to head or a head to tail orientation. The expression cassette contains the gene of interest. The genes in both cassettes are flanked by UTRs of *Plasmodium* origin (chapters 4, and 5; [1]). Circular constructs are used for stable episomal transfection of *P. knowlesi* (chapters 4-5; [1]), similar to other transfection systems [14, 22].

Replacement DNA constructs are based on vectors for selection and integration. They consist of homologous DNA targeting sequences disrupted by a selection cassette. In addition, an expression cassette can be cloned immediately downstream or upstream to the selection cassette. These constructs are designed for site-specific integration by double crossover mechanism (chapters 3 and 5). In *P. knowlesi*, the minimum size of homologous DNA target sequence used was 0.6 kb. The gene for TRAP was knocked out using 1 kb and 0.6 kb 5' and 3' homologous DNA target sequences respectively (Ozwara *et al*., unpublished). Current replacement constructs contain 1 kb of 5' and 3' of homologous DNA targeting sequences as the standard sizes for site-specific recombination in *P. knowlesi*. Prior to electroporation, replacement constructs are linearised at two sites to extract bacterial DNA sequences (chapters 2 and 5), similar to *P. berghei* [19]. However, in *P. falciparum*, circular constructs are used [21].

Insertional DNA constructs are used for site-specific integration of DNA into *P. knowlesi* by single crossover mechanism [9]. The constructs are similar to replacement constructs, except that they have a single internal site-specific homologous DNA segment for gene targeting [14]. The construct is linearised within the targeting sequence, prior to transfection [9]. The structure and processing of constructs for integrating DNA into *P. knowlesi*, *P. berghei* and *P. yoelii* by single cross-over mechanism are similar [9, 14, 22]). Thus far, the structure of *P. knowlesi* and *P. berghei* transfection constructs is identical.

Selectable markers

Transfection was first achieved in *P. knowlesi* using constructs containing *P. berghei* and *T. gondii dhfr-ts* (*tgdhfr-ts*) selectable marker genes [1]. These experiments showed that when *P. knowlesi* parasites were co-transfected with both constructs, the *tgdhfr-ts* construct was

preferentially selected *in vivo* [1]. Subsequently, *tgdhfr-ts* was used as the selectable marker in all stable transfection studies reported in this thesis (chapters 2 to 4).

Although the haploid genome of blood stage *P. knowlesi* parasites has enabled the use of a single selectable marker for stable transfection, additional selectable markers would be required for further genetic manipulation of the same parasite. This may be required to target more than one gene in the same parasite, and to complement knockout parasites. Long term *in vitro* drug screening is now possible using culture adapted *P. knowlesi*, facilitating the screening of new selectable markers for the *P. knowlesi* system. Consequently, development of new selectable markers for stable transfection of *P. knowlesi* is underway (Table 1). Already, a new marker based on *dhfr-ts* genes from human (*hudhfr-ts* [24], flanked by *P. berghei* regulatory DNA sequences has been identified [9]. Other recently identified markers include basticidine S deaminase (*bsd*) from *Aspergillus terreus*, neomycin phosphotransferase II (*neo*) from transposon Tn 5, and viral thymidine kinase [9, 21, 24].

Regulatory DNA sequences

Regulatory DNA sequences for *P. knowlesi* transfection (Table 1) comprises the 5' region that provides both the promoter and the 5' UTR and the 3' region containing the 3' UTR and the transcriptional stop signals. Initial transfection of *P. knowlesi* was achieved using regulatory DNA sequences from *P. falciparum* and *P. berghei* [1]. This study provided the first evidence of heterologous promoter (regulatory DNA sequence) activity in *P. knowlesi*. The use of heterologous regulatory DNA sequences offers advantages for site-specific gene targeting. Integration into the locus of the regulatory sequence might be reduced, and in the process improve gene targeting into the desired loci. In addition, using heterologous regulatory DNA sequences in transfection vectors facilitates generation of shuttle vectors for use across *Plasmodium*.

The experiments in chapter 3 showed that the *pbef-1α* 5' UTR can function in *P. knowlesi*. The *ef-1α* 5' UTR consistently yielded high luciferase activity, suggesting that it is suitable for driving over-expression of genes in *P. knowlesi*. Furthermore, the *pbef-1α* intergenic region has promoter activity in two directions [25], hence it can also be used to drive simultaneous expression of two genes, for instance the gene for the selectable marker, and the gene under study. This could have a significant improvement in the design of constructs for stable transfection of *P. berghei* and *P. knowlesi*, by reducing the size of the constructs.

The *pbama-1*, a stage specific promoter, was shown to work in the same way in *P. knowlesi* as in the homologous system (chapter 2). This indicated for the first time that the

activity of *P. berghei* regulatory DNA sequences in *P. knowlesi* is similar to the homologous system.

4. Transgene expression in *P. knowlesi.*

Transgene expression is a means by which critical aspects of *Plasmodium* biology such as mechanisms underlying drug resistance [26] can be investigated. Stable and transient transfection protocols for *P. knowlesi* have provided the fundamental tools for transgene expression. The two procedures have been used to express four categories of transgenes in *P. knowlesi* (Table 1). These are IFN-γ (chapters 4 and 6), reporter molecules (chapter 3), selectable markers (chapters 2 to 4; [9]) and *P. falciparum* antigen (PFCSP) (A. Beetsma *et al*., unpublished). The categories will be elaborated below, except selectable markers, which were covered in section 3.

Table 1. Parameters for transfection of *P. knowlesi*

Parameter	DNA sequences	References
5' UTRs	*pbdhfr-ts*	[1]
	pbama-1	Chapter 3
	pbef-1α	Chapter 3
	pfhrp3	[1]
	pfhsp86	[9]
	pfcam	[9]
	tgdhfr	[1]
3'UTRs	*pbdhfr-ts*	[1]
	pbs21	A. van der Wel *et al.*, unpublished
	pfhrp2	[1]
	tgdhfr	[1]
Positive selectable markers	*tgdhfr-ts*	[1]
	neo	[9]
	bsd	[9]
	hudhfr-ts	[9]
	pbdhfr-ts	[1]
Negative selectable markers	Thymidine kinase	[9]
Disrupted loci	*csp*	Chapter 2
	trap	H. Ozwara *et al.*, unpublished
	140 kD	Chapter 4
	172 kD	A. van der Wel *et al*., unpublished
	ssu	[9]
Transgene expression	Rhesus IFN-γ	Chapter 4
	Mouse IFN-γ	H. Ozwara, unpublished
	PfAMA-1	A. van der Wel *et al*., unpublished
	PfCSP	A. Beetsma *et al*., unpublished
Reporter gene expression	GFP	Chapter 3
	Luciferase	Chapter 3

Stable transfection procedures for *P. knowlesi* (chapter 2) enabled us to test expression of host cytokines in malaria parasites by generating rhesus IFN-γ-expressing parasites (chapters 4 and 6). Immunoassays demonstrated that transfected *P. knowlesi* released bioactive rhesus IFN-γ. A similar strategy was also used to express mouse IFN-γ in *P. berghei* and *P. knowlesi* (H. Ozwara *et al*., unpublished). These experiments showed for the first time that malaria parasites have the capacity to process higher mammalian proteins correctly. This offers possibilities for investigating the expression of other host immunomodulatory molecules in order to define those that sufficiently immunopotentiate malaria parasites.

In chapter 3, luciferase and GFP reporter molecules were expressed in *P. knowlesi*. Expression of reporters provides an effective means to study both gene expression and protein trafficking in tagging experiments [27, 28]. For instance, parasite molecules can be fused to GFP for elucidating trafficking pathways and their signals [8]. In addition, the ability to easily harvest GFP expressing parasites by fluorescence activated cell sorting as described for *P. berghei* [29] and *P. yoelii* [22] could be adapted to *P. knowlesi* for harvesting specific parasite stages for further analysis.

Transfection of malaria parasites provides a powerful tool for determining gene functions, thus enhancing understanding of parasite biology. Understanding the function of genes of malaria parasite vaccine candidate molecules will facilitate rational design of new vaccines. In recent experiments, A. Beetsma *et al*., (unpublished) successfully expressed *P. falciparum* CSP in *P. knowlesi* sporozoites. This indicates that *P. knowlesi* homologues of vaccine candidate molecules can be knocked out and complemented with molecules from clinically relevant *Plasmodium* species. Phenotypic analysis of the transgenic parasites would enhance the understanding of the function of the vaccine candidate molecule. Furthermore, in the *P. knowlesi*/primate system, host responses to the transgenic vaccine candidate molecule can be determined under natural-chronic and acute-experimental host-parasite interface (chapter 7), which enhances understanding of the vaccine potential of the molecule.

In chapter 3, the *pbama-1* and *pbef-1α* heterologous promoters were identified to function in *P. knowlesi*. The *pbef-1α* promoter drives expression in all parasite blood stages except mature schizonts and the *pbama-1* is only active in mature schizonts [25, 30]. This indicates that the two promoters combined can be used to express transgenes virtually in all blood stages of *P. knowlesi*. Therefore, in principle, genes of stage specific malaria vaccine candidates such as the AMA-1 can be episomally expressed under the *pbef-1α* promoter in all blood stages. If successful, this would over-express the vaccine candidate, possibly generating immunopotentiated parasites.

5. Perspectives.

Studies reported in this thesis have significantly developed *P. knowlesi* as a versatile transfection system. In outlook, certain questions that are relevant to the objectives of the thesis need to be studied. This section outlines some of the issues.

The advent of a simple long term *in vitro* culture system for blood stages has expanded experimental possibilities of the system (chapter 2). However, three aspects of the culture system require further optimisation. Monkeys are still required as erythrocyte-donors for *P. knowlesi* culturing. Adaptation to culturing in human erythrocytes would be more ideal. Failure to achieve this, the parasites will only be maintained in facilities accessible to monkey erythrocytes. Secondly, conditions for inducing *in vitro* gametocyte production are still sub-optimal because long term cultured parasites show a significant decline in gametocyte production. Gametocytes are required for transmission to mosquitoes. In the presence of infectious gametocytes, sporozoites from mosquitoes fed on culture adapted parasites can be used to infect rhesus or human hepatocytes *in vitro* [31], thereby entirely completing the *P. knowlesi* cycle in the laboratory without the need for monkeys (Fig. 2). Finally, although it is possible to adapt *P. knowlesi* to culture in serum free medium (H. Ozwara, unpublished), the potential of this system has not been fully achieved. Using serum free medium would significantly reduce the cost of culturing *P. knowlesi*.

The transfection efficiency of the system is estimated at 1 in 10^6 (Ozwara *et al*., unpublished). Higher transfection efficiencies are required for developing a more robust transfection system. New mechanisms for delivering DNA into the parasites must be tested to identify those that enhance transfection efficiency. One possible approach involves the use of polyamidoamine dendrimers [32]. Using this procedure, the efficiency for transfecting *P. falciparum* was estimated at 1 in 10. Conditions for intracellular transfection of *P. knowlesi* have also not been optimised. These transfection conditions would deliver DNA into all blood developmental stages thus improving transfection efficiency in *P. knowlesi*.

Although the genome of *P. knowlesi* has been sequenced [33], the sequence requires assembling and annotation. This would facilitate DNA microarray studies to identify molecules of interest for isolation of drug and vaccine candidate molecules. The current status of *P. knowlesi* transfection system requires more tools to facilitate identification of more genes of interest from the *P. knowlesi* genome. For example, random insertional mutagenesis system has not been developed for *P. knowlesi* transfection system. The technique can be used to tag the *P. knowlesi* genome. In this manner, the genes whose inactivation can be specifically selected for can be isolated and identified. Transposable

elements such as mariner from Drosophila [34] which shows a simple requirement for TA dinucleotide at its target site could be used in developing an insertional mutagenesis system for *P. knowlesi*.

In vivo studies (chapter 6) show that although 'vaccination' with IFN-γ expressing *P. knowlesi* mediates a delay in the onset of challenge *P. knowlesi* infection, it does not prevent development of a fulminant infection. Other studies [35, 36], showing that more than one cytokine is necessary for protection against malaria. It is necessary to screen for a combination of cytokines that generate immunopotentiated malaria parasites *in vivo*. A combination of cytokines that enhance antigen presentation combined with those that generate TH1 responses is attractive because studies have shown that malaria infection modulates antigen presenting cell functions [37].

CONCLUSION

As a result of the studies described in this thesis, the *P. knowlesi* transfection system is now a more versatile resource for understanding parasite biology. The parasite is phylogenetically close to *P. vivax* and a number of protein homologues exist between the two parasites, allowing determination of function by analogy. Long term *in vitro* cultured parasites expand experimental possibilities for transfection and general understanding of parasite cell biology. *In vitro* and *in vivo* transfection protocols minimise the use of monkeys for the simple process of generating parasites, while retaining the possibility of using either of the protocols as need arises. The natural chronic and experimental acute vertebrate hosts of *P. knowlesi* ensure that host responses to transfected parasites can be determined either to understand mechanisms of protection in a chronic host, or to determine the vaccine potential of a transfection approach in an acute host. Expression of host cytokines such as IFN-γ offers possibilities for using transfection as a tool for developing attenuated and immunopotentiated malaria vaccines. The genome of *P. knowlesi* has been sequenced, indicating that genes for all possible drug and vaccine candidates are accessible. Transfection should play a role in deciphering the function of these molecules and by analogy, similar molecules could be isolated from human malaria parasites and expressed in *P. knowlesi* using shuttle vectors. Subsequently, the drug and/or vaccine potential of selected candidates can be determined using *in vitro* and *in vivo* systems. As a parasite of primates that also infects humans, *P. knowlesi* currently is the only malaria experimental and transfection system available to malaria researchers that combines the benefits of a simple long term *in vitro* culture system

and analysis of host-parasite interaction in natural and experimental host systems similar to human.

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Samenvatting

Malaria is een ziekte die voornamelijk voorkomt bij mensen die leven in ontwikkelingslanden in de tropische en sub-tropische gebieden van de wereld. Malaria infecties worden in het algemeen behandeld met geneesmiddelen als chloroquine. De snelle verspreiding van chloroquine resistentie en het ontbreken van nieuw ontwikkelde goedkope anti-malaria medicijnen onderstreept de noodzaak voor een malaria vaccin. Vaccinatie is de meest kost effectieve manier om malaria te voorkomen, een ziekte die overwegend voorkomt in landen waar budgetten voor gezondheidsbewaking zeer minimaal zijn. Tot nu toe waren er slechts beperkte mogelijkheden tot het uitvoerig bestuderen van structuur/funktie relaties van malaria vaccin kandidaten om inzicht te krijgen in het funktioneren van kandidaat moleculen, een noodzaak voor een rationeel ontwerp van vaccins. Met de komst van transfectie technologieën voor malaria bloedstadium parasieten, zijn er betere methoden beschikbaar gekomen om de funktie van moleculen te bepalen, door parasieten te genereren die het eiwit van onderzoek niet tot expressie brengen, of die gewijzigde vormen van het eiwit tot expressie brengen, en het fenotype van deze parasieten te bestuderen. Bovendien is het mogelijk om immuno-modulerende eiwitten die reaktiviteit van het immuunsysteem bevorderen tot expressie te brengen. Het is dan ook misschien wel mogelijk om uiteindelijk geattenueerde parasieten te ontwikkelen die sterk beschermende afweerreakties induceren en als zodanig gebruikt kunnen worden als levend vaccin. Deze strategie is wellicht veel effectiever dan de subunit vaccin strategie die tot op heden wordt gevolgd. In de studies beschreven in dit proefschrift, zijn protocollen ontwikkeld voor het genereren van malaria parasieten die bepaalde eiwitten niet meer tot expressie brengen, die veranderde vormen van die eiwitten van andere origine tot expressie brengen, en wordt het fenotype van deze mutante parasieten bestudeerd.

Hoofdstuk 1: Inleiding

Malaria transfectie is een robuust genetisch hulpmiddel voor onderzoek naar de funktie van genen. Transfectie systemen zijn ontwikkeld voor de knaagdier malaria parasieten *P. berghei* en *P. yoelii*, de humane malaria parasiet *P. falciparum* en de primaten malaria parasietsoorten *P. cynomolgi* en *P. knowlesi*. Transfectie systemen voor *P. berghei* en *P. falciparum* zijn verder ontwikkeld dan voor de andere malaria transfectie systemen. Het *P. knowlesi*/primaten model is echter het enige experimentele malaria systeem dat de mogelijkheid biedt tot het bestuderen van gastheer-parasiet interacties in de experimentele en natuurlijke gastheer. Om deze reden is verdere ontwikkeling van het *P. knowlesi* systeem

belangrijk. In de introductie van dit proefschrift wordt het malaria probleem beschreven, de verschillende modellen voor transfectie van malaria en het *P. knowlesi* malaria model.

Hoofdstuk 2: *In vitro* en *in vivo* transfectie systeem voor *P. knowlesi*

Om de basis biologie van een parasiet en interactie tussen gastheer en parasiet te kunnen bestuderen is een combinatie van *in vitro* en *in vivo* studies van groot belang. In dit hoofdstuk wordt de ontwikkeling van langlopende *in vitro* kweeksystemen voor *P. knowlesi* beschreven. Bloedstadium parasieten werden aan kweek geadapteerd. *In vitro* geadapteerde parasieten konden weer gemakkelijk *in vivo* groeien en hadden na een enkele passage in een intacte resus aap wildtype karakteristieken terug verkregen. Protocollen voor *in vitro* transfectie en transfectie waarbij het ingebrachte DNA integreert in het genoom van de parasiet werden ontwikkeld gebruik makend van *in vivo* verkregen of *in vitro* geadapteerde *P. knowlesi* parasieten. *P. knowlesi* circumsporozoite eiwit (CSP) knockout parasieten werden gegenereerd door dubbel crossover mechanismen. *In vitro* getransfecteerde en gekloneerde CSP knockout parasieten werden binnen 18 dagen verkregen. Microscopische evaluatie van zich ontwikkelende oocysten in muskieten die CSP knockout parasieten hadden opgenomen bevestigden de afwezigheid van de vorming van sporozoiten, zoals eerder was geobserveerd in *P. berghei* knockout parasieten. Op dit moment is het *P. knowlesi* model het enige malaria systeem dat snelle en precieze dubbel crossover genetische manipulatie procedures combineert met complete *in vitro* en *in vivo* mogelijkheden. Dit biedt de mogelijkheid tot volledige analyse van *P. knowlesi* genotype-fenotype relaties en gastheer-parasiet interacties in een systeem dat nauw is verwant aan de mens.

Hoofdstuk 3: Heterologe promoter activiteit en transiënte transfectie in *P. knowlesi*

De transiënte transfectie voor malaria parasieten biedt de mogelijkheid tot precieze en snelle bestudering van de controle van genexpressie, een aanpak die cruciaal is om te kunnen begrijpen hoe genexpressie plaatsvindt tijdens de ontwikkeling van *Plasmodium* parasieten. Het doel van de studie zoals beschreven in hoofdstuk 3 was om heterologe promoter aktiviteit te karakteriseren na transiënte en stabiele transfectie van de apen malaria parasiet *P. knowlesi*. Het gebruik van heterologe promoters voor de transfectie van *Plasmodium* heeft het voordeel dat tijdens integratie experimenten gerichte integratie van het construct in de locus van interesse vergemakkelijkt wordt. Expressie van luciferase en green fluorescent protein (GFP), reporter moleculen die hun nut bewezen hebben als markers voor genexpressie, werd ook getest in *P. knowlesi*. Expressie van luciferase werd bereikt in *P. knowlesi* door gebruik

te maken van de stadium specifieke promoter van *P. berghei* apical membrane antigen-1 (*pbama-1*) en de constitutieve promoters van *P. berghei* elongation factor-1 alpha (*pbef-1α)* and *P. berghei* dihydrofolate reductase-thymidylate synthase (*pbdhfr-ts)*. Het is van belang regulatie van genexpressie onder controle van heterologe promoter gebieden te analyseren om te bepalen of de regulatie door heterologe promoters op dezelfde manier plaatsvindt als expressie in het homologe systeem. Stabiele integratie experimenten waarbij GFP tot expressie werd gebracht lieten zien dat de *pbama-1* promoter strikt stadium-specifieke regulatie van expressie in *P. knowlesi* behoudt, op dezelfde wijze als in het homologe systeem. Deze studie heeft bovendien de *pbef-1α* promoter geïdentificeerd als een sterke heterologe promoter die goed geschikt is voor integratie en over-expressie van transgenen in *P. knowlesi* bloedstadium parasieten. De hulpmiddelen hier beschreven zullen gebruikt worden om het *P. knowlesi* systeem verder te ontwikkelen om de voordelen verbonden aan dit veelzijdige transfectie systeem ten volle te kunnen benutten.

Hoofdstuk 4: Getransfecteerde *P. knowlesi* parasieten produceren bio-actief gastheer IFN-γ

Het is aangetoond dat recombinant pathogene micro-organismen die gastheer cytokines als IFN-γ tot expressie brengen immuunresponsen kunnen moduleren. Deze immunomodulatie leidde tot een verhoogde mate van bescherming. De expressie van gastheer cytokines door malaria parasieten is echter nog niet bestudeerd. De ontwikkeling van transfectie technologie voor malaria parasieten biedt nu de mogelijkheid tot het tot expressie brengen van gastheer eiwitten zoals cytokines in *Plasmodium*. Indien het mogelijk blijkt gastheer cytokines tot expressie te brengen in malaria parasieten, zou dit de mogelijkheid bieden het vaccin potentieel van een immunomodulerende aanpak te onderzoeken door parasieten te maken die een versterkende werking hebben op het afweersysteem van de gastheer. In dit hoofdstuk wordt het recentelijk ontwikkelde transfectie protocol voor *P. knowlesi* toegepast om de mogelijkheden voor het tot expressie brengen van gastheer cytokines in malaria parasieten te onderzoeken. Langlopende, aan *in vitro* kweek aangepaste *P. knowlesi* bloedstadium parasieten werden getransfecteerd met DNA constructen voor het tot expressie brengen van resusaap (*Macaca mulatta*) IFN-γ onder controle van de *pbama-1* promoter. De getransfecteerde parasieten bleken IFN-γ te produceren. *In vitro* analyse via inhibitie van het celdodend effect van virussen en activatie van *M. mulatta* perifere blood cellen liet zien dat het door de parasiet geproduceerde IFN-γ bio-actief was. Dit is de eerste keer dat aangetoond

is dat het mogelijk is om malaria parasieten te genereren die bio-actieve immunomodulerende gastheer cytokines tot expressie brengen. De expressie van gastheer cytokines door malaria parasieten, zoals in deze studie is aangetoond, biedt een nieuw perspectief om onderzoek te doen naar de mogelijkheid levend geattenueerde malaria vaccins te ontwikkelen die mogelijk een betere afweerreaktie teweeg brengen dan een natuurlijke infectie.

Hoofdstuk 5: Humane monoklonale antilichamen kruisreaktief met primaten antigenen

P. knowlesi is een apen malaria parasiet. Omdat humane en primaten afweersystemen grotendeels vergelijkbaar zijn, reageert een aanzienlijk deel van monoklonale antilichamen, gericht tegen eiwitten van het humane immuunsysteem met gelijkwaardige primaten eiwitten. Er zijn echter slechts weinig van dergelijke monoklonale antilichamen gekarakteriseerd voor hun toepasbaarheid in studies naar het afweer systeem van diverse niet-humane primaten. Hoofdstuk 5 heeft als doel de lijst van antilichamen, beschikbaar voor de karkterisering van het immuunsysteem van de chimpanzee, de resusaap en het doodshoofdaapje, uit te breiden. In totaal 161 monoklonale antilichamen tegen humane T-cel antigenen en cytokine receptoren werden met behulp van FACS getest op perifere bloed mononucleaire cellen van de chimpanzee, de resusaap en het doodshoofdaapje. Deze primaten worden regelmatig gebruikt als experimentele gastheer van malaria. Onze studies identificeerden 105, 73 en 68 nieuwe monoklonale antilichamen die kruisreageerden met de immuunsystemen van respectievelijk chimpanzee, de resusaap en het doodshoofdaapje. De monoklonale antilichamen tegen het systeem van de resusaap zijn een aanvulling op reeds beschikbare antilichamen voor het definiëren van gastheer responsen in de resusapen na infectie met *P. knowlesi*.

Hoofdstuk 6: De respons van resusapen op expressie van IFN-γ door *P. knowlesi*

Het *P. knowlesi* transfectie systeem biedt de mogelijkheid tot analyse van gastheer-parasiet interacties van getransfecteerde parasieten in niet-humane primaten. In hoofdstuk 6 worden gastheer responsen tegen de in hoofdstuk 4 beschreven parasieten die IFN-γ tot expressie brengen gekarakteriseerd. Deze experimenten werden ontworpen om met metingen aan klinische en immuunsysteem parameters de veiligheid en efficacy van infecties met parasieten die cytokine tot expressie brengen in resusapen te bepalen. Bovendien kon de bioaktiviteit van door parasieten geproduceerd IFN-γ worden bepaald. Alle geïnfecteerde dieren ontwikkelden een milde parasitaemie waarvan de piek significant vroeger plaatsvond dan in de controle dieren. Analyse van de klinische chemie en haematologische data van deze apen lieten geen abnormaliteiten aan vitale orgaanfunkties zien. Dit suggereert dat de

hoeveelheid IFN-γ die door de parasiet werd uitgescheiden goed getolereerd werd en dat deze parasieten de veiligheid van de aap niet in gevaar hadden gebracht. Immunologische analyse liet een tweeëneenhalf tot vijfvoudige toename in γδT-cellen zien in dieren die waren geinfecteerd met *P. knowlesi* parasieten die IFN-γ tot expressie brachten in vergelijking met een één tot tweevoudige toename in de controle dieren, suggererend dat de IFN-γ die uitgescheiden werd door de parasieten bioaktief was in resusapen. Alle dieren werden genezen met medicijnen en twee weken later geïnfecteerd met virulente *P. knowlesi* bloedstadium parasieten. Na infectie was er een vijf tot zes dagen langere pre-patente periode in experimentele dieren in vergelijking met controle dieren. Dit suggereert dat *P. knowlesi* parasieten die IFN-γ tot expressie brachten resusapen gedeeltelijk beschermden tegen infectie met homologe parasieten. Bovendien was de *in vitro* proliferatie reaktiviteit van T-cellen één week na infectie significant groter in experimentele apen. Eén week later waren deze responsen echter significant verlaagd, een fenomeen dat samenviel met een toename in parasitaemie. Deze resultaten laten zien dat *P. knowlesi* parasieten die IFN-γ tot expressie brengen even veilig in resusapen zijn als wildtype parasieten en gedeeltelijk een wildtype infectie met behulp van het afweersysteem kunnen moduleren. Bovendien laten deze experimenten zien dat het wellicht mogelijk is om door op de juiste wijze malaria parasieten te genereren die cytokinen tot expressie brengen, het immuunsysteem op een effective manier tot reaktiviteit kan worden aangezet.

Hoofdstuk 7: Het bavianenmodel voor experimentele *P. knowlesi* infectie

Het doel van dit hoofdstuk was om een bavianen (*Papio anubis*) model op te zetten voor de studie naar de reaktie van de gastheer op *Plasmodium knowlesi*. Omdat de baviaan één van de meest gebruikte primaten voor biomedisch onderzoek is, is dit dier aantrekkelijk als experimentele gastheer om gastheer-parasiet interacties van *P. knowlesi* te kunnen onderzoeken. Het gebruik van bavianen in landen waar bavianen van nature voorkomen en in faciliteiten zonder toegang tot andere *P. knowlesi* gastheren, zou de import van resusapen voor *P. knowlesi* onderzoek onnodig maken. Bovendien zijn reagentia voor analyse van het bavianen afweersysteem commercieel verkrijgbaar. Voor deze studie werden bavianen experimenteel geïnfecteerd met *P. knowlesi* bloedstadium parasieten. Alle dieren ontwikkelden of een ernstige dodelijke ziekte die gepaard ging met een bepaalde mate van

funktieverlies van diverse organen en cerebrale malaria, of een milde infectie die werd gekarakteriseerd door een laag niveau van parasieten en vergroting van de milt. Het patroon van parasitaemie dat werd gevonden was vergelijkbaar met dat van resusapen die uit dezelfde parasieten voorraad waren ingespoten. Enkele klinische symptomen in bavianen die waren geïnfecteerd met *P. knowlesi* waren echter vergelijkbaar met infecties van *P. falciparum* in mensen. Deze resultaten hebben voor de eerste keer laten zien dat *P. anubis* volledig gevoelig is voor experimentele infectie met *P. knowlesi* en een geschikt model vormt voor de studie naar gastheer responsen tegen de parasiet.

Experimentele systemen die als model kunnen fungeren voor enige van de complexe interacties die plaatsvinden tussen malaria parasiet en de gastheer, zoals *P. knowlesi* in de baviaan, kunnen van groot belang zijn voor de identificatie en ontwikkeling van nieuwe profylactische geneesmiddelen en geneesmiddelen tegen de ziekte in de mens. Bovendien is de DNA-sequentie bepaald van het genoom van *P. knowlesi*, zodat genen voor alle mogelijke geneesmiddel en vaccin kandidaat moleculen toegankelijk zijn. Gebruik van transfectie technologie zal nuttig zijn voor het bepalen van de funktie van kandidaat moleculen. Ons doel is om bavianen als *in vivo* gastheren te gebruiken om de geschiktheid van geselekteerde kandidaten als geneesmiddel en/of vaccin te bepalen.

Hoofdstuk 8: Algemene discussie

In dit hoofdstuk wordt de huidige staat van *P. knowlesi* transfectie besproken. Protocollen voor langdurige *in vitro* kweek werden ontwikkeld waardoor de mogelijkheden voor transfectie-experimenten vergroot worden. Met de ontwikkelde protocollen voor *in vitro* en *in vivo* transfectie is het mogelijk geworden om het gebruik van apen alleen maar om parsieten te genereren te minimaliseren. *In vivo* experimenten zijn nu alleen nog noodzakelijk in het kader van studies aan parasiet-gastheer interacties. De expressie van gastheer cytokinen zoals IFN-γ biedt nieuwe mogelijkheden voor de ontwikkeling van geattenueerde en immunoreactive malaria vaccins. De DNA-sequentie van het genoom van *P. knowlesi* is bepaald, zodat de genen voor alle mogelijke geneesmiddel en vaccin kandidaat moleculen bekend zijn. Transfectie kan nu een bijdrage leveren aan het ontrafelen van de funktie van deze moleculen. Zo kunnen bijvoorbeeld homologe moleculen worden geïsoleerd uit de humane malaria parasieten en tot expressie worden gebracht in *P. knowlesi*. Vervolgens kan het potentieel van de geselecteerde kandidaten als geneesmiddel of vaccin worden bepaald met behulp van *in vitro* en *in vivo* systemen. Als een primaten parasiet die ook mensen kan infecteren, is *P. knowlesi* momenteel het enige malaria transfectie systeem dat de voordelen

van een eenvoudig langdurig kweeksysteem combineert met de analyse van gastheer-parasiet interacties in natuurlijke en experimentele gastheer systemen die sterk lijken op die van de mens.

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Curriculum vitae

Hastings Ozwara Suba was born on the 16th of March, 1967 in Migori Kenya. In 1987 he finished high school at Maseno National School. The following year, he joined the Faculty of Science, at the University of Nairobi to study Botany and Zoology. He finished undergraduate studies in 1991 having specialised in Zoology. He immediately joined Masters degree programme in the same faculty to study Parasitology. In 1996, he was awarded Master of Science. In 1998, funding for the study described in this thesis was obtained from the Dutch Foundation for Advancement of Tropical Research (WOTRO). This enabled him to conduct PhD research at the Biomedical Primate Research Centre (BPRC) in The Netherlands and the Institute of Primate Research (IPR) in Kenya. The findings of this research form the basis of this thesis. In 2003, he was awarded a 2 year post doctoral grant from WOTRO. He is currently doing the post-doctoral research at IPR.