DECLARATION

I, Teresia Nyawira Kaniaru, do declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

Signature: __________________________ Date: ____________

Teresia Nyawira Kaniaru

Supervisors approval:

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

Dr. Zipporah Ng’ang’a
Department of Zoological Sciences
Kenyatta University, Nairobi, Kenya

Signature: __________________________ Date: ____________

Dr. Michael Gicheru
Department of Zoological Sciences
Kenyatta University, Nairobi, Kenya

Signature: __________________________ Date: ____________

Dr. Hastings Ozwara
Institute of Primate Research, Karen, Kenya

Signature: __________________________ Date: ____________
DEDICATION

To my late father, and loving mum Esther who saw the need for educating me against all odds, and for teaching me the values of hard work.
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CHAPTER ONE
INTRODUCTION

1.1 Background Information

Malaria is a major and growing threat to economic development and public health in approximately 40% of the world’s population living in the tropical and subtropical regions of the world (Breman, 2001). Annually, there are 300-500 million cases of malaria infected people, and up to 2.7 million deaths and Africa accounts for over 90% of the deaths, mainly children under five years of age (Breman, 2001). Furthermore, malaria exerts a heavy economic toll on malaria endemic countries and its people (Greenwood and Mutabingwa, 2002; Sachs and Malaney, 2002). 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 parasites species namely *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. Ovale*. *Plasmodium falciparum* is the most lethal accounting for over 90% of the deaths. Of all the parasitic diseases, malaria is not only the greatest killer, but is also the most widely spread (Breman, 2001)

The global campaign to eradicate malaria was introduced in 1955 after the development of chloroquine and the discovery of insecticidal action of 1,1,1-trichloro-2,2-bis-[p-chlorophenyl]-ethane (DDT; Bruce-Chwatt, 1986), but was faced out by the end of 1960s (Sachs, 2002). The approach has since changed to controlling the disease. With malaria a resurging public health problem, the search
of new methods to combat this disease has been intensified (Gwadz and Green, 1978). Current research is multifaceted, directed towards the development of better means of mosquito control, new chemotherapeutic agents and an effective vaccine (Gwadz and Green, 1978).

Controlling malaria has been far from optimal due to several and often interrelated factors (Sachs and Malaney, 1966; Sachs, 2002). The factors are essentially parasites, mosquitoes, environmental and human related. Malaria parasites have evolved mechanisms such as antigenic variations (Su et al., 1995; Smith et al., 1995) for evading host defenses (Richie and Allan, 2002). The complex life cycle of the Plasmodium, rampant resistance of first line drugs (Wellems and Plowe, 2001; White 1998) has constrained vaccine development and raised the costs of malaria treatments. Insecticide resistance is a major issue preventing mosquito control (Chadre et al., 1999; Della tore et al., 2002). Environmental factors including climatic events like global warming and El-nino (Lundblade et al., 1999; Hay et al., 2002) have very often frustrated malaria control efforts by precipitating epidemics. The benefits of controlling malaria are enormous – it would require bringing down the barriers described above (Ozwarra et al., 2003).

The difficulty of working with human malaria parasites has meant that a significant amount of research has to be performed on different animal models of malaria. Furthermore, these models offer the only means to experimentally
investigate natural host-parasite interactions in vivo (Ozwara et al., 2003). *Plasmodium knowlesi*, a natural parasite of macaques (*Macaca fascicularis*), is a comparative model for understanding many of the important aspects of human malaria (Escalante et al., 1998; Coatney et al., 1971). The accessibility of the entire life cycle of the parasite to experimentation suggests that all aspects of malaria, from biology to host parasite interaction can be studied under controlled conditions. The 24-hour life cycle of the parasite ensures that the data is generated rapidly. *Plasmodium knowlesi* is phylogenetically close to *P. vivax*, sharing many vaccine candidate molecules (Escalante et al., 1998). It is also a parasite for which both the natural and artificial vertebrate hosts are available (Coatney et al., 1971), offering the possibility to study the biology of malaria parasite and its antigens in a natural host – parasite combination and in hosts that are closely related to humans (King et al., 1988). In addition, *P. knowlesi* produces a dichotomous disease profile in the natural host (*Macaca fascicularis*) and the artificial host (*Macaca mulatta*; Gamham, 1966; Coatney et al., 1971), providing ideal opportunities for understanding the mechanisms of immunity to malaria (Butcher, 1996). Furthermore, *P. knowlesi* does infect humans (Chin et al., 1965; Knowles and Gupta, 1932) where a natural infection causes a mild disease (Chin et al., 1965). Recently, *P. knowlesi* was shown to experimentally infect baboons (Ozwara et al., 2003).
The assessment of a candidate vaccine for use in human subjects can be facilitated and expedited by prior testing in non-human primates (Strickland and Kenneth, 1982). These animals are particularly needed because of their evolutionary relationship to man, which is manifested in anatomical, physiological and behavioral resemblances and specific biochemical similarities (WHO, 1988). The baboon is attractive because it is well characterized and is frequently used in biomedical research (King et al., 1988). At the moment, the patterns of immune responses following *P. knowlesi* infection in baboon have not been described. This information would serve as a prerequisite for malaria vaccine development in the baboon model.

The various effector functions of the host immune system in response to malaria infection is complex and are not well understood, although different animal models give insights onto possible immune effector mechanisms (Good and Doolan, 1999). The lack of knowledge of the human immune response is hampering vaccine development (Good and Doolan, 1999). However, it is apparent that different components of the immune response operate at different stages in the parasites life cycle. The pre-erythrocytic stage represents the body’s first encounter with the parasite and the erythrocytic stage is responsible for all the symptoms and pathology of malaria (Good and Doolan, 1999). The exponential growth of parasites in the red blood cells, modification of infected red blood cells in terms of parasite proteins expressed on the cell surface and the concomitant
immune response to the parasite are the aspects that lead to disease manifestations of the clinical disease (Chen et al., 2000).

1.2 Statement of the Problem:

*Plasmodium knowlesi* malaria infections have been described in the natural host *Macaca fascicularis* (Garnham, 1966; Coatney et al., 1971) as well as in *M. mulatta* where it has been experimentally induced (Gwadz and Green, 1978). There is need to expand the primate model of *P. knowlesi* in order to use them in testing for vaccines and drugs. Recently the baboon has been shown to be susceptible to *P. knowlesi* infections and the disease profile and pathology has been determined (Ozwara et al., 2003). It is important to validate the baboon and the *P. knowlesi* system in order to benefit from this system. Although baboons are widely used for research (Moore et al., 2003; Havill et al., 2003; Ochiel et al., 2003), their use in malaria research has been minimal.

1.3 Research Question

What are the immune responses mounted by *P. anubis* in response to infection by *P. knowlesi*?

1.4 Null Hypothesis

*Papio anubis* does not elicit any measurable immune responses against experimental *P. knowlesi* H strain infections.
1.5 Objectives Of The Study

1.5.1 General Objective

To determine host immunological profiles mounted by *P. anubis* during the course of an experimental infection with *P. knowlesi* blood stage parasites.

1.5.2 Specific Objectives

a) To determine parasitaemia development in baboons infected with *P. knowlesi* during a primary and secondary infection.
b) To determine antibody responses in baboons infected with *P. knowlesi* during a primary and secondary infection.
c) To characterise cellular immune responses in baboons infected with *P. knowlesi* during a primary and secondary infection.
d) To evaluate cytokine profile in baboons infected with *P. knowlesi* during a primary and secondary infection.

1.6 Justification

The evaluation of new drugs and vaccines for use in humans requires testing in animal models that develop a disease comparable to that in humans. Similarities in biological mechanisms between human and non-human primates underlie the value of non-human primates as the final test systems preceding clinical trials in humans (WHO, 1988).
The adaptation of human malarial parasites in new world monkeys has provided experimental models for gaining critical insights into the biology, chemotherapy, and immunology of these parasites (WHO, 1988). Of the various species tested, the *Aotus* and *Saimiri* monkeys have been shown to support best the development of *P. falciparum*, *P. vivax* and *P. malariae* (WHO, 1988). In fact the New World monkeys are probably the only available systems for *in vivo* experimentation of the human malaria parasites (Herrera *et al.*, 2002) considering the complexity of using chimpanzees. They therefore represent a valuable resource in human research. Although *Aotus* and *Saimiri* monkeys have a wide distribution in South and Central America, legislation and cost limit their export in countries where they are not found naturally. Moreover, little attention has been paid to the establishment of breeding colonies, and the availability of many species of wild caught *Aotus* and *Saimiri* is limited. Wild caught *Aotus* and *Saimiri* monkeys from some areas (e.g. *Saimiri* from Peru) are heavily infected with filaria, trypanosomes and non-human plasmodial species, treatment of which is difficult or sometimes impossible, hence the sustainability of these animals in immunological studies is suspect (WHO, 1988). New World monkeys also become rapidly immune to further malarial infection or require splenectomy (Richie and Allan, 2002).

*Plasmodium knowlesi* model infects animals on multiple occasions before immunity develops and interactions can be evaluated in an immunological and metabolic environment similar to humans. It has been instrumental in the
discovery and characterization of malaria blood stage vaccine candidate AMA-1 and infact, *P. knowlesi* has been used to identify, develop and evaluate vaccine and drug candidates (Wengelnik *et al*., 2002; Deans *et al*., 1984; Kocken *et al*., 1999). Recently, protocols for genetic modification of *P. knowlesi* have been developed (van der Wel *et al*., 1997; Kocken *et al*., 2002). These are powerful tools for drug and vaccine development because they enable the functions of target drug and vaccine candidates to be determined using *P. knowlesi* genes. More animal models are required in order to benefit fully from advantages offered by this parasite model.

Baboons which are widely distributed over a variety of habitation in Africa (Sinner *et al*., 2001) are fully susceptible to *P. knowlesi* infections (Coatney *et al*., 1971; Ozwara *et al*., 2003). Infact, the disease profile observed in baboons is comparable to that in rhesus monkeys following experimental infection with the same parasite (Ozwara *et al*., 2003), indicating that the virulence of the strain is similar in both models. *Papio anubis* infected with *P. knowlesi* show various clinical characteristics that are also seen in human malaria, including cerebral involvement (Aikawa, 1998; Aikawa *et al*., 1990). The baboon is an attractive experimental model because it is well characterized and is frequently used in biomedical research (King *et al*., 1988).
The use of the baboon will expand the category of primate hosts for use in *in vivo* experiments for host parasite interactions of wild type and transfected *P. knowlesi* parasites. However at the moment baboon immune responses to *P. knowlesi* have not been described. The analysis of the immune responses of the baboon to *P. knowlesi* infections is necessary in its development and validation as a model for the analysis of host-parasite interactions. If the baboon is to be used for testing new methods of vaccine development using *P. knowlesi*, a full understanding of its normal responses to *P. knowlesi* infection is necessary. In addition, the responses will be critical in analyzing the vaccine potentials of an approach under test. The use of the baboons to study *P. knowlesi* infections will specifically find relevance in facilities that are not home to other *P. knowlesi* hosts. These include baboon source countries and primate research facilities with access to baboons. In this study, baseline data in characterizing immune responses in baboons during drug and vaccine development is provided.

This study examined immune responses during the blood stage infection which is an important stage for various reasons. The pre-erythrocytic stage during which the sporozoites travel in the blood after inoculation and then invade and develop within hepatocytes is perhaps the most understood in terms of immunity (Good and Doolan, 1999; Doolan and Hoffman, 2000), and vaccine development is further advanced for this stage than others. The mechanism of immunity required to block parasite transmission by the mosquito is also well understood as
antibodies taken up by the mosquito during its blood meal can prevent gamete fertilization or zygote development (Kaslow, 1997). The blood stage is the stage for which immunity is least well understood and which arguably presents the greatest challenges in terms of vaccine development. It is also the stage that is responsible for all the symptoms and pathology in malaria, the most serious of which are anaemia and cerebral malaria (Good and Doolan, 1999).
CHAPTER TWO

LITERATURE REVIEW

2.1 The Malaria Problem

Malaria remains a major threat throughout the tropics; more than 300 million people are estimated to be infected. Each year the disease kills up to 2.7 million people, the majority being children (Muller and Baker, 1990). Malaria is caused by unicellular protozoa of the genus *Plasmodium*, belonging to the phylum Apicomplexa which are transmitted mainly by mosquitoes of the genus *Anopheles*. Four species of *Plasmodium* infect humans. These are *Plasmodium vivax*, *P. malariae* *P. ovale* and *P. falciparum*. Three of these cause moderate to severe illness yet are seldom fatal. Infection with the fourth species, *Plasmodium falciparum*, however frequently leads to a coma and death. Efforts to control the disease have mainly focused on chemotherapy and vector control. These efforts are seriously hampered by the development of insecticide resistant mosquitoes, rapid emergency of drug resistant parasites and failure of health and mosquito control programmes in developing countries (Van Dijk *et al.*, 1997).

2.2 Malaria Control

One of the themes in malaria research today is to identify the Achilles tendon of the parasite, whether it be for immune intervention, the development of new drugs or for intervention in the vector phase. Luckily enough, new resources and tools are currently emerging which have an enormous potential in the fight against
malaria. The sequencing and annotation of the *Anopheles* (Holt *et al.*, 2002), human (Venter *et al.*, 2001) and *Plasmodium* (Gardner *et al.*, 2002) genomes, has given rise to a number of fields of biology, based on bioinformatics, genomics and proteomics (Florens *et al.*, 2002; Lasonder *et al.*, 2002). These approaches are expected to be an antidote to pessimism on the chances of success in the battle against malaria and may eventually lead to new drugs and vaccines (Gardner *et al.*, 2002; Wirth, 2002). The annotated genome sequence of *Plasmodium* currently in the database is an indication that the genes for all possible drug and vaccine candidates are available. It will now like never before, be possible to compare sequences to define important parasite mechanisms of antigenic variation, control of gene expression, drug resistance and parasite host specificity.

Several studies have shown that malaria vaccines are feasible (Richie and Allan, 2002). Immunisation with irradiated sporozoites partially protects rodents (Nussenzweig *et al.*, 1967), monkeys (Collins and Contacos, 1972) and humans (Clyde, 1990; Egan, 1993; Riekman, 1979). However none of the immune responses identified in humans robustly predicts protection from infection or from disease (Richie and Allan, 2002). Current anti malarial vaccine research efforts largely focus on the development of single parasite molecules, or combinations of a few 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 has not yet induced the required protection in order to manufacture a vaccine.

2.3 The Need For Non-Human Primate Models In Malaria Research

Non-human primates are suited for biomedical research because of their evolutionary kinship to man, which is manifested in anatomical and behavioral resemblance, and specific biochemical similarities (WHO, 1988). In many basic and applied sciences, primates are the only animal models when other models are not susceptible to the disease under study. For example, the neotropical (new world) monkeys are probably the only available system for in vivo experimentation of the human malaria parasites (Herrera et al., 2002), considering the dwindling population of chimpanzees.

Primates have been used in developing adjuvants and vaccine expression systems for use in developing human malaria vaccines (Kocken et al., 1999), and do offer adjunct systems for testing safety and efficacy of malaria vaccines (Stowers and Millers, 2001). Cross-reactivity of human and primate antibodies and surface markers (Stevens et al., 1991) have been identified, allowing characterization of host responses in primates. Furthermore, similarities in epitopes recognized between humans and immunized monkeys have become documented (Herrera et al., 2002). Therefore primates represent a valuable resource for experimental
analysis of host-parasite interactions and for testing candidate malaria drug and vaccines for human use.

2.4 Malaria Parasites Of Non-Human Primates And Their Relevance For Studying Human Malaria.

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 their study. They cannot be maintained in convenient small laboratory animals. Numerous species of rodent (e.g. *Plasmodium berghei*) and avian (e.g. *Plasmodium gallinaceum*) malaria parasites have been widely used as laboratory models to study the biology of *Plasmodia*. One significant drawback of these model organisms is their uncertain phylogenetic relationship with human *Plasmodia*, which calls into question their relevance as biochemical or molecular models (Mons and Sinden, 1990). Despite phylogenetic relationship between *P. falciparum* and avian *Plasmodia* such as *P. gallinaceum* (Escalante et al., 1997), difference in their life cycles, insect hosts and in the immune systems of their vertebrate hosts (Garnham, 1966) limit their usefulness as models. 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 malaria. This is in part due to the close phylogenetic relationships between the non-human primates and human malaria parasite (Escalante *et al.*, 1995). Amongst these are the *falciparum* – like parasites *P. reichenowi*, a chimpanzee malaria parasite, and macaque malaria parasites *P. coatneyi* and *P. fragile* (Garnham, 1966). Other macaque parasites *P. cynomolgi*, *P. knowlesi* and *P. gonderi* are vivax like, while *P. fieldi* and *P. simiovale* are ovale-like. The New World monkey parasite *P. brasilium* and the macaque parasite *P. inui* are regularly used as models for *P. malariae* (Fandeur *et al.*, 2000).

Primate models of malaria offer comparative information useful in understanding biology of malaria parasites, antigen discovery, antigen expression systems, safety and efficacy studies for vaccine development. Predictable patterns of infection, parasitaemia, relapse, and recrudescence, which are essential in the design of statistically valid trials, have been established with these parasites and their monkey hosts.

Primate malaria parasites have contributed a great deal to the understanding of malaria. There is no suitable experimental model for human cerebral malaria. However, *P. coatneyi* and *P. fragile* in rhesus monkeys are useful models for understanding sequestration of mature forms associated with knob formation on
infected erythrocytes (Aikawa et al., 1992; Fujioka et al., 1994). *Plasmodium reichenowi* is commonly used for comparative analysis of *P. falciparum* gene structure and polymorphisms (Okenu et al., 2002; Ozwara et al., 2003) and liver stage development (Daubersies et al., 2002). The study of *P. vivax* type of relapse during liver stage infection has benefited from the study of hypnozoites in *P. cynomolgi* and *P. simiovale* (Shortt and Garham, 2000; Cogswell et al., 1991). *Plasmodium coatneyi* is a suitable model for understanding malaria in pregnancy (Davison et al., 1998; Davison et al., 2000) and new drugs are commonly developed and evaluated using primate models such as *P. cynomolgi* in the rhesus monkeys (Wengelnik et al., 2002; Edstein et al., 1994).

The AMA-1, a leading malaria vaccine candidate (Howell et al., 2001) was identified and first characterized in *P. knowlesi* (Deans et al., 1984; Thomas et al., 1984; Waters et al., 1990). Antigenic variation is one of the mechanisms for parasite evasion of host defence system (Zambrano-villa et al., 2002). This phenomenon was first demonstrated in malaria parasites using *P. knowlesi* (Brown K and Brown I, 1965) and the antigens involved shown on infected erythrocyte surface (Hommel and David, 1981) and subsequently named SICA var (al-Khedery et al., 1999). Primate malaria parasites are used in developing adjuvants for possible use in human malaria vaccination (Khanna et al., 1989). The potential for cytokines as adjuvants for malaria vaccines was demonstrated in primates when sterile protection was obtained in rhesus monkeys inoculated with *P.*
cynomolgi and IL-12 as an adjuvant (Hoffman et al., 1997). Primate malaria parasites are used for developing and testing DNA vaccines and immunization regimens (Rogers et al., 2002). The role of the spleen on parasite’s virulence was demonstrated using splenectomised monkeys infected with P. knowlesi (Barnwell et al., 1982). In developing alternative vaccination approaches, Mitchell, Brown and Co-workers (Mitchell et al., 1975; Brown et al., 1970) attempted developing attenuated blood stage parasite vaccines using P. knowlesi merozoites. Irradiated sporozoite vaccination was tested several years using primate malaria parasites (Gwadz et al., 1979; Collins and Contacos, 1972). Finally, primate malaria parasites have been widely used to identify candidate antigens and characterize host response against blood stage (Deans et al., 1988), liver stage (Sharma et al., 1986), multistage (Rogers et al., 2001) and transmission blocking vaccines (Gwadz and Koontz, 1984). Although the proper study of mankind is man, non-human primates are likely superior over clinical trials for identifying better vaccine antigens (Heppner et al., 2001). New World monkeys are currently used to test safety and efficacy of human malaria vaccines, screening of anti-malarial drugs and the understanding of pathogenesis (Herrera et al., 2002). Although the full validation of vaccines will require more systematic human trials, non-human primates have and will continue to be very valuable for evaluation of potentially protective malaria antigens for human testing (Ozwara et al., 2003).
2.5 Problems Associated With Using Non-Human Primates For Malaria Research

The limited supply of some primates can severely restrict or preclude their use in research. Some source countries have placed embargoes on export of primates (King et al., 1988) and other primate species are considered endangered (Kennedy et al., 1997). Primates are also expensive to purchase and maintain experimentally, and the animal rights movement holds the view that research with animals and more so primates is unnecessary, inhumane and unethical. There are also strong criticisms on the use of New World primates as a critical path for malaria blood-stage vaccine development (Heppner et al., 2001), which include a rapid acquisition of effective blood-stage immunity, limiting the ability to model vaccine effects or efficacy in semi-immune human population (Jones et al., 2000). Few strains have been adapted to the monkeys excluding clone 3D7, the most prevalent allele (Tariabe et al., 1987) and the lack of cerebral malaria, a critical indicator of severe disease (WHO, 1990).

In view of decreasing availability of primates and challenges involving primate experiments certain measures need to be observed. Bleeding programs and biomedical research facilities should be improved in countries of primate origins (WHO, 1988; Herrera et al., 2002), experiments involving primates should be subjected to the highest available ethical and scientific scrutiny, and these procedures should be standardized. In addition, most primates in research should
not be sacrificed except where necessary, and studies that compromise their use in subsequent investigations should be minimized. The minimum number of animals for statistically valid results should be used and where possible tissues and clinical specimens should be shared. Finally, primates should be used as the final test systems in as much as the procedures can allow (WHO, 1988; King et al., 1988).

2.6 Plasmodium knowlesi

2.6.1 Life Cycle Of Plasmodium knowlesi

The life cycle of *Plasmodium knowlesi* 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), undergo many rounds of replication and transform into liver schizonts. The infected hepatocyte rupture releasing merozoites which infect circulating erythrocytes immediately. In the cyclical patterns of development termed intra-erythrocytic schizogony (blood stage development) that follows, the parasites develop into rings, trophozoites and schizonts, releasing more merozoites that continue to invade, colonize 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 (Hegner, 1938). 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 3 days post inoculation if the infection is heavy. Mature microgametocytes (7μm) are smaller than the macrogametocytes (8.5 μm) and stains brick red with giemsa compared to the light blue colour of macrogametocytes. A mosquito takes up gametocytes 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 multinuclear oocyst. A mature oocyst filled with sporozoites, ruptures releasing sporozoites into the haemocoel, from where they migrate into the salivary gland, ready to infect a vertebrate host during the next blood meal (Garnham, 1966; Gwadz and Green, 1978).

The mosquito hosts of *Plasmodium knowlesi* include *Anopheles dirus, A. balabacensis* and *A. hackeri* (Garnham, 1966; Coatney et al., 1971), all of which are found in Asia. Other possible vectors include *A. freeboni* and *A. maculatus* (Coatney et al., 1971). Development in the mosquito (sporogony) takes 10-12
days at a temperature of 26-28°C while the exo-erythrocytic schizogony phase lasts 5.5 days.

2.6.2 Animal Models Of *P. knowlesi*

The natural vertebrate hosts for *P. knowlesi* are *Macaca fascicularis* and *M. nemestrina*. Experimental infections 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 baboons (Garham, 1966; Coatney *et al.*, 1971; Collins *et al.*, 1978; Dutta *et al.*, 1978; Dutta *et al.*, 1981; Dutta *et al.*, 1982; Langhorne and Cohen, 1979). In baboons, the infection has been induced in *Papio cynocephalus*, *P. doguera*, *P. jubieleaus* and *P. papio* (Ozwara *et al.*, 2003). The parasite also infects humans (Chin *et al.*, 1965; Chin *et al.*, 1968; Knowles and Gupta, 1932; Ciuca *et al.*, 1955). In humans, *P. knowlesi* causes mild infection, seldom exceeding 1% parasitaemia. However, infection could get virulent on repeated passages in humans (Ciuca *et al.*, 1955). In the natural host, 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 animal in 7 to 14 days post infection (Garnham, 1966). It requires several infections and cure in order for *M. mulatta* to develop immunity against *P. knowlesi* (Barnwell *et al.*, 1982, Voller and Rossam, 1969, Miller *et al.*, 1977). Infections of Olive baboons (*P. anubis*) with *P. knowlesi* is either acute or chronic (Ozwara *et al.*, 2003).
Animals with acute infection develop multiple organ dysfunction and cerebral involvement. Chronically infected animals are classified as having mild malaria, with moderate to low level of clinical symptoms observed in acutely infected animals, and their peak parasitaemia not exceeding 3% (Ozwara et al., 2003).

The availability of natural and experimental hosts of *P. knowlesi* offer 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 (King et al., 1988). In addition the dichotomous disease profile of *P. knowlesi* (a chronic infection in natural hosts and an acute disease in artificial hosts) provides the opportunities of understanding the mechanisms of immunity to malaria (Butcher, 1996). Mechanisms of immunity in an experimental *P. knowlesi* infection have not been characterized in *P. anubis*, the most commonly used baboon for biomedical research (Ozwara et al., 2003).

### 2.6.3 Plasmodium 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 cannot be maintained in convenient small laboratory animals. Although there is proximal phylogenetic relationship between *P.*
*falciparum* and avian *Plasmodia* such as *P. gallinaceum* (Escalante et al., 1997), differences in their life cycles, insect hosts and in immune system of their vertebrate hosts (Clyde et al., 1973) limit their usefulness as models for human malaria. A good laboratory model should be relevant to human malaria and offer the possibility 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 non-human primates and human malaria parasites (Escalante et al., 1995).

*Plasmodium 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 (King et al., 1985). Secondly *P. knowlesi* is phylogenetically close to *P. vivax*, sharing many vaccine candidate molecules (Escalante et al., 1989). Thirdly the entire genome has recently been sequenced to a five-fold coverage (*http://www.sanger.ac.uk/projects/P-knownlesi*), availing more gene sequences for analyzing 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.
2.7 Immunity To Malaria

Immunity to malaria is a complex but intensively studied subject as new approaches for the development of agents having immunizing potential are being undertaken (Brown and Nossal, 1986; Miller et al., 1986). Concurrent with these efforts are studies aimed at defining host parasite relationships, which might reveal potential targets for intervention. These include identifying and characterizing particular host immune responses which suppress parasite growth or actually kill *Plasmodia* as well as determining how such responses can be enhanced or made more selective on their activity (Allison and Eugui, 1983; Deans and Cohen, 1983; Howard and Barnwell, 1984). Immunity to malaria is established under natural conditions but it involves repeated infections, takes years to develop and may not be absolute, in that parasitaemia may persist in the absence of clinical disease (Neva, 1977). It is not known with certainty how *Plasmodia* establish stable relationship with their hosts nor how immune responses eventually clear *Plasmodia* from host tissues or prevent uncontrolled parasite growth (William and Long, 1988).

Humans have interacted with malaria parasites for millennia (Bruce-Chwatt, 1952), suggesting that the malaria parasites has had the ample time to adapt to, and evolve with the human host. In the interaction between host and the parasite, the malaria parasite invades the human host, and the host mounts an immune response against the parasite. At one extreme, a small group of individuals rapidly develops
an immune response and sterilizes their infections. At the other extreme, there is an effective immune response, and these individuals develop hyper-parasitaemia and die. Most individuals however fall in between the two extremes of hyper-parasitaemia and sterilization of their infection (Bruce-Chwatt, 1952). The immune response however plays a major role in controlling parasite replication, but unfortunately also contributes to the pathologic changes associated with malaria (Miller et al., 1994).

2.7.1 Acquired Immunity

In endemic areas, most individuals develop an immune response that controls parasite replication but does not eliminate the parasite from blood. These individuals by lowering the parasite burden, and presumably by modifying the host immune response to be less destructive decrease the clinical impact of malaria and are termed as immune (Bruce-Chwatt, 1952; Lucas et al., 1969). Numerous epidemiological studies conducted in areas of stable malaria transmission report an age dependent increase in *Plasmodium* specific immune response as well as an age related decrease in malaria dependent morbidity (Wahlgre et al., 1986; Deloron et al., 1987; Hogh et al., 1991; Al-Yaman et al., 1995; Warsame et al., 1997).

During the first months of a child's life passive immunity of the mother confers some protection. However parasite numbers soon increase and the mortality in hyper-endemic areas is highest during the first few years of life. By school age,
children have developed considerable degree of active immunity (Bruce-Chwart., 1952; Lucas et al., 1969). This gradually acquired immunity is manifested by lower parasite densities, fewer clinical complications, and enhanced parasite specific immune responses (Marsh and Greenwood, 1992). This immunity is short lived however and in the absence of repeated infections, previously immune individuals, who have spent less than a year away from malaria endemic areas are once again susceptible to clinical disease (Marsh and Greenwood, 1992). Similarly subjects living in hypo endemic areas of malaria transmission acquire immunity so slowly that almost every infection leads to symptomatic disease (Luxenburger et al., 1996). Most immune individuals have parasites present in their blood despite mounting a vigorous anti parasite immune response. These individuals generally display delayed type hypersensitivity reactions (a measure of cell mediated immunity) to malaria antigens (Weindanz and Long., 1988). In addition their peripheral blood mononuclear cells (PBMC) proliferate in response to malaria antigens, and their CD4+ T cells secrete a variety of cytokines (Troye_Blomberg et al., 1994). Antibodies purified from malaria immune individuals passively protect recipients from on going infections (Cohen et al., 1961). Anti- parasite antibodies also are readily detected in the sera of immune individuals, and these antibodies inhibit the replication of P. falciparum parasites in vitro (Jensen et al., 1982) Collectively these studies indicate that immune individuals mount a vigorous anti parasite response.
The inability to sterilize malarial parasites from blood together with the observation that immune individuals mount an apparently effective anti parasite immune responses raises the question of why most immune human subjects do not kill all the parasite targets in their blood. This question can be restated in the light of co-involvement of humans and *Plasmodium* as “what are the molecular mechanisms the parasite has evolved to prevent its destruction and at the same time not overwhelm the host?” Possible mechanisms include antigenic variation by the parasite, polymorphism of the parasite proteins, competition between protective and non-protective responses and the ability of the parasite to manipulate the host responses.

Antigenic variation of parasite antigens during the cause of an infection is a mechanism often postulated to explain the inability of immune individuals to completely clear parasites from blood (Brown and Brown., 1965). Another mechanism is that an individual is continually infected with different clones of malarial parasites (Howard, 1989). The immune response against the initial infecting clone suppresses the replication of that parasite clonotype and of subsequent clones infecting the individual. However the immune response to a newly invading clone is not sufficient to allow complete removal of the new clone from the circulation. Recognition of proteins of the newly invading parasite clone may be poor because glycoproteins contain peptides of the initial parasite clone. Alternatively, its possible that the peptides from a newly invading parasite clone
bides better to the MHC (major histocompatibility complex) resulting to weakening of the immune response against the initial parasite clone. Either way, the response against one or more of the clones is weak, and this clone or clones may establish chronic infection. Such a competitive response has been reported for the liver stage of malaria (Gilbert et al., 1998).

Although antigenic variation and polymorphism are considered by many investigators to explain the hosts inability to totally clear malaria parasites from circulation, other factors may be important such as competition between protective and non-protective immune responses (Brown and Brown, 1965; Miller et al., 1965). During the initial immune response against the parasite, multiple antibody reactivities are found in the serum of the infected host. Although some of these antibodies may be protective most appears to have little if any function in protection (Day and Marsh, 1991). In fact certain of these non-protective antibodies may actually inhibit the activities of protective antibodies (Patino et al., 1997).

The malaria parasite expresses proteins with homology to host proteins. The hosts immune system may detect and down regulate these potentially detrimental auto reactive responses (Perrin et al., 1988; Schofield and Tachado, 1996). The down regulated immune system allows some parasites to escape destruction and establish chronic infection. The malaria parasite choice of RBCs (Red blood cells)
as its hosts cell may explain why certain components of the immune system respond but do not appear to kill the parasite. The number of CD8+ T cells are elevated in the blood of humans infected with *P. falciparum* as well as in the spleens of mice infected with *P. chabaudi*. The mature RBC lacks the golgi apparatus and cannot process proteins. Thus in RBC, the malaria proteins are not presented in the context of MHC class I or II. Therefore CD4+ and CD8+ T cells can not directly target parasitised RBCs through the MHC pathway. The case report of a patient who had asymptomatic *P. malariae* for at least forty years (Vinetz *et al.*, 1998), provides evidence for an equilibrium between the individual and the parasite.

2.7.2 Waning Immunity To Malaria

Memory responses capable of activating protection in malaria wane so rapidly after infection has cured. Based on the current understanding of the immune system, memory responses should be activated in people with malaria. The observed lack of immunity in previously immune people returning to an endemic area after a protracted absence conflicts with our understanding of immunological memory. One possible explanation for the loss of protective memory responses in malaria is that there is a recall response in immune individuals who have left endemic areas in protracted period, but this immune response has reverted to being similar in nature to the acute responses of a naive individual (Hay *et al.*, 2002). Thus an immune person returning to the same endemic area may be as susceptible
to the complications of malaria as children or visitors. In contrast to humans, a mouse exhibits a marked memory response to malaria that is protective. This memory response is CD4+ T cell dependent, and occurs at a time when the cells are polarized into a Th2 phenotype (Hay et al., 2002).

2.7.3 Immune Mediated Pathology

The severe complications of *P. falciparum* malaria; cerebral malaria, anaemia, and respiratory distress occur at a parasitaemia ranging from low to high (Gupta et al., 1994). The lysis of RBC contributes to anaemia but the occurrence of anaemia at low parasitaemia suggests that the host is also removing parasitised RBC at a greater rate in infected than in uninfected individual (Gupta et al., 1994).

Pathogenesis in cerebral malaria may occur due to sequestering parasitised erythrocytes, possibly in combination with parasites rosettes and blockage of cerebral capillary vessels, which may lead to tissue hypoxia, coma and death. While most patients who have recovered from cerebral malaria have no apparent neurologic deficits, some individuals do have psychological problems due to effects of cerebral malaria on the cerebral nerve tissue. Clinical reports note that survivors of cerebral malaria frequently develop depression, have impaired memory, show personality changes and are more prone to violence (Varney et al., 1997). However, anoxia of the brain during cerebral malaria would most likely lead to irreversible brain damage throughout the brain, not necessarily
psychological disorders. In addition, several studies report that not all patients with cerebral malaria have sequestration (Jerusalem et al., 1983; Sengers et al., 1971), thereby supporting alternative mechanisms such as inflammation. Also patients with malaria may still die from cerebral malaria even though treatment has removed parasites from blood (Horstmann et al., 1985). The occurrence of cerebral malaria and respiratory distress at low parasitaemia suggests that the physical presence of the parasite (vascular plugging) is not mediating disease in these individuals. Rather, an inappropriate response may be causing the pathological changes associated with malaria. Infection with P. falciparum leads to increased serum concentrations of inflammatory cytokines TNF-α (tumour necrosis factor alpha), IL-1β (interleukin), IFN-γ (interferon), IL-10 and TNF receptors have been detected in the sera of falciparum patients (Kern et al., 1992; Mordmuller et al., 1997).

2.7.4 Immunity To Blood Stage Malaria Infections

2.7.4.1 Humoral Immune Responses

Elevated levels of immunoglobulin are a hallmark of malaria infections (Cohen et al., 1961; Rosenberg, 1978). Levels of IgG isotypes are increased, supporting the role of T cells in the production of elevated levels of immunoglobulin. Plasmodium specific antibody is important in protection against malaria. The passive transfer of malaria specific antibodies into humans (Cohen et al 1961; Bouharoun-Tayoun et al., 1990), as well as in non human primates infected with
*P. falciparum* (Romero, 1992), provides *in vivo* evidence for the protective role of antibodies against the *P. falciparum* parasite. The IgG fractions of immune sera have been shown to contain the protective activity (Sabchareon *et al*., 1991). How antibodies function in protection is controversial because experimental evidence exists for several different mechanisms. Firstly, antibodies may contribute to protection by preventing the parasite from binding host cells or by blocking the invasion of RBCs by merozoites (Udeinya *et al*., 1983, Wahlin *et al*., 1994). Another potential role for antibodies in protection is to mediate antibody dependent phagocytosis involving FcR bearing cells such as mononuclear cells (Bouharoun- Tayoun *et al*., 1990; Bouharoun-Tayoun *et al*., 1995; Groux and Gysin, 1990).

The results from studies of animal models of malaria also indicate that antibodies are crucial for clearance of malarial parasites from blood. B cell deficient animals suffer from malaria that is prolonged in duration and fatal when injected with a non fatal parasite (Rank and Weidanz, 1976; van der Heyde, 1994).

2.7.4.2 Cell Mediated Immunity

Although humans with malaria show delayed type hypersensitivity reactions to malarial antigens, it is difficult to determine whether this measure of cell mediated immunity contributes to protection. T cells might participate in several ways in naturally acquired immunity. Since RBCs do not express either class I or class II
MHC molecules, it seems very unlikely that T cells could have direct classical cytotoxic effects on infected RBCs or that parasite peptides could be presented directly by infected RBC to induce T cell activation. However, it is certainly possible that T cells might be stimulated by malaria antigens processed by professional antigen-presenting cells to secrete cytokines which might have direct or indirect effects on parasite killing (Bouharoun-Tayoun et al., 1995).

Additionally, T cells may provide help for antibody production.
CHAPTER THREE
MATERIALS AND METHODS

3.1 Parasites

*Plasmodium knowlesi* H strain (Chin et al., 1965) blood stage parasites were used for inducing baboon infections. The original parasite inoculum was PKI (A+), previously cloned by micromanipulation and passaged in baboon (Bamwell et al., 1983). These parasites were retrieved from liquid nitrogen and quickly thawed at 37° in a water bath, washed twice in 3.5% NaCl and twice in RPMI 1640 (Sigma, UK) by spinning at 1200 revolutions per minute (RPM) for 10 minutes. The pellet was then transferred into a starting culture medium consisting 2.5% packed cell volume (PCV), 20% baboon serum and the rest RPMI 1640 having 20μg/ml of gentamycin (Sigma, UK). The parasites were cultured overnight before being inoculated into baboons (Ozwara et al., 2003).

3.2 Animals

Adult baboons (*Papio anubis*, weight range = 12-23kg) of either sex originating from Kajiado district of Kenya were used. Prior to the experiments, all animals were screened and determined to be free of infection with *Plasmodium* by Giemsa-stained thick blood film. All the animals were fed on a standard diet for non-human primates, and water was provided *ad libitum* (Olobo et al., 1990). The institutional animal care and use committee (ACUC), and the institutions scientific and ethical review committee (ISERC) of the Institute of Primate Research approved the experiments.
approved the experiments. Six baboons were selected and housed individually in single cages in a bio-containment facility to avoid possible transmission of *P. knowlesi* between experimental baboons.

### 3.3 Animal Infection, Observation And Sampling

Animals were sedated using ketamine hydrochloride (Vetelar, Parnke Davies co., Ponypool Germany) at 10mg/kg body weight, and inoculated intravenously with 1 x 10^6 *P. knowlesi* blood stage parasites in 1ml of RPMI 1640 medium (Invitrogen, UK). Prior to inoculation, 13mls of blood was obtained from each animal, 10ml was obtained in Alsevers solution and used for peripheral blood mononuclear cells preparation and the rest was processed for serum. This was used to get pre-infection values for IFN γ levels, antibody titres, recall proliferative responses and cellular phenotypes. These formed the baseline values over the other subsequent values obtained from each animal in the other sampling points. Animals were then bled on day 7 and day 14 post infection. Depending on the parasitaemia profile, animals were grouped as either acute or chronic. Chronically infected animals were the animals that were able to control parasitaemia levels to below 2% from a peak of about 5%, while in acutely infected animals, parasitaemia levels rose steadily after infection to above 5% and were not able to control it. Acutely infected animals were treated when parasitaemia reached above 5% and chronically infected animals were treated on day 11 post infection irrespective of parasitaemia. Treatment was done using pyrimethamine (5-[4-chrophenyl]-6-
ethyl-2,4-pyrimidinediamine; Sigma Aldrichemie, UK) at 1 mg/kg body weight once per day for three days.

Finger pricks were taken from each animal on a daily basis from day three up to day 14 post infection and used to prepare blood film. Chronically infected animals were then re-challenged two weeks later after treatment and confirmation of cure. Finger pricks and bioassay similar to those done for the initial infection were repeated. The general status of the animals was monitored by regularly determining weight, appetite and general behavior. Weight of the animals was measured on a sedated animal at the bleeding time points. Appetite was evaluated by closely monitoring the feeding habits of the animals.

3.4 Processing Baboon Blood For Serum

The method used was as done by Ozwara (Ozwara et al., 2003). Blood from a healthy baboon was collected aseptically in serum in tubes. It was then kept at room temperature for three hours before spinning at 2000 RPM for 10 minutes (Sorvall, Germany). All subsequent spinning for this study were done in this centrifuge. The serum was then aliquoted into cryovials and stored at -20°C. Serum that was used for parasite culturing was prepared in a similar way but was heat inactivated in a water bath at 56°C for 30 minutes, and stored at -20°C until needed.
3.5 Processing Of Blood For Parasite Culture

The method used for processing blood for parasite culture was as done by Ozwara (Ozwara et al., 2003). Aseptically collected heparinised baboon blood was spun at 1500 RPM for 10 minutes and the plasma removed. The remaining red cell pellet was washed three times with sterile Alsevers solution at 1500 RPM for 10 minutes. After last wash, the Alsevers solution was removed and an equal volume of RPMI 1640 put into the red cell pellet resulting in 50% PCV, which was stored at 4°C and used within 2 weeks.

3.6 Parasitaemia Determination

This was determined from a peripheral blood thin smear. A field was divided into four. At least 2000 RBCs on a thin smear were counted. This was done by counting erythrocytes from a quarter of each field and multiplying by four. The number of infected erythrocytes in a minimum of 2000 erythrocytes was also counted.

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\text{Percentage parasitaemia} = \frac{\text{total infected erythrocytes}}{\text{number of erythrocytes counted}} \times 100.
\]

3.7 Antigen Preparation

One hundred milliliters of heparinised blood was obtained from one baboon, which had a parasitaemia of 6.4%. Most nucleated cells were removed using Ficoll-hypaque (ICN/Cappel, UK) by centrifugation at 3000 RPM for 30 min. The
Ficoll-hypaque was then washed off from the red blood cells three times using an equal volume of RPMI 1640 and spinning at 1500 RPM 10 minutes to concentrate the parasites. The supernatant was discarded and 2ml of the topmost brown layer scooped into a separate tube. This had a parasitaemia of 13.2% and was used in preparing the crude whole parasite antigen as follows; The cells were lysed using 0.2% saponin (ICN/Cappel, UK), incubated for 10 minutes at room temperature and then spun at 3200 RPM for 10 minutes. The parasite pellet obtained was washed twice with phosphate buffered saline (PBS) by spinning at 1500 RPM for 10 minutes. The parasites were then homogenized, aliquoted at 1x10^6 parasites in 100μl of PBS and stored in -70°C until required.

3.8 Peripheral Blood Mononuclear Cell (PBMC) Preparation

The method of Gicheru et al., 1995 for PBMC isolation was applied. Peripheral blood was obtained from the femoral vein and diluted in an equal volume of sterile Alsevers solution. Peripheral blood mononuclear cells were isolated over Ficoll-hypaque by centrifugation at 3000 RPM for 30 minutes. A ratio of 4ml of diluted blood to 3ml of Ficoll-hypaque was used. Cells at the interface were aspirated and washed in Alsevers solution and then in complete RPMI 1640 medium containing 10% heat inactivated foetal bovine serum (FBS), 2-Beta mercaptoethanol (5x10^-5 M), L-glutamine (5mM/ml), and gentamycin (100μg/ml). Cell viability was determined by trypan blue staining.
3.9 Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA method was as done by Gicheru et al., 1995.

3.9.1 ELISA For Antibody Detection

Polystyrene Micro ELISA plates (Dynatech laboratories, Sussex, UK) were coated overnight with 100μl of crude and solubilized whole parasite homogenised antigen at a concentration of 1x10^6 parasites/ml diluted in bicarbonate buffer, pH 9.6. Excess coating buffer was flicked off and non-specific binding sites were blocked with 3% bovine serum albumin (BSA Sigma, UK) in PBS for 1hr at 37°C. Unbound BSA was washed off six times with 0.05% Tween 20 (Sigma, UK) in PBS. Fifty microlitres of undiluted serum samples were then dispensed into the wells and incubated for 2 hour at 37°C. Control wells received 50μl of the blocking buffer. Unbound serum was washed off six times as above and 50μl of alkaline phosphate conjugated rabbit anti monkey IgG (Cappel, Organon Teknika, Or,USA) diluted 1/2000 was added and followed by incubation for 1 hour at 37°C. Unbound conjugate was washed off as above before adding 50μl of p-nitrophenyl phosphate substrate (pNPP, Sigma, UK, final concentration 1mg/ml) in 10% diethanolamine buffer. The plates were incubated at 37°C for 30 minutes in the dark. Optical density (O.D) was read at 405nm in a microplate reader (Dynatech laboratories, UK). In assaying for IgM antibodies in serum, 100μl of horse radish peroxidase conjugated rabbit anti monkey IgM (Cappel Organon Teknika, OR, USA) diluted 1/2000 was added and incubated for one hour at 37°C. One hundred microlitres Orthophenyldiamine (OPD) (Sigma, UK, final
concentration of 0.4μg/ml) in phosphate citrate buffer was used as the substrate. Optical density (O.D) was read at 450nm after 30 min of incubation.

3.9.2. ELISA For Interferon Gamma Detection

Purified PBMC were adjusted to 4x10^6/ml in complete medium (RPMI 1640 with 10% heat inactivated FBS, 2-mercaptoethanol (5x10^{-5}M), L-glutamine (5mM/ml), and gentamycin (100μg/ml) and stimulated with either Con A or crude P. knowlesi whole parasite antigen prepared as described above. Culture supernatants were pooled from triplicate wells after the stimulation of PBMC. The concentration of IFN γ in the supernatants produced after the 72 hours of stimulation, and serum samples was determined in a sandwich ELISA as follows.

Polystyrene micro-Elisa plates (Dynatech laboratories, Sussex UK) were coated overnight with 50μl of monoclonal antibody which was rabbit anti-human recombinant IFN γ (2μg/ml; Genzyme corporation MA,USA). The wells were washed 6 times with washing buffer (1%BSA and 0.05% Tween 20 in PBS). Non specific binding sites were blocked with 200μl of 3% BSA in PBS for 1 hr at 37°C. Excess blocking buffer was flicked off, and 50μl of test samples added. Wells where blocking buffer alone was added (blank wells) served as negative controls. These were incubated for 2hrs at 37°C and then washed as described above. 50μl biotinylated anti-human IFN γ (Mab-7B6-1-Biotin: MABTECH, Sweden) was then added at a dilution of 1:200 per well and incubated for 1hr at
37°C. Washing was done as before and 50μl of streptavidin (ALP-PQ MABTECH, Sweden) was added at a dilution of 1:10,000 to each well, and incubated for 1hr at 37°C. Fifty microlitres of p-NPP in 10% diethanolamine buffer was added to each well and plates incubated at 37°C for 30 minutes in the dark. The plates were read at an Optical density (O.D) of 405nm in a microplate reader. Optical density readings for the blank wells were subtracted from O.D readings of the sample wells to get the final O.D reading.

3.10 Preparation Of Slides For Immunohistochemical Staining
Two hundred microlitres of 1 x 10^5 cells/ml was spun on to slides at 600 RPM for 5 minutes in a cytopsin (Shadon, UK). The cells were then dried using light from 40 watts bulb (Phillips, Kenya) placed 1½ inches above the slides for three hours. Slides were then lapped in aluminium foil and self-indicating silica gel and then preserved at -70°C until used.

3.11 Recall Proliferation Assay
Purified PBMC were adjusted to 4x10^6/ml in complete RPM1 1640 medium. Cell suspension (50μl) was distributed to each well of the 96 well flat bottomed microtitre plates (Nunc, Roskilde, Denmark). One hundred microlitres of crude P. knowlesi whole parasite antigen (1x10^6 parasites) or concanavalian A (Con A; Sigma UK) at a concentration of 10μg/ml were added to the wells. Control wells received 100μl of complete RPM1 1640. Cultures were set up in triplicates.
The cultures were then incubated at 37°C in humidified atmosphere containing 5% CO₂ for 72 hours. The cells were pulsed with 0.5μCi of ³H-thymidine (5mCi/mg: Amersham, UK) in 20μl RPMI 1640 medium and a further incubation was carried out for 18-24hrs. Cells were harvested onto fibre glass filters by using a Titertek cell harvesters (Flow laboratories, UK). The filters were air dried and immersed in scintillation fluid (PPO, POPOP, Triton x-100, Germany). Incorporation of ³H-thymidine into DNA was determined by liquid scintillation spectrophotometry. Results were expressed as stimulation index (SI) that was obtained by dividing the proliferation of test cultures by proliferation of control cultures.

3.12 Immunohistochemical Staining Of Cells

The method of Modlin et al., 1995 for cell staining was used with some modifications which included use of Mayers haematoxylin as the counterstain. The slides were retrieved from -70°C and left at room temperature for at least 20 minutes before staining began. One slide, in which the primary antibody was omitted, was included as a control for non-specific staining. All incubations were done at room temperature.

The cells were fixed in chilled 100% acetone for 4 minutes. This was followed by washing the slides as follows: First in cold PBS, then PBS at room temperature and finally in PBS at 37°C, 5 minutes for each wash. The cells were incubated in hydrogen peroxide – azide mixture for 10 minutes. From here onwards, all
incubations were done in moist chamber. Excess azide mixture was removed by washing twice in cold PBS and at room temperature. Non-specific binding of the primary antibody was blocked by incubating the cells for 15 minutes in 3% BSA in PBS with thiomersal (Sigma, UK). Excess BSA was washed off, once in cold PBS and twice in PBS for 5 minutes each time. Twenty five microlitres of primary antibody was applied to the cells and incubated for 2 hours at room temperature. Excess antibody was flicked off, followed by washing twice in cold PBS for 5 minutes each time. The secondary antibody (biotin labeled goat anti mouse IgG) diluted 1:50 was applied to the cells and incubated for 25 minutes. Unbound secondary antibody was flicked off and washed using PBS once, followed by warm PBS for 5 minutes each round. Twenty five microlitres of streptavidin conjugated horseradish peroxide diluted 1:40 was applied to the cells and incubated for 15 minutes. Excess unbound streptavidin horseradish peroxide was flicked off, followed by washing once in PBS at 37°C for 5 minutes each round. Colour development was done using chromogen substrate mixture 3-amino-9-ethyl carbazole (AEC Sigma, UK); dissolved in dimethylformamide (Sigma, UK) and mixed with 30% hydrogen peroxide for 5 minutes in the dark, to avoid destruction of reaction product (red in colour). Excess stain was washed off with acetate buffer. The cells were then counterstained with Mayers haematoxylin (Sigma, UK) for 1 minute. Excess haematoxylin was washed off under running water for 2 minutes. The cells were mounted in glycerol-sodium chloride mountant. Cover slips (Chance, UK) were placed on and the edges sealed with nail
varnish (Luron, UK). The slides were allowed to dry at room temperature for 30 minutes before counting positive cells.

Cells were counted under a microscope. A minimum of 200 cells counted was used to calculate the percentage of positive cells (Olobo, 1992). The positive cells had red surface showing the chromogen substrate reaction product. Negative cells only had blue colour of the counterstain. The X40 objective was used for counting cells.

The monoclonal antibodies used were as follows:

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu 2a</td>
<td>CD8</td>
<td>Becton Dickinson, USA</td>
</tr>
<tr>
<td>Leu 3a</td>
<td>CD4</td>
<td>Becton Dickinson, USA</td>
</tr>
</tbody>
</table>

6.1.2 Secondary Infection

Animals with chronic parasitaemia were re-infected with parasitaemia by day six post infection (Table 4.2). Parasitaemia levels were much lower than during the primary infection (Pan 200%) whose peak during the primary infection was 2000 parasites/μL. Therefore parasitaemia levels were much lower than during the primary infection. Thereafter parasitaemia levels were much lower than during the primary infection. The second infection developed some parasitaemia during the primary infection.
CHAPTER FOUR
RESULTS

4.1 Parasitaemia

4.1.1 Primary Infection

All the baboons inoculated with *P. knowlesi* H strain blood stages developed patent parasitaemia with all the blood stages being manifested (Figure 4.1; Plate 4.1-4.5). Those that had acute parasitaemia had patent parasitaemia by day four post inoculation while chronically infected animals had patent parasitaemia by day five inoculation (Figure 4.1; Table 4.1-4.2). Acutely infected animals had parasitaemia that systematically increased to over 6%, reaching as high as 9.1% in some baboons at the time of treatment (Plate 4.2; Figure 4.1). Chronically infected animals had peak parasitaemia levels ranging from 1.5%-5%, which there after decreased to less than 2% parasitaemia on average (Table 4.2).

4.1.2 Secondary Infection

Animals with chronic parasitaemia were re-infected and developed patent parasitaemia by day six post infection (Table 4.2; Figure 4.2). Peak parasitaemia levels were much lower than during the primary infection, apart from one animal (Pan 2698), whose peak during the primary infection was lower than in the secondary infection. Thereafter parasitaemia levels were reduced to sub patent levels by day 14 post re-infection (Figure 4.2; Table 4.2). Animals that had developed acute parasitaemia during the primary infection were withdrawn from
the experiment due to technical reasons unrelated to the experiment and euthanised.

Figure 4.1: Parasitaemia profiles of baboons infected with *P. knowlesi* during the primary infection.
Figure 4.2: Parasitaemia profiles of baboons infected with *P. knowlesi* during the secondary infection
Table 4.1: Disease status of baboons infected with *P. knowlesi* H strain during primary infection

<table>
<thead>
<tr>
<th>Baboon number</th>
<th>Disease status</th>
<th>Day for patent parasitaemia</th>
<th>Day post inoculation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan 2730</td>
<td>Acute</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Pan 2574</td>
<td>Acute</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Pan 2677</td>
<td>Acute</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Pan 2601</td>
<td>Chronic</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Pan 2669</td>
<td>Chronic</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Pan 2698</td>
<td>Chronic</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>

*Day post Inoculation when the infection was scored as acute or chronic

Table 4.2: Comparison of parasitaemia in baboons during primary and secondary infections with *P. knowlesi*.

<table>
<thead>
<tr>
<th>Baboon number</th>
<th>Parameter</th>
<th>Primary infection</th>
<th>Secondary infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan 2669</td>
<td>Day of patent parasitaemia</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Peak parasitaemia</td>
<td>5%</td>
<td>2.39%</td>
</tr>
<tr>
<td></td>
<td>Treatment parasitaemia*</td>
<td>1.3%</td>
<td>&lt;&gt;</td>
</tr>
<tr>
<td>Pan 2601</td>
<td>Day of patent parasitaemia</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Peak parasitaemia</td>
<td>2.6%</td>
<td>1.5%</td>
</tr>
<tr>
<td></td>
<td>Treatment parasitaemia*</td>
<td>1.94%</td>
<td>&lt;&gt;</td>
</tr>
<tr>
<td>Pan 2698</td>
<td>Day of patent parasitaemia</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Peak parasitaemia</td>
<td>1.5%</td>
<td>2.62%</td>
</tr>
<tr>
<td></td>
<td>Treatment parasitaemia*</td>
<td>0.1%</td>
<td>&lt;&gt;</td>
</tr>
</tbody>
</table>

*Treatment parasitaemia recorded at onset of treatment.
<> Parasitaemia below detectable levels
Plate 4.1: Different life stages of *P. knowlesi* in erythrocytes

Key-  
Broken arrow points at the ring stage
Thin arrow points at a trophozoite
Broad arrow points at the schizont stage

Plate 4.2: RBCs from an acutely infected baboon (Pan 2574) at day 8 post infection.
Plate 4.3: A case of multi infection of an RBC by *P. knowlesi*

Plate 4.4: RBCs infected with ring stage of *P. knowlesi* parasite (Pan 2677)
4.2 Clinical Symptoms

Onset of patent parasitaemia was followed by loss of appetite in all animals as measured by decreased food intake and marginal weight losses (data not shown). Baboons with acute infection developed severe clinical symptoms and were characterized as having severe malaria. These included remaining in a sitting position in the cage (apathy) with ruffled fur. There was reduced ocular tension and skin turgor indicating dehydration. 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. After re-infection, these animals remained asymptomatic. They appeared normal, fed well, were playful and weights remained as before with minor fluctuation.
4.3 Gross Pathology

At euthanasia, baboons with severe malaria were remarkably similar in the quality of gross appearance, varying only in the degree of manifestation of the pathologic changes. As a general feature, all tissues particularly the mesentery, were of a yellow-tan appearance. These animals presented with severe acute haemolytic anaemia manifested by an increase in the number of reticulocytes (Plate 4.2 and 4.6). Baboons with mild malaria had similar indications but at a low level of manifestation than in the severely infected animals. However mucus membranes were pale with a yellowish tinge but not extended to the mesentery. In all infected baboons, the spleen was pronouncedly enlarged. Lungs had patchy consolidations 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.
Plate 4.6: RBCs from an acutely infected baboon (Pan 2677), two days after treatment.
Key - Arrow denotes reticulocytes recognisable by their large size and the faint purplish grey colour (polychromatic)

4.4 Antibodies Against *P. knowlesi*

All baboons produced anti *P. knowlesi* IgG and IgM antibodies upon infection with *P. knowlesi* blood stage parasites (Figure 4.3 and 4.4). The anti *P. knowlesi* IgM responses increased in baboons with acute and chronic infection throughout the infection period. The IgM response was higher in acutely infected animals than in the chronically infected animals (Figure 4.3). Anti *P. knowlesi* IgG responses rose slowly upon infection (Figure 4.4). The increase in IgG responses however was higher in chronically infected animals than in acutely infected animals (Figure 4.4). Immunoglobulin G levels had increased by 18% in acutely infected animals at day 14 post infection, whereas in chronically infected animals, the increase at day 14 post infection was 48%, and 72% at day 42 post infection.
Figure 4.3: Mean IgM responses of baboons infected with *P. knowlesi*. 
Figure 4.4: Mean IgG responses of baboons infected with *P. knowlesi*

4.5 Interferon Gamma Responses

All the animals produced IFN-γ by day 2 post-inoculation, with the infected animals showing a steady increase in level. The IFN-γ produced by chronically infected guinea pigs increased with primary infection reaching a peak at day 4 (Figure 4.5). The IFN-γ levels in chronically infected animals were much higher than in acute infection.
4.5 Interferon Gamma Responses

All the animals produced IFNγ by day 7 post infection (Figure 4.5). Acutely infected animals showed a steady increase in serum IFNγ (Figure 4.5). Although the IFNγ produced by chronically infected animals also rose in the course of the primary infection reaching a peak at day 14 (Figure 4.5), the levels in acutely infected animals was much higher at the two sampling points (Figure 4.5). Upon treatment, the IFNγ levels in chronically infected animals subsided and then rose.
one week later, only to decrease again at day 42 post infection (Figure 4.5).

IFNγ detected in blast supernatant increased in both acutely and chronically infected animals upon infection (Figure 4.6). The levels in acutely infected animals reached peak at day 7, and then decreased (Figure 4.6). In the chronically infected animals, IFNγ in the supernatant increased gradually to reach a peak at day 42 post primary infection (Figure 4.6). However the amounts of IFNγ produced by cells from acutely infected animals remained higher than in those from chronically infected animals (Figure 4.6).
4.6 Lymphocyte Proliferation

PBMC from the baboons showed mitogen and antigen specific (Figure 4.7) proliferation, with the highest stimulation index (SI) seen from the chronically infected animals by day 42 post primary infection $10.06 \pm 2.74$ (mean $\pm$ S.D). Antigen proliferation observed in PBMC from acutely infected animals was lower than in chronically infected animals at day 14 post inoculation (Figure 4.7). Upon re-infection, antigen specific proliferation increased in chronically infected animals (Figure 4.7) 14 days post re-infection.

Figure 4.7: Antigen specific recall proliferative responses of PBMC from *P. knowlesi* infected baboons.
Plate 4.7: Immunohistochemical staining of baboon PBMC using anti human CD4 monoclonal antibody.

Key: A CD4+ T cell.

4.7 Cellular Phenotypes

Using anti human CD4 and CD8 monoclonal antibodies, profiles of baboon CD4+ and CD8+ T cells (Plates 4.7 and 4.8) were determined.

4.7.1 CD4+ T Cells

CD4+ cells increased in numbers in both the acutely and chronically infected animals after infection (Figure 4.8 and 4.9). In chronically infected animals, the increase in CD4+ T cells was higher than in acutely infected animals in all the sampling points after infection (Figure 4.9). However, the numbers of CD4+ T cells in these chronically infected animals decreased by day 28 post inoculation.
Upon re-infection, the numbers rose by day 42 post inoculation (Figure 4.8 and 4.9).

Figure 4.8: Mean CD4 + T cells in *P. knowlesi* infected baboons
4.7.2 CD8+ T Cells

CD8+ T cells varied in the different animals and at different times. In acutely infected baboons, there was a gradual decrease during the early post inoculation (Figure 4.9). In the chronically infected baboons, there was little variation during the primary infection and autoinfection.

![Graph showing comparison of CD4+ T cells in acute and chronically P. knowlesi infected baboons.](image)

Figure 4.9: Comparison of CD4+ T cells in acute and chronically *P. knowlesi* infected baboons.
4.7.2 CD8+ T Cells

CD 8+ T cells varied in the different animals and at different sampling points. In acutely infected baboons, there was a gradual increase between day 0 and day 14 post inoculation (Figure 4.10). In the chronically infected animals, there was very little variation during the primary infection and secondary infection (Figure 4.10).

Figure 4.10: Comparison of CD8+ T cells in acute and chronically P. knowlesi infected baboons
Plate 4.8: Immunohistochemical staining of baboon PBMC using human anti human CD8 monoclonal antibody.

Key- Intact arrow demonstrates a CD8+ T cell

Broken arrow demonstrates a CD8 negative cell
CHAPTER FIVE

DISCUSSION

In this study immune responses mounted by experimental \( P. \) knowlesi infection in olive baboons (\textit{Papio anubis}) is presented for the first time. All olive baboons were successfully infected with an inoculum size \( 1 \times 10^6 P. \) knowlesi parasites. Our results shows that \textit{P.} anubis is fully susceptible to experimental \( P. \) knowlesi H strain infection since all infected animals developed patent parasitaemia. This is in support of earlier work where baboons were shown to be susceptible to experimental \( P. \) knowlesi infection (Ozwara \textit{et al.}, 2003)

The disease profile was either severe or mild. In baboons with severe \( P. \) knowlesi infection, patent parasitaemia was noted much earlier than in the mildly infected animals and immunological profiles differed substantially between the two groups of animals. The disease profiles reported here have also been seen in rhesus monkeys which get severe infections (Ozwara \textit{et al.}, 2003; Ibiwoye \textit{et al.}, 1993; Rosen \textit{et al.}, 1968) and \( P. \) knowlesi infected \textit{M. fascicularis} which produce chronic self regulating infection. The precise mechanism that predisposes a dual outcome in the parasitaemia profile and immunological profile warrants further investigations. Ozwara \textit{et al.}, 2003).

Our experiments are the first to show that \( P. \) knowlesi infected baboons can be treated and re-infected. One phenomenon in malaria is that the immune response is
never able to eradicate the infection (Chen et al., 2000). However, for the first time, we have shown that mildly infected *P. anubis* are able to bring down the parasites to sub patent levels upon re-infection. It would have been important to re-infect the acutely infected animals and see the parasitaemia and immunological profile upon re-infection but the fact that mildly infected animals were able to eliminate parasites from peripheral circulation upon re-infection suggests that protection is associated with prior exposure. Our data is in agreement with earlier studies by Voller and Rossan (1969) and Richie and Saul (2002) which indicated that protection is associated with pre-exposure.

The antibody levels increased during the infection. Immunoglobin M titres increased steadily and remained higher than the IgG levels in both the acutely and chronically infected animals. There is evidence suggesting that *P. knowlesi* infections in monkeys show antigenic variations (Brown and Brown, 1965). This antigenic variation may partly account for this persistently raised IgM levels seen in the *P. knowlesi* infected baboons. More over, IgM responses are mounted faster than IgG especially in primary infections where IgM seen to boost the initial response (Roitt, 1988). This also explains the high IgM titres detected and low IgG response demonstrated. The rise in IgG levels was gradual during the infection period, reaching a peak at day 42 post infection in the chronic animals. This is in agreement with the fact that more time is required for IgG to develop
and that specific IgG response is detected much later after infection, and persists for a longer time (Roitt, 1988).

Acutely infected animals developed higher parasitaemia levels as compared to the chronic animals during the primary infection. Chronic animals were infact able to control parasitaemia levels to below 2%. Whereas the IgG levels had increased by 18% in acute animals at day 14 post infection, IgG responses in chronic animals had increased by 48% at !4 days post infection and 72% at 42 days post infection. The fact that chronic animals were able to control the parasitaemia during the primary infection and even able to eliminate the parasites from peripheral circulation upon re-infection supports the role of specific antibodies in protection against *P. knowlesi* infected baboons. This is agreement with other studies for example in humans blood stage protection is partly mediated by antibodies, although IFNγ and T cell proliferation in response to blood stage antigens are also associated with protection (Miller et al., 1998; Good et al., 1998). In addition there is growing evidence for the protective role of IgG in *P. falciparum* (Christophe et al., 2000). Passive transfer of IgG has provided protection against *P. falciparum* blood stage in South American monkeys (Faudeur et al., 1984; Groux and Gysin, 1990), and in humans (Bouharoun-tayoun et al., 1990; Cohen et al., 1961)). Further human antibodies efficiently inhibit *in vitro* *P. falciparum* merozoite proliferation (Bouharoun-Tayoun et al., 1990), and mediate opsonisation of infected erythrocytes (Groux and Gysin, 1990).
During the primary infection, proliferative cellular responses were generally low in the two groups. This may be due to the short time of the infection, which may not have been enough for immunological memory to develop. The chronic animals showed a higher proliferation response at day 14 post inoculation when compared with acute animals. This increased even more upon re-infection. It therefore clearly demonstrates that there is immunologic memory elicited by baboons upon infection with *P. knowlesi*. Since antigen induced proliferative responses are pre-dominantly mediated by T cells, these results suggest T cells reactivity in *P. knowlesi* infected baboons. These results are in agreement with other studies in humans and chimpanzees where lymphocyte specific proliferative response was demonstrated (Pombo *et al*., 2000; Taylor *et al*., 1985).

Upon infection, IFNγ production increased rapidly in acutely infected animals so that by day 7 post inoculation one animal Pan 2730 had an O.D of 0.787 compared to the chronic animals which had an O.D of 0.101 ± 0.024 (mean ± SD). The chronically infected animals upon re-infection were able to maintain a low parasitaemia level and similarly the IFNγ level remained relatively low. At day 14 post re-infection, these animals had managed to bring down the parasites from the peripheral circulation to sub patent levels as shown by absence of parasites from a thick smear, and at this time the O.D of serum IFNγ in these chronically infected animals was 0.030 ± 0.031 (mean ± S.D) which was relatively very low.
The role of cytokines in the regulation of immune responses against *Plasmodium* infections and pathogenesis of malaria has been extensively studied in rodent malaria models but there are few studies on cytokine studies in primates especially after experimental malaria (Chunfu et al., 1999). In one study in which human volunteers were infected experimentally with *Plasmodium falciparum*, there was increased production of IFNγ (Harpaz et al., 1992). In another study, *P. Coatneyi* in infected Rhesus monkeys demonstrated the increase of IFNγ production occurring in the early stage of infection, and is thought to have been associated with the pathologic changes that led to death of the infected animals (Chunfu et al., 1999). This observation was supported by Riley (1999) who suggested that low amounts of IFNγ may be associated with resolution of infection. Excessive production of cytokines by human host has an adverse effect on disease progression (Chen et al., 2000). High circulating levels of IFNγ and TNFα are more often found in patients with severe malaria than in uncomplicated cases (Kwiatkoski et al., 1990). The fact that in our study, levels of circulating IFNγ were three fold higher in severely infected animals than they were in the chronic animals may be in support of these earlier observation, therefore resistance or susceptibility to malaria may depend on induction of appropriate levels of IFNγ and possibly other pro-inflammatory cytokines like TNF α as suggested by others (Riley, 1999)
There were slight differences in pre infection C4D+ and CD8+ T cells. This may be explained by the individual variations between the baboons and the small sample size. There was increase in the number of CD4+ T cells upon infection in both acutely and chronically infected animals. The increase was significant in the chronic animals, which was not the case with the acutely infected animals. Although immunity to malaria is complex and not properly understood (Good and Doolan, 1999), our studies show that CD4+ T cells, whether acting as effectors or as helpers are an important immune mechanism against the erythrocytic stage of malaria infection. The considerable increase in the CD4+ T cells especially with the chronic animals as opposed to the acutely infected animals is in support of this. Good and Doolan (1999) reported that polyclonal populations of CD4+ T cells specific for malaria parasites in mice could adoptively transfer protection, controlling parasite density very effectively.

CD4+ T cells may not only act as helpers for antibody responses, but also as effector cells, given their ability to inhibit parasite growth in vitro (Fell et al., 1994). Furthermore, IFNγ production by CD4+ T cells in response to specific erythrocytic antigens is associated with protection against malaria re infection (Luty et al., 1999). T cell secretion of IFNγ may also help induce cytophilic IgG blood stage specific antibodies and assist in antibody dependent cellular inhibitory mechanisms (Bouharoun-Tayoun et al., 1990). There was very little variation in CD8+ T cells after infection of baboons with P. knowlesi. These cells do not seem
to be activated by the blood stage *P. knowlesi* infection. This may probably be due to the fact that the *P. knowlesi* antigens are not presented to these cells in association with MHC class I molecules which is necessary for activation of CD8+ T cells. Pombo and others (2002) have also reported similar findings in humans experimentally infected with ultra low doses of *P. falciparum* blood stage parasites.
CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

a) Olive baboons develop either acute or chronic malaria during a *P. knowlesi* infection, and the levels of production of immune effector molecules differed in these two groups of animals. *Plasmodium knowlesi* infected baboons can therefore be used as a model to analyse mechanisms of host response during acute and chronic malaria in humans.

b) The immunologic profile seen in both the acutely and chronically infected baboons showed that there are immune responses that were associated with chronic infection as well as with acute infection.

c) Specific antibody responses were important in immunity to blood stage malaria infection.

d) There was antigen induced proliferation of lymphocytes in baboons infected with *P. knowlesi*. The responses were higher during the secondary response as demonstrated in recall proliferative responses.

e) Interferon gamma was produced by baboons upon infection with *P. knowlesi* blood stages. The levels of production were higher in acutely infected than in chronically infected animals.

f) CD4+ T cells were activated in the host baboon upon infection with *P. knowlesi* blood stages.
g) CD8+ T cells did not seem to be activated in baboons upon infection with *P. knowlesi* blood stages. These cells may not be important in immunity to blood stage malaria infection in baboons.
6.2 RECOMMENDATIONS

a) More aspects of immune responses in baboons infected with *P. knowlesi* blood stages need to be looked at especially on cytokines such as TNFα, IL-1 and prostaglandins. Production of these pro-inflammatory cytokines has been shown in humans with severe malaria as compared with uncomplicated malaria. To be able to use baboons as a model, it would be important to show whether these aspects compare in both humans and baboons.

b) It would be important to re challenge acutely infected baboons and see the immune profile. This would help us see whether these animals are able to resolve the disease or still suffer from severe disease after re infection. It would be important to see whether this compares with humans who get infected and cured many times in their life time.

c) The mechanisms leading to the dual outcome of infections (acute or chronic) should be investigated. It is important to understand the factors leading to susceptibility or resistance to malaria in a suitable animal model.
REFERENCES


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http://plasmodb.org/.

http://www.sanger.ac.uk/projects/p.knowlesi/.


APPENDICES

Appendix I

BUFFERS AND REAGENTS

Carbonate – Bicarbonate buffer (pH 9.6)

Na₂CO₃ 1.59g  
NaHCO₃ 2.93g  
NaN₃ 0.2g

Make up to 1 litre distilled water, store at 4°C for not more than 2 weeks

Phosphate buffered saline (PBS 1 X) (pH 7.2 – 7.4)

NaCl 8.0g  
KH₂PO₄ 0.2g  
Na₂HPO₄.12H₂O 2.9g  
KCl 0.2g  
NaN₃ 0.2g

Make up to 1 litre with distilled water, store at 4°C

0.05% Tween 20 in PBS (pH 7.2 – 7.4)

NaCl 8.0g  
KH₂PO₄ 0.2g  
Na₂HPO₄.12H₂O 2.9g  
KCl 0.2g  
NaN₃ 0.2g  
Tween 20 0.5ml

Make up to 1 litre with distilled water and store at room temperature

PBS with Thiomersal (pH 7.2 – 7.4)

NaCl 7.02g  
NaH₂PO₄.1H₂O 1.38g
K₂HPo₄  6.96g
Thiomersal  19.8mg

Make up to 1 litre with distilled water and store at room temperature.

10% diethanolamine buffer (pH 9.8)
Diethanolamine  97ml
Distilled water  800ml
NaN₃  0.2g
MgCl₂.6H₂O  100mg

1 M HCl is added until pH is 9.8 and total volume made up to 1 litre.
Store at 4°C in the dark. Remove sufficient amount 1-2 hours before the substrate solution is to be used and allow to warm to room temperature.

Asevers solution
Dextrose  10.25g
NaCl  2.1g
Trisodium citrate  4.0g
Double distilled water  500ml

Sterilize by filtering through 0.2μm pore size filter.
Store at 4°C.

2-mercaptoethanol (diluted)
2 mercaptoethanol  0.39ml
Double distilled water  10ml

Filter sterilize through 0.45μm pore size filter. Store at -20°C.

Complete RPMI 1640 (pH 7.2)
RPMI 1640  445ml
FBS 50ml
L-glutamine 5 ml
Gentamycin 1ml (50mg/ml)

Diluted 2-mecarptoethanol 500μl
Filter using 0.45μl pore size filter

Giemsa stain
Giemsa stain 4ml
Methanol 4ml
Double distilled water 92ml
Store at room temperature

H2O2/Azide solution
Stock solution
H2O2 – 30%
Azide – 1%
PBS – 10 x
Always prepared fresh from stock solutions:
Double distilled water 40ml
PBS 5ml
Azide 5ml
H2O2 500μl

0.05M Acetate buffer (PH5.1)
Stock solution A
CH3C00H (acetic acid glacial): to 500ml of double distilled water add 7.5ml of acetic acid and store at 4°C
Stock solution B

$\text{CH}_3\text{CooNa}$ (sodium acetate): dissolve 17g in 500ml of double distilled water to give 0.25M solution.

To make acetate buffer, mix 24ml of A with 76ml of B and dilute using 400ml of double distilled water to give 0.05M solution.

3-amino – 9 – ethylcarbozole (AEC)

Fleshly prepared less than 2 minutes before staining and kept at $28^\circ\text{C}$. 10mg dissolved in 2.5mls of dimethylformamide and 50ml acetate buffer added. This is filtered on paper. 100μl of 30% $\text{H}_2\text{O}_2$ is added just before use.

NaCl-Glycerol Mountant (pH 9.5)

Glycerol 45ml
0.9% NaCl 5ml
Tris base 500mg
Mix this base and NaCl before adding the glycerol.

Phosphate citrate buffer

Citric acid 0.1M
$\text{Na}_2\text{HPo}_4$ 0.2M
Distilled water 50ml
Appendix II

Olive Baboon: A juvenile and an adult olive baboon