CYTOLYTIC ACTIVITY OF CD4+ AND CD8+ T LYMPHOCYTES IN Plasmodium falciparum MALARIA SUSCEPTIBLE AND RESISTANT INDIVIDUALS IN A MALARIA ENDEMIC AREA OF WESTERN KENYA.

JOHN MICHAEL OBOR ONG'ECHA, BSc. (Hons.)
156/7481/91

A Thesis in partial fulfilment for the award of Master of Science (MSc.) degree in Immunology of Kenyatta University.

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DECLARATION

I John M. O. Ong'echa, hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

Signed

Date

John M. O. Ong'echa

I Professor A. S. S. Orago, hereby declare that this thesis has been submitted for examination with my approval as University supervisor.

Signed

Date

Professor A. S. S. Orago

I Dr. Walter R. Weiss, hereby declare that this thesis has been submitted for examination with my approval as second supervisor.

Signed

Date

Dr. Walter R. Weiss
DEDICATION

I wish to express my sincere appreciation to my supervisors, Professor William Ouma and Dr. Peter Were, for their guidance and encouragement during my studies. Special thanks go to their respective institutions, the Department of Brewing and Distilling Technology, Makerere University, and the Department of Food Science and Technology, University of Agriculture and Applied Sciences, for providing the necessary facilities and assistance. Professors William Ouma and Peter Were, and Assistant Professor John Ouma, for their invaluable advice and guidance throughout my studies.

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ABSTRACT

Acquired immunity in malaria is both species- and stage-specific, with both B- and T-cells being involved. Cell-mediated immunity by T-cells involves cytokine production and the direct killing of infected hepatocytes and/or sporozoites. This cytolytic ability is effected by cytotoxic T lymphocytes (CTL). One epitope recognized by the CTL (Th3R) spans the amino-acid sequence 368-390 of the circumsporozoite (CS) protein on the surface of the sporozoites and infected hepatocytes. This epitope has significant antigen diversity, thus posing a potential problem in producing a pre-erythrocytic vaccine.

In this study, peripheral blood lymphocytes (PBL) from adult male residents of a malaria endemic area were expanded for six days using rIL-2 and malaria synthetic peptides representing the 368-390 amino-acid sequence of the \textit{Plasmodium falciparum} CS protein. The effector cells were then tested against Epstein-Barr virus (EBV) lymphoblastoid targets pulsed with or without the peptides. Malaria specific lysis was determined as the difference between lysis in the presence of a peptide and lysis in the absence of a peptide. Specific lysis of 10% or more was considered as a positive response. Samples from 40 individuals were assayed. Ten individuals (25%) showed CTL activity, 13 individuals (32.5%) showed negative responses and the remaining individuals (42.5%) had 'indeterminate' results. There was no relationship between CTL responses and susceptibility or resistance to \textit{P. falciparum} re-infection (U=61.5; $U_{0.05(2)}^{9,13}=89$; $P>$
However, there was cross-reactivity of the 368-390 peptide variants. The CTL responses were MHC-restricted, with the short peptide 127 (368-379) being the optimal size for the CTL epitope.
CHAPTER ONE

1.0. INTRODUCTION AND LITERATURE REVIEW

1.1. Human malarial infections

1.1.1. Human malaria parasites

Malaria infections are caused by protozoan micro-organisms belonging to the family Plasmodiidae within the order Coccidiida. They fall within the sub-order Haemosporidiidae which comprises various parasites found in the blood of reptiles, birds, and mammals. Members of the genus *Plasmodium*, have an asexual multiplication [schizogony] within the parenchymal cells of the liver of the vertebrate host while sexual division [sporogony] takes place within the midgut of female mosquitoes of the genus *Anopheles*. There are about 120 known species of plasmodia with at least 22 species found in primate hosts. The four generally recognized species of malaria parasites of man are *P. malariae*, *P. vivax*, *P. falciparum*, and *P. ovale* (Bruce-Chwatt, 1985). Of these four species, *P. falciparum* causes lethal malaria while *P. vivax* and *P. ovale* are responsible for relapsing malaria (Kumar *et al.*, 1992) due to the formation of hypnozoites within the hepatocytes (Fig. 1). *P. falciparum* infections result in high mortalities if untreated in children, non-immune adults and gravid patients, while the other species rarely cause death (Howard, 1986). *P. falciparum* is the predominant species in tropical Africa and now accounts for over 40% of all reported cases of malaria in the rest of the world as reported by World Health Organization (WHO, 1991).
The life cycle of the malaria parasite. (Adapted from Hospital Practice, September 15, 1990, by permission of HP Publishing, New York)
1.1.2. Epidemiology and global impact of human malaria

According to current estimates (WHO, 1993a), over 40% of the world population is at risk of contracting malaria. The global incidence of malaria is estimated at 270-480 million clinical cases per year, with 140-280 million cases being in children under 5 years old (WHO, 1993b). Furthermore, 1.4 - 2.8 million deaths occur every year with 1 million being in children under the age of 5. Other reports indicate that 56% of the world's population live in malaria endemic areas (Peters, 1990). Africa alone accounts for more than 90% of all malarial infections worldwide (Marsh, 1992) and has most of its area classified as being holoendemic (WHO, 1992). Malaria infections occur mostly among rural human population living in lowlands and coastal areas (Marsh, 1992). This appears to be due to the belief that cool highland temperatures lengthened the duration of the extrinsic cycle of the parasite to exceed its life span, thus prohibiting stable transmission (Metselaar, 1959). However, recently, malaria epidemics have been reported in highland areas of Kenya (Khan et al., 1991), some with temperatures as low as 12°C (Anthony et al., 1992). The prevalence of malaria is not homogeneous as differences have been observed within different parts of the same villages in West Africa (Greenwood, 1989).

1.1.3. Malaria in children

The brunt of malarial mortalities is mainly borne by children and pregnant women. It has been established that pregnant women and children below 5 years of age have a physiologic depression of the immune system and an underdeveloped immune system respectively (Weinberg, 1984). Severe hypoglycaemia is a particular problem in pregnant women.
and African children with severe malaria (Marsh, 1992). Other reports indicate that 1-2 million children under the age of 5 years die of malaria annually in Africa (Greenwood et al., 1991). At the global level, between 2-5 million deaths due to malaria occur annually (Greenwood et al., 1991). Although malaria-specific death rates are not known for most areas, Greenwood et al. (1987), reported that approximately 10 deaths/1000/year occur in the 1-4 year age-group of The Gambian children. In stable endemic areas children were resistant during the first 6-months post-partum, after which they become susceptible to severe malaria and sometimes death (Marsh, 1992). After the age of 5 years, the frequency of clinical disease diminishes and malaria-specific mortalities become rare. Marsh et al. (1989) observed that during the period of maximum susceptibility to clinical disease, children were still parasitaemic most of the time although clinically well. Resistance during the first few months after birth has been attributed to a probable passive transfer of protective maternal antibodies (Chizzolini et al., 1991), although other factors such as persistent haemoglobin F [HbF] could also play a role (Weatherall and Clegg, 1981).

1.1.4. Malaria and pregnancy

Women living in holoendemic malarious areas frequently develop increased prevalence and severity of malaria infection during pregnancy (Brabin, 1985) with the infection being 4-12 times, more common in pregnant women compared with non-pregnant women, contributing to reduced birth weight by between 55-650g (Hutter, 1987). Malaria is also the most important cause of abortion in hyperendemic areas (Akinboye and Ogunrinade, 1987). The primigravidae developed more severe and
frequent complications (Kaseje et al., 1987). Brabin (1983) observed that the peak prevalence of infection occurs at 13-16 weeks gestation and infants, especially first-borns to infected mothers, have lower birth-weights and are at increased risk of prenatal and neonatal infections (WHO, 1984). Resistance to malaria during pregnancy was regained with increasing parity, although the precise mechanism through which this was achieved is still unknown. In high transmission areas the maternal placenta was more parasitized than the peripheral blood (McGregor, 1987), with the placental infection being more frequent and heavier in primigravidae (McGregor et al., 1983). Furthermore, McGregor (1987) noted that, the new uteroplacental vasculature generated in the primigravidae during pregnancy, were immunologically naive tissues having had no previous exposure to malaria, thus allowing for parasite colonization. During infected pregnancy, local immunity develops and immunologic memory of infection is retained in the uterus post-partum to protect future infections. However, recent studies by Rasheed et al. (1992) showed that the placental intervillous mononuclear cells contain fewer T cells, with low mean CD4:CD8 cell ratio and more monocytes/macrophages compared with those of the peripheral blood. This could account for the observed immunoinssufficiency. Suppressed T cell proliferation was also reported in primigravidae and pregnant women in general (Riley et al., 1989). In addition, corticosteroids which suppress cell-mediated immunity, were increased in primigravidae and in malaria-infected pregnancies relative to other pregnant women (Vleugels et al., 1987). However, malaria reduces birth-weight in endemic areas despite differences in parasite densities and placental involvement, suggesting that
instead of interfering with nutrient transport to the foetus, malaria alters a systemic process that regulates foetal growth (Nosten et al., 1991).

1.1.5. Malaria in non-immune individuals

The non-immune or naive individuals are those who have never been exposed to malaria infections in their life time hence, have not been able to develop acquired immunity to subsequent infections. These include mainly those who reside in non-malarious regions. In such individuals at any age, acute malaria infection virtually implies a clinical case (Marsh, 1992).

1.1.6. Pathology and clinical manifestations of malaria in humans

Clinical manifestations of P. falciparum malaria infections can be either mild or severe depending on the stage of the disease and the immune status of the infected individual. The mild clinical states include paroxysms of fever, aches, nausea, hepatosplenomegaly, and hypergammaglobulinaemia (Bruce - Chwatt, 1985). Some of the severe manifestations and complications of falciparum malaria as recognized by the WHO include cerebral malaria, severe anaemia, renal failure, hyperthermia, respiratory distress and hypoglycaemia. Of these severe complications, cerebral malaria is the most common clinical manifestation and cause of death (WHO, 1986). However, severe anaemia, another complication with high mortality figures, is responsible for more deaths than cerebral malaria in some areas of tropical Africa (Greenwood et al., 1991). Cerebral malaria may present as fever and headache, diarrhoea and intermittent hallucinations, comatose with renal failure, sepsis, pneumonia, and adult respiratory distress syndrome (Hoffman, 1991). The
aetiology of cerebral malaria has been ascribed to rosette formation and/or cytoadherence in the cerebral microvasculature (Carlson, 1993). The clinical course and outcome of falciparum infection can be influenced by socio-economic factors such as occupation and educational level (Banguero, 1984). This is so considering that prompt drug treatment is essential in falciparum malaria in which the interval between the onset of severe symptoms and death is only 2.8 days (Greenwood et al., 1987). Also critical, is the observation that over 50% of deaths due to cerebral malaria in hospitals occur between 12-48h. after admission (Kwiatkowski et al., 1990). This has been explained in part by the fact that P. falciparum parasitization leads to a series of interlinked pathophysiological processes which, once initiated have their own momentum, and it is not unusual for mortalities to occur after appropriate anti-malarial therapy has had time to work and even clear parasitaemia (Hoffman, 1991).

1.1.7. Vectors of malaria parasites

The principal vectors of human malaria in sub-Saharan Africa are the Anopheles gambiae Giles s.l. complex and An. funestus Giles s.l. (Wekesa et al., 1992). The prevalence of these vectors vary from one region to another although most studies have demonstrated An. gambiae complex as the main vector with An. funestus playing a secondary role (Beier et al., 1990). It was recently reported that the An. gambiae s.l. complex comprises the predominantly anthropophilic An. gambiae and the partially zoophilic An. arabiensis (Petrarca et al., 1991).

1.1.8. Parasite resistance to chloroquine
Armed with new drugs and potent pesticides, the WHO declared in 1955 that malaria was soon to be eradicated in a 5-year programme of intensive spraying combined with aggressive chloroquine treatment for the infected people. However, by late 1960s, the once potent pesticide Dichloro-diphenyl-trichloroethane (DDT) fell out of favour and chloroquine lost its punch forcing WHO to embrace the more modest goal of control (Bruce - Chwatt, 1985). Thus, the worldwide resurgence of malaria and the number of people affected by this disease, which is growing at an alarming rate, is due to limited effectiveness of anti-malarial drugs and insecticides. According to a review by Hoffman (1991), *P. falciparum* resistance to chloroquine, once the ideal drug for the prevention and treatment of malaria, was first reported in Thailand and Colombia in the late 1950s. By 1990, chloroquine-resistance had been reported from all malarious areas of the world except for the Islands of Hispaniola in the Carribean, Central America above Panama, and the Middle East. The emergence of chloroquine resistance in Africa was documented for the first time in Kenya in the late 1970's (Wernsdorfer and Payne, 1991). Chloroquine resistant *P. falciparum* is widespread today in Africa, with its distribution not being homogeneous (Wernsdorfer, 1991). It has been further reported that *P. vivax* also shows chloroquine resistance, albeit of low incidence (Wernsdorfer, 1991).

1.2. Protective immunity to malaria

Studies in malaria endemic areas have shown that some individuals are more susceptible to infection by *P. falciparum* than others (Hill, 1992).
This resistance could be mediated by either the innate or the acquired arms of the immune system.

1.2.1. Innate immunity

This is the immunity an individual has right from birth, and is also known as natural immunity. In many malaria endemic areas, a variety of genetic red cell [erythrocyte] disorders have been shown to confer protection against malaria infections.

1.2.1.1. Sickle-cell trait

The most studied disorder, the sickle cell trait, characterized by an abnormal haemoglobin genotype [HbAS], has recently been shown to confer more than 90% protection in African children against cerebral malaria and severe malaria-related anaemia, although its effect on parasitaemia levels was less pronounced (Hill et al., 1991). The protection effected by this trait is more marked against the disease itself than against malaria infection (Willcox et al., 1983) as individuals can acquire P. falciparum malaria, but display reduced fatality and severity of the disease. Although this trait is favoured by evolution due to the protection it confers against severe malaria in its heterozygous form, the homozygous state results in high mortalities in children (Carlson, 1993). Many studies have been carried out on the protective nature of the trait, however, the precise mechanism of this protection is still unknown. Several workers have proposed different hypotheses trying to explain the mechanisms behind this protection. Luzzatto et al. (1970) suggested that parasitized erythrocytes bearing the AS genotype sickled in the spleen and are removed by phagocytosis. Pasvol et al. (1978) in in vitro studies observed
that *P. falciparum* grew less readily in erythrocytes with AS genotype than in those with AA genotype under conditions of low oxygen tension. Later on, Orjih *et al.* (1985) proposed that under conditions of oxygen stress, AS genotype erythrocytes sickled and released ferriprotoporphyrin IX which is toxic to malaria parasites. Bayoumi (1987) concluded that the protective effects were due to the fact that individuals having this abnormal genotype had enhanced immune responses. This suggestion was supported by the finding that cell-mediated immune responses to malaria antigens in subjects with HbAS were higher than in those with HbAA genotype (Bayoumi *et al.*, 1990). Another hypothesis put forward was that erythrocytes having the AS genotype infected with *P. falciparum* did not bind as well as AA genotype, to endothelial receptors or to non-infected erythrocytes [rosetting]. However, studies by Udomsangpetch *et al.* (1993) did not find any association between haemoglobin genotypes and rosette formation. Sicard *et al.* (1979) observed the existence of a casual relationship between the occurrence of HbE and resistance to malaria but the details of this observation by subsequent workers is still eagerly awaited. The presence of HbF was also reported to play a role in the reduction of parasitaemia, especially in children under the age of 6-months (Weatherall and Clegg, 1981). Furthermore, the persistence of this trait in adults, following a failure to switch to the production of normal HbAA during the first 5 years of life, could also play a role in the protection against severe malaria. This protection was suggested to be due to the impairment of *P. falciparum* growth in presence of HbF (Pasvol *et al.*, 1977) possibly due to increased intra-erythrocytic oxidative stress (Friedman, 1979).
1.2.1.2. **Blood groups and malaria infections**

Recent studies in The Gambia (Hill, 1992) revealed that blood group O confers protection against cerebral malaria compared to blood groups A and B. Carlson and Wahlgren (1992), observed that the protection against cerebral malaria conferred by blood group O phenotype could be mediated via impaired rosette formation [the spontaneous binding of uninfected erythrocytes to *P. falciparum* -infected erythrocytes]. Results in support of this suggestion were provided by Udomsangpetch *et al.* (1993) who observed a very strong association between rosette formation and ABO blood group, with group A and B erythrocytes forming rosettes more readily than group O. Carlson (1993), suggested that the impairment of rosetting may hamper parasite growth, enhance parasite clearance by phagocytosis and prevent the pathogenesis of cerebral malaria by preventing sequestration in the brain microvasculature. Studies elsewhere have indicated that rosette formation protects against phagocytosis by leucocytes (Ruangjirachuporn *et al.*, 1992). Luzzi *et al.* (1991a) suggested that the observed increases in phagocytosis of parasitized erythrocytes in thalassaemia and sickle cell trait could possibly be due to impaired rosetting capacity.

Hadley *et al.* (1986) reported that the invasion of erythrocytes by malaria parasites is dependent on binding of parasite proteins to receptors on the erythrocyte surface. Both *P. vivax* and the simian parasite *P. knowlesi* invade only human erythrocytes expressing the Duffy blood group while *P. falciparum* invades human erythrocytes irrespective of Duffy blood group (Holder, 1994). Thus, the basis for the observed *P. vivax* resistance in blacks has been ascribed to the Duffy blood group negative genotype FyFy (Miller *et al.*, 1976). Susceptibility of both Duffy
blood group-negative and positive individuals to *P. falciparum* infection has been explained in part by the observation that: unlike *P. vivax* which requires the Duffy blood group antigen for erythrocyte invasion, *P. falciparum* binds specifically to sialic acids on erythrocyte glycophorin (Camus and Hadley, 1985). However, Mitchell *et al.* (1986) observed that some *P. falciparum* strains could invade sialic acid-deficient erythrocytes.

### 1.2.1.3. Thalassaemia traits

Individuals with the β-thalassaemia trait are protected against *P. falciparum* infection and against *P. falciparum* malaria-related severe complications (Willcox *et al.*, 1983). Mild forms of α-thalassaemia have also been observed to confer protection against malaria, albeit at a lower degree (Flint *et al.*, 1986). Protection in α- and β-thalassaemia was suggested to be due to accelerated immune clearance by phagocytosis (Brockleman *et al.*, 1987). Recently, Luzzi *et al.* (1991b) reported enhanced immunoglobulin binding to the surface of parasitized erythrocytes. This opsonizing effect supports the phagocytic role and has been shown to facilitate phagocytosis of infected erythrocytes (Yuthavong *et al.*, 1990). The involvement of rosetting in the protection against malaria has been supported by the observation that erythrocytes from thalassaemic individuals have reduced rosetting capacity when compared with erythrocytes from normal individuals (Carlson 1993).

### 1.2.1.4. Glucose-6-phosphate dehydrogenase [G6PD] deficiency

This is an enzymopathy of erythrocytes which has been observed to provide resistance to malaria (WHO, 1986). The immunologic involvement in the protective effects of G6PD deficiency was recently adduced by the
observation that ring-infected G6PD-deficient erythrocytes were preferentially phagocytosed by human monocytes (Giribaldi et al., 1992).

1.2.1.5. Iron deficiency

Another factor which has been associated with protection against malaria is malnutrition, especially iron deficiency. Oppenheimer et al. (1986) observed increased susceptibility to clinical malaria following correction of the iron deficiency in Papua New Guinea. Although Ahmad et al. (1985) reported a correlation between weight-for-age and parasitaemia in children, the role of iron deficiency in protection is still debatable, more so, given that the effects of nutritional factors are difficult to separate from other closely related social factors in this kind of study (Greenwood et al., 1991).

1.2.2. Acquired immunity

Acquired immunity is mediated by two branches of the immune system, the humoral and the cell-mediated responses. The former is mediated mainly by antibodies with B-cells as the principal cells while the T-cells are the most important effector cells in the cell-mediated responses. However, the delineation into the two separate branches is simplified for easier understanding as most of the effector functions of the two branches are usually intertwined. For example, T-cells are central to the development of both humoral and cell-mediated responses (Hodes, 1989).

Acquired immunity to malaria develops gradually and slowly after repeated exposures (McGregor et al., 1956), and is both species- and stage-specific (Howard, 1986). This immunity is not absolute, as parasitaemia
persists in the absence of clinical disease (Neva, 1977). The specificity of acquired immunity to malaria is proposed to be due to antigenic diversity displayed by the various species and stages, and is the possible cause of the slow development of protective immunity in individuals living in malaria endemic areas (Howard, 1987). Troye-Blomberg and Perlmann (1994) concluded that the slow development of immunity to malaria in humans could be due to parasitic suppression of the specific and non-specific immune responses of children. Elsewhere, it has been proposed that the slow development of protective immunity could partly be due to the polymorphism of the T-cell epitopes (Udhayakumar et al., 1994). Similarly, Ho and Webster (1989), hypothesized that the immune response to malaria antigens consisted of a series of primary immunizations with rare secondary responses, hence, the gradual build up of protective immunity.

1.2.2.1. Immunity to sporozoites and hepatic stages

1.2.2.1.1. Sporozoite antigens

Sporozoites have a developed protein coat known as the circumsporozoite protein [CSP]. The CSP, a 51 KDa membrane protein (Zavala et al., 1983), constitutes the major surface antigen of the sporozoite stage of malaria parasites (Nussenzweig and Nussenzweig, 1989), and was first identified on P. berghei by Yoshida et al. (1980). It is also present (although in small amounts) in other developmental stages such as the erythrocytic forms (Atkinson et al., 1989), and in blood schizonts of plasmodia (Cochrane et al., 1989). The surface membrane of salivary gland sporozoites are uniformly covered with the CSP, the main protein
synthesized by the mature infective stage of the parasite (Yoshida et al., 1981), while present only in small quantities on non-infective sporozoites from oocysts (Aikawa et al., 1981).

The gene for the CSP of *P. falciparum* has been cloned and has been shown to encode a protein of 412 amino acids. Of these polypeptides, 40% are included in 41 tandem repeated tetrapeptides: 37 of which are Asparagine-Alanine-Asparagine-Proline [NANP] and 4 are Asparagine-Valine-Asparagine-Proline [NVNP] (Dame et al., 1984). At the flanks of the repeat region, there are two non-repeat sequences, regions I and II, located in the N- and C-terminal regions respectively. The non-repeat regions are relatively conserved in the CSP of all plasmodial species (Dame et al., 1984).

Recently, a new sporozoite surface antigen, the sporozoite surface protein 2 [SSP2], was reported in *P. yoelii* (Hedstrom et al., 1990). A *P. falciparum* homologue of PySSP2 was suggested by Robson et al. (1988) to be the thrombospondin-related anonymous protein [TRAP]. Following the characterization of the gene encoding the PySSP2, it was observed that SSP2 bore striking similarities to TRAP (Rogers et al., 1992a). Studies on the characterization of *P. falciparum* SSP2 by Rogers et al. (1992b) confirmed that TRAP is *P. falciparum* SSP2. The PfSSP2 was also reported to be a 90KDa protein (Rogers et al., 1992b) and shares sequence homology with CS exclusively in region II (Hedstrom et al., 1990).

1.2.2.1.2. Sporozoite infectivity

In highly endemic regions, nearly 100% of the people become infected at biting rates of approximately one infected bite per 24h. (Hoffman et al., 1987). Other reports indicate that except for highly
endemic areas, the number of sporozoites inoculated by a single mosquito is small, probably fewer than 100 (Beier et al., 1991). The observed speed and efficiency of infection and uniqueness of the target cell led Cerami et al. (1992a) to suggest that hepatocyte invasion is mediated by specific ligands on the parasite surface membranes for receptors on the host hepatocytes. Although the precise mechanism of hepatocyte invasion was not known, it was suggested that sporozoites could enter hepatocytes directly from the circulation (Shin et al., 1982) or by first being recognized by Kupffer cells (Meis et al., 1983) or endothelial cells prior to entering hepatocytes. However, recent findings by Cerami et al. (1992b) suggest that the ligand for the liver receptor is the sulphated glycoconjugate binding (SGB) motif contained within region II of the CS, and that no binding to Kupffer cells or endothelial cells is involved. They also observed that antibodies to CS repeats prevent invasion, but not attachment to target cells as earlier reported (Hollingdale et al., 1982). More recently, the hepatocyte receptors were demonstrated to be heparan sulfated proteoglycans [HSPGs] (Frevert et al., 1993) with a molecular weight of 400-700 KDa.

1.2.2.1.3. Sporozoite immunogenicity

Sporozoites are immunogenic and capable of eliciting protective immune responses in several host species including humans (Miller et al., 1986). Immunization of animals and humans with radiation attenuated sporozoites provides solid sterile immunity to malaria (Egan et al., 1993). This immunity has been reported to induce humoral (Charoenvit et al., 1987) and cellular (Malik et al., 1991) immune responses against the CSP.
1.2.2.1.4. **Sporozoite antibody responses**

Malaria infection rapidly induces an increase in immunoglobulin synthesis (Cohen *et al.*, 1961) with high titres of antibodies being reported in residents of malarious areas (McGregor, 1974). Following an acute, clinical *P. falciparum* infection, the antibody (IgM and IgG) responses against the *P. falciparum* CSP antigen are irregular and short-lived (Brown *et al.*, 1991). Petersen *et al.* (1992) also reported that the antibody variability could even be observed within and between individuals naturally primed by repeated infections and long periods of subclinical, low level persistent parasitaemia. Most anti-sporozoite antibodies detected in humans and other hosts immunized with sporozoites were directed against the repeat regions of the CSP (Zavala *et al.*, 1983). Protection against sporozoites was partly mediated by antibodies since monoclonal antibodies and immune sera were shown to neutralize sporozoite infectivity in animals and in cultured cells (Miller *et al.*, 1986). However, Freeman *et al.* (1980) observed that only a small portion of the immunoglobulin is parasite-specific. The observed immune suppression in acute malaria infections (Greenwood *et al.*, 1982) was suggested to be due to polyclonal B-cell activation, which has been proposed to result in depressed specific responses to other pathogens or to vaccinations (Greenwood, 1984). The humoral immune response to the CSP, has been reported to be thymus-independent (Schofield, 1990) and primarily produced low affinity, non-neutralizing antibodies (Schofield, 1991). Although most CSP antibodies were directed against the repeat region (Zavala *et al.*, 1983), recent studies have demonstrated the presence of CS antibodies directed against epitopes outside the repeat region in adult residents of malaria endemic areas. Del Giudice *et al.* (1988) reported the
presence of naturally acquired antibodies to epitopes in the N-terminal, while antibodies to the C-terminal flanking region of the CSP of *P. falciparum* was reported by Stuber *et al.* (1990). The antibody responses to the repeat-free flanking regions, induced by natural infection, was short-lived and of low-to-moderate magnitude (Brown *et al.*, 1992). Although the precise function of the CSP antibodies has not been elucidated, monoclonal antibodies against the major repeat region of the CSP can prevent liver infection in rodent malaria (Charoenvit *et al.*, 1987). Mazier *et al.* (1986) showed that antibodies raised against the CSP of *P. falciparum* appeared to inhibit sporozoite attachment to, entry into, and development in human hepatocytes. It was observed that boosting of CS antibodies developed poorly, and that the antibody levels may not be sufficient to confer protection in natural infection, since serum antibody half-life was less than one month (Webster *et al.*, 1988). Significant anti-CS antibody titres were observed to develop slowly after years of exposure although no association could be demonstrated between presence of antibody and protection (Riley *et al.*, 1990). Furthermore, apparently immune adults have been reported to lack these antibodies (Quakyi *et al.*, 1989). Other studies in malaria endemic areas have shown little correlation between naturally acquired anti-sporozoite antibodies and resistance to infection (Pang *et al.*, 1988).

1.2.2.1.5. Sporozoite T-cell responses

It has been consistently reported that immunity induced by irradiated sporozoites is strongly dependent on cell-mediated immune responses to sporozoites or exoerythrocytic antigens (Verhave *et al.*, 1978). Although antibodies contribute to some immune protection, priming of T-
cells was important for the development and maintenance of immunity to malaria (Good and Miller, 1989). It was also reported that efficient production of anti-malaria antibodies required an intact and functioning T-cell system (Weidanz and Long, 1988). Similarly protective immunity induced by irradiated *P. berghei* sporozoites was mediated by antigen-specific T-cells (Egan *et al.*, 1987). These cells were suggested to participate in cytotoxic or lymphokine-mediated killing of erythrocytic parasites in the liver (Ferreira *et al.*, 1986). The observation by Miller *et al.* (1986), that B-cell depleted mice could be successfully immunized with radiation-attenuated sporozoites suggested that T-cells alone may be sufficient for sporozoite-induced immunity in mice.

Human proliferative helper (Zevering *et al.*, 1990) and cytotoxic T lymphocyte [CTL] domains were reported to map to the same general region, although fine differences did occur (Doolan *et al.*, 1991a). These epitopes, [domains] were shown to be the flanking regions of the CSP (Good *et al.*, 1988). Studies among malaria immune adults in The Gambia identified two T-cell epitopes [Th2R (326-345) and Th3R (361-380)] located outside the repeat region of the *P. falciparum* CSP (Good *et al.*, 1987). These two epitopes were recently reported to be polymorphic, their polymorphism being highly restricted in certain geographic regions (Doolan *et al.*, 1992). Similarly, Shi *et al.* (1992) reported that parasite isolates from high endemic areas exhibited greater antigenic diversity than those from low endemic areas, with some variant sequences being unique to particular geographical areas.

There are two major T-cell subsets [CD4+ and CD8+ cells] that play some role in immunity to malaria. The CD8+ T-cells are antigen-specific cytotoxic cells recognizing antigens in association with the Major
Histocompatibility Complex [MHC] class I molecules, while the CD4+ T-cells are central regulatory cells responsible for the induction of cytotoxic T-cells and for antibody production in response to antigens recognized in association with class II molecules. Recently, another T-cell subset, the γδ cells, were reported in acute *P. falciparum* infections (Ho *et al.*, 1990) although their function(s) is yet to be defined. Lymphocyte subset analysis in acute *P. falciparum* infection has consistently reported a high incidence of inverted CD4:CD8 ratios due to an absolute rise in CD8 sub-populations (Gunapala *et al.*, 1990; Orago and Facer, 1991).

1.2.2.1.5.1. The role of CTL

Protective immunity against pre-erythrocytic stages of malaria is now thought to be mediated primarily by CTL (Hoffman *et al.*, 1994). These CTL have been reported to express either the CD8 (Malik *et al.*, 1991) and/or CD4 (Moreno *et al.*, 1991) surface markers.

1.2.2.1.5.1.1. CD8+ CTL

CSP-specific and class-I-restricted CD8+ CTL have a major role in protective immunity against plasmodial infections (Romero *et al.*, 1989). Weiss *et al.* (1990) observed that the role played by the CTL in sporozoite-induced immunity in the rodent model is directed at the liver stages of the parasite. Immunity developed in mice vaccinated with irradiated *P. berghei* or *P. yoelii* sporozoites was shown to be dependent on the presence of CD8+ T-cells as injection of these mice with anti-CD8 monoclonal antibodies abrogated the protection (Weiss *et al.*, 1988). Hoffman *et al.* (1989b) also reported that CD8+ T-cells from mice immunized with irradiated *P. berghei* sporozoites lysed sporozoite-
infected hepatocytes *in vitro*, in an MHC class-I-restricted manner. Furthermore, it was observed that oral immunization of mice with an attenuated *Salmonella typhimurium* recombinant containing the *P. berghei* CS gene induced specific and protective CD8+ T-cells (Aggarwal *et al.*, 1990). The epitopes recognized by the CD8+ CTL from immune mice were reported to be short C-terminally located amino acid sequences of the CSP of both *P. berghei* and *P. yoelii* (Hoffman *et al.*, 1990). Doolan *et al.* (1991a) observed that some individuals with sporozoite exposure contain peripheral blood mononuclear cells [PBMC] which can be expanded to form CS-specific CTL with only a small percentage having peripheral blood precursors. CD8+ CTL against the CSP of *P. falciparum* has been isolated from protected volunteers immunized with *P. falciparum* irradiated sporozoites (Malik *et al.*, 1991) or from individuals naturally exposed (Sedegah *et al.*, 1992). The adoptive transfer of some CD8+ T-cell clones into naïve mice was observed to inhibit the development of the intrahepatocytic stages of the parasites (Rodrigues *et al.*, 1991). It was recently reported that both protective and nonprotective CTL clones do not differ in their fine specificity as earlier reported (Casanova *et al.*, 1991), but in their expression of certain adhesion molecules, specifically, a CD44 and VLA-4 molecules (Rodrigues *et al.*, 1992).

1.2.2.1.5.1.2. CD4+ CTL

Recently, exposure to sporozoites was reported to induce class II restricted cytotoxic CS-specific CD4+ T-cells in humans (Moreno *et al.*, 1991), which recognize an epitope in the *P. falciparum* CSP different from the C-terminal epitope recognized by the human CD8+ CTL (Malik *et al.*, 1991). Murine CD4+ CTL clones were observed to confer extensive
protection against *P. berghei* sporozoite challenge upon passive transfer to naive mice (Tsuji *et al*., 1990). Following the observation that human liver parenchymal cells could express class II [HLA-DR] molecules during the course of transplantation reaction (Steinhoff, 1990) and viral infections (Van den Oord *et al*., 1986), it has been proposed that the CD4+ CTL could probably function in the immune protection by lysing infected hepatocytes after recognizing class II MHC/CS peptide complexes on the parenchymal cell surface (Moreno *et al*., 1991).

1.2.2.1.6. Responses to infected hepatocytes

The malaria epitopes on the surface of infected hepatocytes have been proposed to induce antibody-dependent cellular cytotoxicity [ADCC]. A heat-shock-like protein, HSP 70-1, expressed on the surface of *P. falciparum* -infected hepatocytes, was the target of ADCC mediated by the non-parenchymal cells of the liver (Renia *et al*., 1990). The non parenchymal cells involved could either be Kupffer and/or natural killer cells, with the former being proposed to function by producing IL-6 which has been reported to exert its antimalarial effect through L-arginine-dependent cytotoxicity (Nussler *et al*., 1991), synthesis of acute phase proteins (Pied *et al*., 1989) and by the induction of an oxidative burst (Pied *et al*., 1991).

1.2.2.2. Immunity to asexual blood stages

1.2.2.2.1. Asexual blood stage antigens

A large number of potentially protective antigens have been identified in the asexual stages. These antigens exhibit antigenic diversity
and variation (Oaks et al., 1991). Holder (1988), described a precursor to major merozoite surface antigen [PMMSA] which refers to a group of asexual blood stage antigens of malaria synthesised during schizogony and expressed on the surface of merozoites. These antigens include the 195 KDa (Howard et al., 1984), p195 (Holder et al., 1985), p190 (Hall et al., 1984) and gp185 (Howard et al., 1985) in *P. falciparum*. The gp 195 is also called merozoite surface antigen-1 [MSA-1], merozoite surface protein-1 [MSP-1] or PMMSA. The MSA-1 is a 195-KDa glycoprotein processed at the time of schizont rupture to generate the majority of the antigens on the mature merozoite surface (Holder, 1988). Smythe et al. (1988) described another surface antigen [MSA-2], a 45-KDa polypeptide, which like MSA-1 appears to be anchored in the merozoite membrane by a glycosylphosphatidylinositol [GPI] moiety. Antigenic variation has been demonstrated in the asexual erythrocytic stages of the human malaria parasite, *P. falciparum*, with the variant antigen, PfEMP-1, being expressed on the surface of infected erythrocytes (Biggs et al., 1991). Another *P. falciparum* antigen, the Pf155/RESA has been reported to contain two major tandem repeats: a C-terminus of 5 tandem repeats of the sequence Glu-Glu-Asn-Val-Glu-His-Asp-Ala followed by over 30 tandem repeats of Glu-Glu-Asn-Val and the other repeat block at the centre of the molecule contains the sequence Asp-Asp-Glu-His-Val-Glu-Glu-Pro-Thr-Val-Ala (Favaloro et al., 1986). A glutamate-rich *P. falciparum* antigen, GLURP, is present in the exoerythrocytic schizont and in the erythrocytic schizont (Borre et al., 1991). Two schools of thought have arisen as to the origin of the antigens expressed on the surface of *P. falciparum*-infected erythrocytes. Newbold and Marsh (1990) proposed that these antigens were derived from the parasite, while Sherman and
Winograd (1990) suggested that these were host proteins resulting from parasite-induced modifications.

1.2.2.2. Antibody responses to asexual blood stages

It has been shown that antibodies are important for the clearance of parasite loads in both animal and human blood stage infections (Berzins et al., 1991). Cavacini et al. (1990) reported that full parasite clearance in *P. chabaudi* infection could only be achieved in the presence of B cells or antibodies, and individuals living in malaria-endemic areas had PMMSA-specific antibodies (Hall et al., 1984). The MSA-1 is immunogenic and has been proposed to be the target of protective immunity (Chang et al., 1992). Riley et al. (1992) observed that the prevalence of antibodies to all regions of the MSA-1 molecule increased with age, the highest prevalence being directed against the regions of the molecule that are highly conserved between parasite isolates. Human T and B cell domains in the MSA-1 have been localized to the conserved regions towards the N-terminal of the protein (Sinigaglia et al., 1988b). Chang et al. (1992) also observed that conformation dependent antibodies against the conserved C-terminal domain are important for protection. Monoclonal antibodies to the merozoite surface antigens inhibited parasite growth and merozoite re-invasion *in vitro* (Perrin et al., 1981). Furthermore, active immunization with merozoite surface protein purified from parasites could induce protective immunity against human malaria in various monkey models (Siddiqui et al., 1987). Similarly antibodies against antigens from merozoite organelles involved in invasion, inhibit parasite growth *in vitro* (Wahlin et al., 1984) and immunization with these antigens induced protective immunity in animal model systems (Oka et al., 1984).
Biggs et al. (1992) demonstrated agglutinating antibodies to the PfEMP-1 antigen which could inhibit the cytoadherence of infected erythrocytes to venular endothelium. An association of high antibody titres against GLURP$_{489-1271}$ fusion protein with low parasitaemia was reported in Liberian children 2-9 years of age (Hogh et al., 1992). The Pf155/RESA contains the previously defined immunodominant B and T cell epitopes (Perlmann et al., 1989) and antibodies to defined Pf155/RESA sequences have been associated with immune protection (Petersen et al., 1990). Recently, immunization of humans with a synthetic peptide Spf66, comprising three blood stage antigens conferred protection, the highest being among children aged 1-4 years (77.2%) and adults older than 45 years (67.0%) (Valero et al., 1993).

Although antibodies play an important protective role against erythrocytic malaria, accumulating evidence suggests that this humoral immunity is helper T cell dependent, relying on IL-4 production by activated CD4+ T cells (Troye-Blomberg et al., 1990). Suss et al. (1988) also observed that CD4+ T cells were the most important mediators of blood stage protection in the P. chabaudi system, while Boucharoun-Tayoun et al. (1990) reported that protection of the host was significantly correlated to phagocytosis of P. falciparum-infected erythrocytes. However Lelchuk and Playfair (1985) concluded that phagocytosis of infected host erythrocytes per se was not sufficient to confer protective immunity in rodent malaria.

1.2.2.2.3. T-cell responses to blood-stages

The CD4+ T-cells were suggested to play a major role in the control of blood-stage infections. Meding et al. (1990) observed that susceptible
mice infected with *P. chabaudi chabaudi* were protected by the CD4+ T-cells, mainly the TH1 type producing IFN-γ and IL-2. It had earlier been reported that the TH1 type CD4+ T-cells are activated during the acute phase of infection while after the control of the infection the CD4+ T-cells of TH2 type, responsible for antibody formation, are the major antigen-specific CD4+ T-cells (Langhorne *et al.*, 1989). Although a precise role for CD8+ T-cells has not been described in blood-stage immunity, Podoba and Stevenson (1991) reported that the CD8+ T-cells were involved in the development of blood stage immunity to *P. chabaudi* during the later stages. This occurred after activation by CD4+ T-cells (Weidanz *et al.*, 1990). Recently, Weiss *et al.* (1993) suggested that the role of CD4+ T-cells was not in the effector arm directly killing the parasite, but by inducing other immune effector mechanisms.

In humans, the existence of malaria specific, but functionally distinct subsets of CD4+ T-cells [TH1 and TH2] has been demonstrated. Berzins *et al.* (1991) demonstrated the protective role of antibodies in erythrocytic malaria in humans which, was T helper cell dependent (Troye-Blomberg *et al.*, 1990), relying on IL-4 production by activated CD4+ T-cells [TH2].

Although it has been observed that the number of γ/δ T-cells are increased during *P. falciparum* infections (Ho *et al.*, 1990) their precise role has not been ascertained. Being MHC-unrestricted, these cells could play an important role in the primary response to malarial infection by having a direct cytotoxic potential, thus killing parasitized erythrocytes.

1.2.2.3. **Immunity to sexual blood-stages**

In *P. falciparum* parasites several antigens have been identified on the surface of both male and female gametes having molecular weights
230-KDa (Carter et al., 1984); 48-KDa (Kumar and Carter, 1984) and 45-KDa (Vermeulen et al., 1985). A 25-KDa protein has also been reported on the surface of activated female falciparum gametes, zygotes and ookinetes (Kaslow 1990; Kaslow et al., 1989; Lal et al., 1990). Monoclonal antibodies to these antigens have been reported to block fertilization and subsequent zygote development (McGregor, 1987). Mouse antibodies against a Pfs 25 recombinant vaccine have been shown to inhibit P. falciparum development in An. freeborni mosquitoes (Kaslow et al., 1991).

1.2.2.4. The role of cytokines in protective immunity to malaria

1.2.2.4.1. IL-1

Low doses of IL-1 have been reported to inhibit the in vitro development of hepatic stages of P. falciparum and P. yoelii through the formation of C-reactive protein which bind sporozoites at an early phase of infection (Pied et al., 1989). However, following the observation that IL-1 enhanced binding of human malaria parasites to endothelium (Berendt et al., 1989), it may play a role in the pathophysiology of cerebral malaria.

1.2.2.4.2. IL-2

Kremsner et al. (1990) reported a decreased concentration of IL-2 in patients with acute P. falciparum malaria accompanied with high parasitaemia and elevated soluble IL-2R [sIL-2R] expression in non-immune individuals. The overproduction of sIL-2R was proposed to be due to imbalance in the production of IL-4 and IL-5 by T-cells following Loughnan and Nossal's (1989) report that IL-4 regulated the expression of the β-chain IL-2R [p75] while IL-5 controlled the expression of the α-chain
IL-2R [p55] on murine B-cells. The maliariostatic effect of varying doses of recombinant IL-2 on *P. falciparum* *in vitro* has been recently reported (Orao and Facer, 1993).

1.2.2.4.3. IL-6

IL-6 was reported to interfere with hepatic development of *P. falciparum* parasites (Pied *et al.*, 1991). However, it was observed that concentrations of circulating IL-6 and TNF-α correlated with parasitaemia and severity of infection in adult residents of a malarious area (Butcher *et al.*, 1990).

1.2.2.4.4. IFN-γ

IFN-γ had a protective role in murine *P. berghei* infections (Ferreira *et al.*, 1986). Schofield *et al.* (1987) observed that rIFN-γ collaborated with CD8+ T-cells and antibodies to protect *P. yoelii*-infected rats against sporozoite challenge. Recent work by Orao and Facer (1993) showed that rIFN-γ retarded the growth of *P. falciparum* *in vitro* cultures, the inhibition being due to defective schizont maturation rather than merozoite invasion. rIFN-γ has been demonstrated to be an effective adjuvant if administered intraperitoneally or subcutaneously with lysates of blood-stage *P. yoelii* in mice (Playfair and De Souza, 1987).

1.2.2.4.5. TNF-α

rTNF-α was reported to prevent the development of sporozoites within hepatocytes following sporozoite invasion (Ferreira *et al.*, 1986). Hviid *et al.* (1988) showed that TNF-α inhibited the growth of *P. falciparum* blood-stage malaria parasites *in vivo* but not *in vitro*.
Furthermore, it has been observed in experimental malaria (Clark and Chaudhri, 1989) and human malaria (Grau et al., 1989; Kwiatkowski et al., 1990) that the presence of TNF-α in host serum above some threshold leads to tissue injury which are predominantly associated with acute *P. falciparum* infections.

1.3. Malaria vaccine development

The resurgence of malaria and the increasing reports of drug-resistance by malarial parasites makes the need for an affordable and effective malaria vaccine for humans more urgent than ever. *P. falciparum* and other malaria parasites have a complex life-cycle (Fig. 1), that presents a major obstacle for vaccine development. Although immunity to malaria is stage-specific, Quakyi *et al.* (1992) suggested that effective resistance may not be stage-specific. Thus, a polyvalent or cocktail vaccine incorporating important target antigens from all the three stages of the parasite may be required.

Malaria vaccine development is now focussed on three types of distinct vaccines (Siddiqui, 1991) with each being directed against one of the stages of the life-cycle of the malaria parasite. These are anti-parasite vaccines rather than "anti-disease" vaccines (Troye-Blomberg and Perlmann, 1994), and include:

i. Preerythrocytic stage vaccines

ii. Asexual blood stage vaccines

iii. Sexual blood stage vaccines

1.3.1. Preerythrocytic stage vaccines
The preerythrocytic stages include the sporozoites in the bloodstream and the parasites developing within the hepatocytes. These vaccines are meant to prevent infection and plasmodial growth in the liver.

Solid sterile immunity to malaria infection has been reported in animals and humans immunized with radiation attenuated sporozoites (Egan et al., 1993). However, the use of whole sporozoites for immunization is not feasible because of the impracticality of producing enough parasites for large scale vaccine manufacturing. Hence, the current malaria vaccine research now geared to the construction of subunit vaccines either as synthetic peptides or recombinant proteins and more recently, DNA vaccines. However, induction of protective immunity has not been possible with recombinant or synthetic vaccine based on CSP alone (Hoffman et al., 1991) as that achieved when irradiated sporozoites are used.

Most of the anti-sporozoite vaccines have been based on the repeat region [NANP] of the CSP (Nussenzweig and Nussenzweig, 1989). The first subunit *P. falciparum* vaccine tested in humans was a recombinant CSP [R32tet32] expressed in *E. coli* (Young et al., 1985). About 15-30% of the adult population in a malaria endemic area failed to develop CS antibodies to this vaccine (Hoffman et al., 1987). Riley et al. (1990) also reported no association between the presence of anti-CS antibody and protection.

An additional sporozoite surface protein [SSP2], has been characterized (Rogers et al., 1992a). Following the observation that mice immunized with *P. yoelii* SSP2 and *P. yoelii* CSP were completely protected against challenge with *P. yoelii* parasites (Khusmith et al., 1991) the SSP2 is a promising additional immunogen in designing a sporozoite vaccine.
Several proteins expressed on the surfaces of infected hepatocytes have been reported with a potential role as vaccine immunogens in designing a preerythrocytic stage vaccine. Renia et al. (1990) reported a heat-shock-like protein [HSP70-1], a part of the C-terminal fragment of the *P. falciparum*, as the first malaria antigen expressed on the surface of infected hepatocytes. Another antigen expressed on the surface of infected hepatocytes is the liver stage antigen-1 [LSA-1] (Zhu and Hollingdale, 1991).

1.3.2. Asexual blood stage vaccines

The vaccines against the merozoite stage have received much attention since this stage is responsible for the morbidity and mortality of malaria. Several merozoite antigens have been identified as potential vaccine candidates. These include; MSP-1 (Holder, 1988), MSA-2 (Smythe et al., 1988), PfEMP-1 (Biggs et al., 1991), Pf155/RESA (Favaloro et al., 1986), GLURP (Borre et al., 1991), and RAP-1 (Brown and Coppel, 1991).

Recently a *P. falciparum* asexual blood-stage synthetic vaccine, SPf66, was reported to confer a protective efficacy of 33.6% against first or only episodes and 50.5% against second episodes in a phase III trial (Valeró et al., 1993). The SPf66, a polymeric synthetic peptide consisting of 45 amino acids, is derived from portions of three *P. falciparum* blood-stage antigens: 55.1, 83.1, and 35.1 - joined together by the CSP repeat sequence [PNANP] (Millet et al., 1993).

1.3.3. Sexual blood stage vaccines

These vaccines are also known as transmission-blocking vaccines, because they induce production of antibodies that prevent transmission
from the vertebrate host to the mosquito vector. Several candidate immunogens for malaria transmission blocking immunity have been reported. These include the genetically restricted 230-KDa gamete surface antigen (Quakyi et al., 1987), 48/45-KDa, and 40/10-KDa surface proteins (Kaslow, 1990). However, a 25-KDa antigen [Pfs 25], expressed predominantly by zygotes and ookinetes, is reported to be widely immunogenic and lacks antigenic diversity (Kaslow et al., 1989).

1.3.4. Difficulties in malaria vaccine development

1.3.4.1. Inadequate Knowledge

The major difficulty in developing an effective malaria vaccine is the lack of knowledge or definition of the components of natural immunity to malaria. There is still very little insight into what constitutes the mechanisms of protective immunity and what immunogens are suitable targets for protective immunity.

1.3.4.2. Adjuvants

The commonly used adjuvant in human vaccines, aluminium hydroxide, is less efficient, hence the need to identify much more efficient adjuvants. Alving and Richards (1990) have identified a promising adjuvant system for human use, a monophosphoryl lipid A [MPLA] incorporated into liposomes containing the antigen and are adsorbed onto aluminium hydroxide. Another promising adjuvant delivery system is based on vehicles called "immunostimulatory complexes" [ISCOMS], which are stable particles comprising the immunogen, the saponin Quil A and lipids (Morein et al., 1987). Also of interest is the use of live vectors in which selected genes from the pathogen is inserted, e.g. the oral use of
attenuated *Salmonella typhimurium* transfected with the CSP gene from *P. berghei* protects mice against sporozoite challenge (Aggarwal *et al.*, 1990).

### 1.3.4.3. Polymorphism

The high polymorphism of the parasite antigens and the human population complicates the vaccine development strategies. Qari *et al.* (1991) reported that there was polymorphism in both the repetitive and nonrepetitive regions of the CSP of *P. vivax*. Sequence analysis of CS epitope from *P. falciparum* parasites in malaria endemic areas has revealed presence of polymorphism in the TH1R-N1 [103-123], TH2R [326-343] and TH3R [361-380] regions (Shi *et al.*, 1992). Studies by Lockyer and Schwartz (1987) on *P. falciparum* CSP revealed that polymorphism was restricted to the nonrepeat T-cell immunodominant regions of the molecule. The TH1R-NI region was proposed to contain the putative hepatocyte binding site and the proliferative T cell determinant (de la Cruz *et al.*, 1987). A greater antigenic diversity was reported for the TH3R region [CTL epitope] than for the TH1-N1 and TH2R regions. de la Cruz *et al.* (1988) have shown that polymorphism in the TH2R and TH1R affects proliferative and helper responses. Recently, Udhayakumar *et al.* (1994) showed that polymorphism in the CTL epitope abrogated CTL recognition. Since it is only feasible to develop synthetic or recombinant vaccines, the ideal vaccine should be one which can stimulate the desired responses in most individuals if not all. However, most peptides are reported to have limited abilities to bind or be presented in association with different HLA molecules. That MHC-restriction is a limiting factor in the development of synthetic peptides and recombinant proteins as vaccines was demonstrated by Schofield and Uadia (1990) who observed that antibody
responses to sporozoites was not MHC-restricted unlike the use of synthetic or recombinant antigens.

1.3.4.4. Use of carrier molecules for sub-unit vaccines

Since most of the parasite-derived synthetic or recombinant antigens are naturally poor immunogens, different proteins have been used as carriers to enhance their immunogenicity, so as to potentiate their use as vaccines. Following the observation by Que et al. (1988) that, the immune response in rabbits immunized with CSP peptides conjugated to different bacterial protein carriers depended on the nature of the carrier protein, Troye-Blomberg and Perlman (1994) suggested that the carrier molecules played an important role of positioning the epitopes for optimal processing and presentation to the immune system. Lussow et al. (1990) also reported that the use of purified protein derivative [PPD] conjugated to CSP peptide [Asn-Ala-Asn-Pro] in Bacille-Calmette-Guerin [BCG] primed mice overcame H-2 restriction of the antibody response and the need for an adjuvant. Due to the natural exposure of humans to Mycobacteria, and the common use of PPD and BCG, their potential use as carrier system has the possible risk of inducing autoimmunity. Hence, more insight on their role in relation to autoimmunity is required.

1.4. Rationale for this study

Since the desired vaccine should have T- and B-cell epitopes that can be recognized and kept in memory during the generation of the immune response, it is of great importance to fully define the epitopes involved, immunogens recognized and the particular cells involved.
CTL play a very important protective role against the sporozoites and the liver stages. Following the observation that peptide 368-390 from the 7G8 \textit{P. falciparum} CS protein could induce cytolytic activity (Malik \textit{et al.}, 1991), more insight into the nature of this induction is needed. Recent observation by Malik \textit{et al.} (1993) that CTL induction against the \textit{P. falciparum} CS protein could be achieved without adjuvants, gives prospects for the development of an effective human vaccine based on the induction of CTL.

Previous studies in western Kenya by Hoffman \textit{et al.} (1989a) showed that volunteers immunized with irradiated \textit{P. falciparum} sporozoites produced CTL against peptide 368-390 of the \textit{P. falciparum} [7G8 strain] CSP. Sedegah \textit{et al.} (1992) tested 11 individuals whose lymphocytes had shown proliferation to stimulation with peptides 361-380, 371-390, or 368-390 and because 9 individuals were resistant to malaria in the previous study. The results showed that in 4 of the 11 individuals there was peptide-specific, genetically restricted CTL activity, with this activity being eliminated or reduced by depletion of CD8+ T-cells but not CD4+ T-cells.

This study was therefore carried out with two aims. First, to understand the relationship between circulating CTL and resistance to malaria infection by looking at a group of volunteers who had been observed to be relatively resistant to reinfection with malaria and another group that easily succumbed to reinfection with malaria. Second, to determine the effect of polymorphism in the CTL epitope on cross reactivity following the identification of polymorphic forms in the CTL epitope of \textit{P. falciparum} isolated from western Kenya (Shi \textit{et al.}, Unpublished data) and the recent observation that antigenic diversity
within the CTL epitope on the CSP of *P. falciparum* abrogated CTL responses (Udhayakumar *et al.*, 1994). This study tested the CTL responses using these peptide variants with particular emphasis on the Kenyan variants.
2.0. **OBJECTIVES**

2.1. **Main Objective**

To demonstrate if there is any relation in the *in vitro* CTL responses with susceptibility and resistance to malaria re-infection in a malaria endemic area.

2.2. **Specific objectives**

2.2.1. To transform B-cells from all the volunteers with Epstein-Barr Virus [EBV] supernatant to obtain the long term B-cell lines to act as the CTL targets.

2.2.2. To determine CTL responses to the different variants of the 368-390 aminoacid sequence of the CSP using PBMC from the volunteers.

2.2.3. To determine CTL responses to selected variants of the 368-390 amino-acid sequence of the CSP using depleted-CD4 or depleted-CD8 T cell populations.
2.2.4. To determine whether the CTL responses are HLA-restricted or not.

2.2.5. To determine the fine-mapping of the CTL epitope by using shorter peptides spanning the 368-390 aminoacid sequence of the CSP.

2.2.6. To determine if there is any correlation between the CTL response and susceptibility to *P. falciparum* infection.
CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study area

The study was undertaken at Saradidi, a P. falciparum-malaria holoendemic area located 10Km south of the Equator in Siaya District, of Nyanza Province in western Kenya. It is 13Km from the northern shores of Lake Victoria and approximately 65Km from Kisumu town. It covers an area of about 225Km² with an average population density of about 250 persons per Km². It is about 1100m above sea level with hot and humid climate [average temperatures=26.2°C]. It has two seasons of heavy rainfall, the long rains being in March-May and the short rains in October-November, the annual rainfall being about 15mm. Almost 99% of the residents in this area belong to the Luo ethnic group (Kaseje and Spencer, 1987).

The breakdown of human malaria infection by parasite species in the study area was previously reported to be 87.1% P. falciparum; 1.6% P. malariae; 0.4% P. ovale; and 11.0% mixed, with the parasites being transmitted by the An. gambiae complex [An. gambiae and An. arabiensis Patton] and An. funestus (Spencer et al., 1987). Studies at Saradidi by Beier et al. (1990) showed that An. gambiae accounted for 89% of mosquitoes collected. These studies also observed that this area had sporozoite rates of about 12%, being among the highest ever reported.
3.2. **Study subjects**

3.2.1. **Inclusion and exclusion criteria**

The volunteers were life-long, male adult residents of Saradidi. Those included in the study were 20 individuals who were observed to be susceptible to *P. falciparum* reinfection, and 20 other individuals who were found to be relatively resistant to reinfection during previous studies by Hoffman *et al.* (1986; 1989a).

Before the study commenced, all volunteers underwent a physical examination exercise and individuals found to have either a positive serological test for HIV-1, or abnormal total leucocyte counts, or anaemia, or other forms of chronic illnesses were excluded from this study. Those included in the study were also expected to receive all their general health care [including malaria treatment] at the Saradidi Rural Health Project clinic, a Walter Reed project. This was designed to allow for full monitoring of the volunteers to prevent them from taking prescriptions which could have negative effects in achieving the aims of the study.

3.2.2. **Consent**

All volunteers gave written informed consent to be included in the study, after reading the consent forms [Appendix 1] in a language they understood best. The recruitment was completely voluntary with the volunteers having the right to withdraw from the study whenever they so wished.

3.2.3. **Ethical considerations**

The protocol for this study was approved by the Kenya National Ethical Review Committee at the Kenya Medical Research Institute and by
the Human Subject Review Board, Office of the Surgeon General United States Army.

The volunteers donated 30-40ml of blood three to four times during the study at monthly intervals. Donating 30-40ml of blood at a monthly interval did not pose any risk to healthy individuals from previous studies (Hoffman et al., 1986, 1989a).

3.2.4. Determination of *P. falciparum* malaria resistant and susceptible individuals

The volunteers were followed for a period of 12 weeks during a peak malaria period after first being treated of malaria using a standard dose of quinine and doxycycline. During this time, a weekly blood-smear was made from each individual and the time when each individual became positive for *P. falciparum* infection noted. The week when half of the volunteers had recorded positive *P. falciparum* infections was assigned an infection index 1.0. Each individual was assigned an infection index which was calculated as follows:

\[
\text{Infection index} = \frac{\text{week when an individual first became positive for } P. \text{ falciparum malaria}}{\text{week when half of the volunteers became positive for } P. \text{ falciparum malaria}}
\]

Infection indices above or equal to 1.7 were considered "resistant" while indices below 1.7 were considered "susceptible" as previously defined by Hoffman et al. (1989a). Thus, the most "resistant" and "susceptible" individuals were those having the highest and lowest infection indices respectively.
3.3. Specimen acquisition and processing

3.3.1. Blood collection

30-40ml of blood was received from each individual into heparinized 60ml syringes. Each syringe was clearly labelled with the volunteers name and study number. The samples were then transported for about 45 minutes by road to the Walter Reed laboratory located within the Vector Biology and Control Research Centre [VBCRC], Kemri, Kisian.

3.3.2. Separation of leucocytes from whole blood

This was achieved by using the density gradient centrifugation method described by Boyum (1968), with modifications. Briefly, 50ml tubes were clearly labelled with the volunteers name and study number. About 10ml of Lymphoprep™ (Density 1.077 ± 0.001 g/ml) [Nycomed Pharma AS, Oslo, Norway] was put into each tube and blood [two times the volume of Lymphoprep] layered over the Lymphoprep using pipettes into corresponding tubes. The tubes were then capped and centrifuged in a Beckman GS-6R centrifuge [Beckman Instruments Inc., USA] at 150g or 1500 revolutions per minute [r.p.m.] for 30 minutes at room temperature [25°C] without brakes.

After 30 minutes, the tubes were carefully removed from the centrifuge and taken into a sterile hood [Nuaire, Inc. Plymouth, USA] where theuffy coat containing the peripheral blood mononuclear cells [PBMC] together with a third of the Lymphoprep and plasma layers were transferred into labelled sterile tubes using sterile transfer pipettes. The cells were then washed two times with physiological saline [pH 7.0 ; 25°C] by adding two to three times the volume of the cell suspension. The first
wash was done at 150g or 1500 r.p.m. for 10 minutes while the second wash was done at 120g or 1200 r.p.m. for 10 minutes, with the supernatant being aspirated off at the end of each wash and the resulting pellet resuspended again. After the second wash, the pellet was resuspended in 1-4ml of cell culture medium [C-RPMI-1640-10] and a viability count done.

3.3.3. Cell culture medium

The PBMC meant for CTL induction [effector cells] were cultured in RPMI-1640 supplemented with 5% heat-inactivated foetal bovine serum [FBS], 5% heat-inactivated human AB+ serum, 2mM L-glutamine, 100µg/ml streptomycin, 100U/ml penicillin, and 5 x 10⁻⁵ M 2-mercaptoethanol. This was referred to as complete cell culture medium [C-RPMI-1640]. For the B-cell lines the medium was RPMI-1640 supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 100µg/ml streptomycin, 100U/ml penicillin, 5 x 10⁻⁵ 2-mercaptoethanol, 1% Sodium pyruvate, and 1% Non-essential amino acids. This was referred to as complete-RPMI-1640-10 [C-RPMI-1640-10].

3.3.4. Viable cell counts

The cell suspension was mixed well by gently tapping the 50ml tube several times at the bottom. A one in ten dilution of the cell suspension in 0.2% [w/v] trypan blue was made in a clean test tube. The test tube was then mixed well and the resulting cell suspension used to charge a clean improved Neubauer haemocytometer. The haemocytometer was left to stand for one minute before the viability count was done under a phase contrast microscope using objective x10. The cells in the four squares
excluding the dye were counted and the resulting counts divided by four, then multiplied by the dilution factor [10] and by a factor of 10^4 to give the total number of PBMC ml^-1 of the cell suspension. The counting was finished within 2-4 minutes after cell suspension in the dye.

3.3.5. Immortalization of B-cells

3.3.5.1. Virus production

The cell line B95.8, obtained from American Type Culture Collection (Rockville, Maryland), was used to produce the Epstein-Barr virus [EBV]. Briefly, the vial containing the cells was quickly thawed by pressing between the hands. The cell suspension was transferred into a 15ml centrifuge tube and 10ml of C-RPMI-1640-10 added. This was then centrifuged at 100g or 1000 r.p.m. for 10 minutes, after which the supernatant was discarded while the pellet was re-suspended in 10ml of C-RPMI-1640-10. The cell suspension was transferred into a 25cm^2 culture flask and placed in a 37°C incubator (Nuaire Instruments Inc., Washington D.C.) with a humid atmosphere of 5% CO_2 in air. When the cells started growing (medium turning yellow), the cultures were passaged twice per week by removing half of the culture medium and replacing with same volume of fresh medium. The cultures were then expanded up to 250ml in 175cm^2 flasks and on reaching a cell density of 5 x 10^5 ml^-1 the cultures were left undisturbed for 7 days. The medium was then harvested, centrifuged at 200g or 2000 r.p.m. for 15 minutes at 20°C and the resulting supernatant filtered through a 0.45µm pore filter. This retains cells and large particles but not the enveloped infectious virus particles. The filtered supernatant was then aliquoted into 2ml Nunc vials and stored at -196°C in a liquid nitrogen tank until use.
3.3.5.2. **B-cell transformation and culture**

After PBMC isolation and viability count as described above in 3.3.2. and 3.3.4. respectively, 7-10 x 10^6 cells in 2.5ml of C-RPMI-1640-10 was put in a sterile labelled 50ml centrifuge tube. 2.0ml of EBV supernatant was then added and incubated at 37°C waterbath for 2h.. 5ml of C-RPMI-1640-10 and 5μg of cyclosporin A was added to each tube. The resulting 10ml cell suspension was transferred into a 25cm² tissue culture flask and incubated in a humidified 37°C incubator with 5% CO₂ in air. The cultures were checked for blast formation after day 4 of culturing and any flask showing blast formation was split into two flasks after shaking the original flask gently to mix the cells. An equal amount of C-RPMI-1640-10 was added to the cell suspension in the two flasks and then re-incubated. The transformed B-cells were maintained as long term cell lines by weekly removing two thirds of the cell cultures and replacing with equal amount of fresh C-RPMI-1640-10, until use.

The CTL recognize antigens in association with MHC class I or II molecules. The B-cell lines were grown to provide target cells which were matched to the effectors. This allowed for matched CTL responses to be compared to mismatched responses, in which targets from a different person were used. This gave an indication if the response was HLA-restricted or not.

3.3.6. **Effector cells for CTL assay**

The method described by Sedegah *et al.* (1992) was used, with modifications. Briefly, after PBMC isolation (3.3.2.), 3 x 10^6 cells were stimulated *in vitro* for 6 days in 24 well culture plates (Coster Corporation
Cambridge, MA, U.S.A.) in a final volume of 2ml of C-RPMI-1640 medium in the presence of 5µg/ml of a *P. falciparum* CS peptide spanning the amino acid sequence 368-390. The plates were incubated at 37°C in a humidified chamber in the presence of 5% CO₂. Two days after culture initiation, human rIL-2 (Cetus Corporation, Merryville, C.A.) at 50U/ml, anti-CD3 monoclonal antibody G19.4 (0.05µg/ml) and anti-CD28 monoclonal antibody (0.01µg/ml) were added to each well. After 6 days of culture, CD8+ or CD4+ T cells were depleted from portions of the effectors.

3.3.6.1. Depletion of CD4+ and CD8+ effector T cells

The CD4+ and CD8+ T cells were depleted from the effector cell populations using magnetic Dynabeads M-450 [Dynal A.S, Oslo, Norway] coated with anti-CD4 or anti-CD8 monoclonal antibodies to deplete the CD4+ and CD8+ T cells respectively.

The effector cells were removed from the 24 well plates, using sterile transfer pipettes, and transferred into 15ml centrifuge tubes. The tubes were spun at 50g or 500 r.p.m. for 10 minutes at 25°C. The pellet was washed two times in wash buffer [PBS +2% FCS]. After the final wash the pellet was resuspended in 1ml of the wash buffer and a viability count done.

3.3.6.1.1. Magnetic Dynabeads M-450

Dynabeads M-450 CD8 and Dynabeads M-450 CD4 were used to deplete CD8+ and CD4+ T cells respectively. The ratio of beads to cells was 10:1, with all the depletion processes being done at 4°C. The desired number of beads was removed from the vial and transferred into a washing tube containing 5ml of the wash buffer and, washed three times.
This was done by placing the washing tube containing the beads on a magnetic particle concentrator [Dynal MPC-6, Dynal Inc., Norway] for one minute and the fluid aspirated off. After the final wash, the beads were resuspended in the wash buffer, in a volume equal to that originally pipetted from the vial. The beads were then added to the cells and the tubes mixed gently for 30 minutes. The bound cells were isolated by placing the tubes in the magnetic particle concentrator for 2-3 minutes. The unbound cells were removed in the supernatant and put in a sterile tube which was again placed in the magnetic particle concentrator for 2-3 minutes. Finally, the supernatant containing the negatively selected cells was removed, spun at 50g or 500 r.p.m. for 10 minutes and a viability count done.

3.3.6.1.2. Degree of depletion

The degree of depletion was compared with the whole PBMC. Briefly, whole PBMC, CD4 depleted, and CD8 depleted cell populations were stained using anti-CD8 and anti-CD4 monoclonal antibodies coupled to fluorescent isothiocynate [FITC] and the percentage of stained cells determined using a Facscan machine. CD8-depletion was observed to reduce CD8+ cells from 30% to 2% while enriching CD4+ cells from 40% to 81%, while depletion of CD4+ cells reduced CD4+ cells from 40% to 1.4% and enriched CD8+ cells from 25% to 82%.

3.3.7. Target cells for CTL assay

B-lymphoblastoid cells transformed with EBV and maintained as long term lines (Sugden and Mark, 1977), were used as the targets. 48h. before the CTL assay, the targets were passaged in C-RPMI-1640 and human
rIFN-γ [Genzyme, Cambridge, MA] added at 150U/ml. One day before the
CTL assay, the EBV targets were spun at 50g or 500 r.p.m. for 10 minutes
[25°C] and a viability count done. 1 x 10⁶ cells /ml was plated in 24 well
plates in a total volume of 1ml/well. To each well was added 150U of
human rIFN-γ, and 0.01μCi of tritiated thymidine [Dupont NEN Products,
Boston, MA]. To some of the wells, different malaria peptides [10μg/ml]
were added, while to some no peptide was added. The plates were then
incubated for 16-18h. in a humidified incubator at 37°C and 5% CO₂ in air.

3.3.8. CTL assay

Matzinger's (1991) JAM test was used to determine cell lysis. Briefly, after 16-18h. incubation, the target cells were removed from the
plates using transfer pipettes and put in 15ml tubes. 10ml of C-RPMI-
1640-10 was added to each tube and spun at 50g or 500 r.p.m. for 10
minutes. The supernatant was then discarded while the pellet was
resuspended in 1ml of C-RPMI-1640 and malaria peptides added at
10μg/ml to targets pulsed with peptides in 3.3.6. above. 5 x 10³ target
cells, either autologous or HLA-mismatched [heterologous], were added to
round-bottomed 96-well plates. Varying numbers of effector cells were
then added [in triplicates] to some of the wells containing the target cells,
spun at 25g or 250 r.p.m. for 2 minutes and harvested 6h. later using a cell
harvester [LKB Wallac 1295-001, Skatron, Norway]. Some wells containing
only the target cells were harvested at 0h. and others at 6h.. The cells
were harvested onto printed fibre glass filtermat A [Wallac Oy, Finland]
which were air-dried. The fibreglass filtermats trap intact DNA while
small pieces of fragmented DNA are washed through (Matzinger, 1991)
The notches on the dry filtermats corresponding to the wells on the 96-
well plates were removed and each notch put in a clean vial [Kimble glass, Vineland, NJ]. To each vial was added 1ml of a cocktail Bio-Safe NA scintillation fluid [Research Products International Corp. Illinois, USA]. The vials were then cocked and loaded onto racks and the amount of beta radiation determined using liquid scintillation counter [LKB Wallac 1214 Rackbeta, Wallac Oy, Finland] attached to a Facit printer [Japan] and a computer [Bios model TTLA, Daewoo Electronic Co. Ltd., Korea]. The readings were saved in floppy discs and a print-out got from the printer. The data was got as the average of the replicates used.

3.3.8.1. Determination of malaria specific lysis

Target lysis in the presence of a particular type of effector cell population was compared between peptide pulsed targets and targets without peptide. These were calculated as follows:

1. % Lysis with malaria antigen[Ag] = \[
\frac{\text{Experimental release with } Ag \ - \ \text{Spontaneous release with } Ag}{\text{Background release} \ - \ \text{Spontaneous release with } Ag}\times 100
\]

2. % Lysis without malaria Ag = \[
\frac{\text{Experimental release without } Ag \ - \ \text{Spontaneous release without } Ag}{\text{Background release} \ - \ \text{Spontaneous release without } Ag}\times 100
\]

3. % Ag Specific Lysis = \{ % Lysis with malaria Ag - % Lysis without malaria Ag \}
4. Experimental release was defined as the amount of beta radiation obtained at a given E:T ratio after 6h. incubation.

5. Spontaneous release was defined as the amount of beta radiation obtained from wells containing targets only after 6h. incubation.

6. Background release was defined as the amount of beta radiation obtained from filtermats corresponding to wells which did not have any cells.

7. Spontaneous killing was determined as the percent decrease in beta radiation of a given target between times 0h. and 6h. of incubation.

3.3.8.2 Malaria peptides

The peptides used in this study were synthetic peptides, synthesised using Fmoc chemistry and had purities of over 90% homogeneity as determined by reverse-phase high pressure liquid chromatography [HPLC] (Udhayakumar, personal communication). The peptides represented polymorphic sequences of the CTL epitope in different P. falciparum isolates from Brazil [BRA], Papua New Guinea [PNG], The Gambia [GMB], and Kenya [Table 1.]. These peptides comprised 23 amino-acids each and represented the residues 368-390. A shorter peptide [127], comprising 12 amino-acids at the amino terminal end of this epitope, was also used. The amino-acid sequences in Table 1. were in single letter codes, where the single letters represented the amino-acids shown in Appendix III.
Table 1: The amino acid sequence of CTL epitopes in the CS protein of *P. falciparum* in different field isolates and their geographical distribution.

<table>
<thead>
<tr>
<th>NO</th>
<th>Amino Acid sequence of CTL epitope in various field isolates</th>
<th>% Geographical distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BRA</td>
</tr>
<tr>
<td>347</td>
<td>KPKDELDYENDIKEKIKCKEKS</td>
<td>90.5</td>
</tr>
<tr>
<td>P2</td>
<td>Q</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>QCS</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>S</td>
<td>9.5</td>
</tr>
<tr>
<td>P5</td>
<td>V</td>
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</tr>
<tr>
<td>P6</td>
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<td></td>
</tr>
<tr>
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<td>QN</td>
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<td>P9</td>
<td>AD</td>
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</tr>
<tr>
<td>P10</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>348</td>
<td>QI</td>
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</tr>
<tr>
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</tr>
<tr>
<td>350</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>351</td>
<td>QGI</td>
<td></td>
</tr>
</tbody>
</table>

* The data on Kenyan isolates is unpublished. This data was obtained from Dr. Shi Ya-Ping at CDC.
3.3.8.3. **Mixed Lymphocyte Reaction (MLR) assays**

Since there was no positive (peptide) control for malaria CTL assays, an MLR assay was run concurrently with the malaria CTL assays. The MLR, having a strong CTL response, was meant to ascertain that the negative malaria CTL assay results were true negatives, and not due to some experimental factors. Briefly, PBMC from two individuals were used. First, monocytes/macrophages were isolated from each individual by incubating their PBMC [3 x 10^6 cells in 1ml of C-RPMI-1640] in 24 well culture plates for 2h. at 37°C, humidified incubator having 5% CO₂ in air. The isolated monocytes/macrophages were then used to stimulate the other persons effector cells (PBMC). On the second day of culture, human rIL-2, anti-CD3 and anti-CD28 monoclonal antibodies were added in the same way as for the malaria effector cells. The cells were stimulated for 6 days and on the seventh day the assay was done in the same way as for the malaria CTL except that the targets were never pulsed with any antigen (peptide).

Alternatively, an individual was considered negative if his assay showed no any response while another person, whose assay was also done at the same time and conditions, showed a positive response. Any malaria antigen specific killing equal to or greater than 10% was arbitrarily considered positive or significant while those less than 10% were considered negative or insignificant. All negative numbers were represented as zero.

3.4. **Statistical analysis**
The relationship between CTL responses and susceptibility or resistance to *P. falciparum* infection was analysed using the Mann-Whitney test based on the infection indices.
CHAPTER FOUR

4.0. RESULTS

4.1. Thymidine incorporation assay

Previous CTL reports in human malaria research employed the use of the standard Chromium $^{51}$Cr release assay (Malik et al., 1991; Sedegah et al., 1992). This study used tritiated thymidine as the labeling reagent, with the JAM test (Matzinger, 1991) being applied to determine the degree of cell lysis. The JAM test measures the DNA retained by living cells, unlike the $^{51}$Cr release assay which is based on plasma membrane disintegration and the consequent release of cytoplasm. The JAM test was reported to be faster, more sensitive, easier to set up, less expensive and safer than the current standard $^{51}$Cr release assay (Matzinger, 1991). However, the JAM test had a shortcoming in that the targets had to be in growth phase to ensure sensitivity.

4.2. CTL responses

4.2.1. PBMC effectors

4.2.1.1. Optimal peptide concentrations

4.2.1.1.1. Volunteer 1

In the CTL assay, peptides were added at two stages: i). to the effectors for CTL induction; and ii). to the targets (during pulsing) for target recognition. For CTL induction, two peptide concentrations were tested: 10μg/ml and 5μg/ml. The resulting effector cells were tested against targets pulsed with peptides 347 and 127 at 30μg/ml and 10μg/ml...
concentrations. Although the CTL assay for the two peptide concentrations for CTL induction was done at different E:T ratios, [60:1, 30:1; and 80:1, 40:1] (Fig. 2), there was a positive response for both peptide concentrations with no big differences between the two concentrations. Subsequent CTL induction stages employed the use of 5μg/ml of the peptide. For target recognition [pulsing], there was no big difference in the CTL response when 30μg/ml or 10μg/ml of the peptide was used. Hence, in subsequent assays 10μg/ml of the peptide was used. In CTL induction, use of peptide 347 resulted in greater response [15.4-28.3%] (Fig. 2) than when peptide 127 was used at corresponding concentrations and E:T ratios [0-13.8%] (Fig. 3).

**Conclusion**

The longer peptide 347 may be optimal for CTL induction than the shorter peptide 127.

4.4.1.1.2. Volunteer 2

When all the Kenyan variants were tested in volunteer 2 using 347 stimulated effector cells, there was over 10% antigen specific lysis for peptides 349 and 351 (Fig. 4). However, when responses to the peptide variants were relatively compared, there was virtually no response to peptides P2, P8, and 348. When these peptides were tested against heterologous targets, there was virtually no response to any of the peptides since the highest response was less than 7%. (Fig. 5) while a response was considered positive if the specific lysis was equal to or greater than 10%.

**Conclusion**

i). The glutamine amino-acid change at position 5 in the peptides P2, P8, and 348 may have an abrogative role in CTL recognition.
Fig. 2

CTL Response to CS Protein of *P. falciparum* in Volunteer 1 using 347-stimulated effector cells

![Graph A](image1)

![Graph B](image2)

**Peptides**
- 347 (30 μg/ml)
- 347 (10 μg/ml)
- 127 (30 μg/ml)
- 127 (10 μg/ml)

A: 10 μg/ml of 347 used to induce CTL
B: 5 μg/ml of 347 used to induce CTL
CTL Response to CS Protein of *P. falciparum* in Volunteer 1 using 127-stimulated effector cells

**Peptides**
- 347 (30 µg/ml)
- 347 (10 µg/ml)
- 127 (30 µg/ml)
- 127 (10 µg/ml)

**Legend:**
- 5 µg/ml of 127 used to induce CTL
- 10 µg/ml of 127 used to induce CTL
CTL Response to CS Protein of *P. falciparum* in Volunteer 2 using 347-stimulated effector cells against autologous targets.

![Graph showing antigen-specific lysis (%) for different E:T ratios and peptides](image)

**Peptides**

- 347
- P2
- P4
- P8
- 348
- 349
- 350
- 351
CTL Response to CS Protein of *P. falciparum* in Volunteer 2 using 347-stimulated effector cells against heterologous targets

![Graph showing CTL response to CS protein](image)

**Peptides**
- Dark gray: 347
- Gray: P2
- Light gray: P4
- Black: P8
- Dark gray: 348
- Black: 349
- Light gray: 350
- Dark gray: 351

E:T Ratio

80:1 40:1 20:1

Antigen Specific Lysis (%)
ii). The glutamine amino-acid change at the same position in peptide 351 may be compensated for by the glycine change at position 7 since peptide 348 having similar changes as peptide 351, except for the glycine change, abrogated recognition.

iii). The CTL response appeared MHC-restricted since there was significant positive responses only to autologous but not heterologous targets.

4.4.1.1.3. Volunteer 3

When 347 stimulated effectors were tested against 347 or 351 pulsed targets, there were positive responses to both peptides [347 = 13.3% ; 351 = 10.6%] when autologous targets were used. However, when heterologous targets were used, there was no any positive response [347 = 1.6% ; 351 = 0%] (Fig. 6).

**Conclusion**

i) The three amino-acid changes in peptide 351 may not have significant abrogative role in CTL recognition (in contrast to reports by Udhayakumar *et al.*, 1994).

ii) The CTL response was MHC-restricted.

When this volunteer was encountered for the second time, the role of peptides 347 and 351 in CTL induction was compared (Fig. 7). From the results, use of either peptide in inducing CTL gave some degree of positive response. However, when peptide 347 was used to induce CTL, it gave a greater response to both 347 and 351 pulsed targets [12.4% and 0.6% respectively] as opposed to the use of peptide 351 [347=6.7% ; 351=9.8%].
Fig. 6

CTL Response to CS Protein of *P. falciparum* in Volunteer 3 using 347-stimulated effector cells

A: Autologous targets
B: Heterologous targets
CTL Response to CS Protein of *P. falciparum* in Volunteer 3 using 347- and 351-stimulated effector cells

**A**

<table>
<thead>
<tr>
<th>E:T Ratio</th>
<th>347</th>
<th>351</th>
</tr>
</thead>
<tbody>
<tr>
<td>80:1</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>40:1</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>20:1</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>E:T Ratio</th>
<th>347</th>
<th>351</th>
</tr>
</thead>
<tbody>
<tr>
<td>80:1</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>40:1</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>20:1</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

A: stimulation with 347  
B: stimulation with 351
Conclusion

i) Peptide 347-induced CTL to recognize both peptide 347 and 351 pulsed targets.

ii) Peptide 351-induced CTL are not as effective as peptide 347-induced CTL in recognizing either peptide 347 or 351-pulsed targets.

4.4.1.1.4. Volunteer 4

When 347-stimulated effector cells were tested against 347- or 127-pulsed targets there was a positive response for both peptides. However, peptide 127-pulsed targets had greater response [22.3%] than the 347-pulsed targets [13.4%] (Fig. 8.).

Conclusion

The shorter peptide 127 could be the most optimal size of the epitope required for recognition, unlike the longer peptide 347.

4.4.1.1.5. Volunteer 5

In this volunteer, the effector cells were stimulated with peptide 347 for 6 days and the assay carried out on day 7 as described in 3.0. However some of the effectors were re-stimulated further with the same peptide for 6 more days before the CTL assay was carried out. As shown in Fig. 9, it was observed that there was no positive response during the first week of stimulation, while following the second week re-stimulation, positive responses for both 347- and 127-pulsed targets were recorded.

Conclusion

One week stimulation appeared not be the optimal duration for successful CTL induction.
CTL Response to CS Protein of *P. falciparum* in Volunteer 4 using 347-stimulated effector cells
CTL Response to CS Protein of *P. falciparum* in Volunteer 5 using 347-stimulated effector cells

**A:** One week stimulation

**B:** Two weeks stimulation

<table>
<thead>
<tr>
<th>Peptides</th>
<th>E:T Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>347</td>
<td>100:1</td>
</tr>
<tr>
<td>127</td>
<td>50:1</td>
</tr>
<tr>
<td>347</td>
<td>25:1</td>
</tr>
</tbody>
</table>

The CTL responses in this individual were significantly higher than in the other volunteers and in comparison with the other volunteers, the responses differed only in the peptides used.
4.4.1.6. **Volunteer 21**

When effectors from this individual were stimulated using peptide P8 and tested against targets pulsed with peptides 347, P2, P3, P8, and 351, it was observed that there was positive responses to all the peptides (Fig. 10). All these peptides except peptide 347 had the glutamine amino acid change at position 5. The responses were relatively higher for the peptides having this change \[ P2 = 15\% ; P3 = 13.8\% ; P8 = 20.3\% ; 351 = 17.0\% \] than for the peptide 347 \[ 10.2\% \] which had a glutamic acid at position 5.

**Conclusion**

Effector cells induced with the peptide P8 tend to recognize more, targets pulsed with peptides bearing the glutamine change than that having the glutamic amino acid change.

4.4.1.6. **Volunteers 6, 7, 8, and 9**

The CTL responses in this individuals, although positive, were quite inconsistent in that responses could be obtained for some E:T ratios but not others. These responses differed very much from those observed in the other positive volunteers [1-5].

4.4.1.7. **Volunteers 10-20, 22 and 23**

When CTL assays were run using samples from these volunteers, they showed negative responses compared to the positive controls performed during the same experiments.
CTL Response to CS Protein of *P. falciparum* in Volunteer 21 using P8-stimulated effector cells

![Graph showing CTL response to CS protein of *P. falciparum* in Volunteer 21 using P8-stimulated effector cells. The graph displays the percentage of antigen-specific lysis at different E:T ratios (80:1, 40:1, 20:1) for peptides 347, P2, P3, P8, and 351.]
4.4.1.2. Positive controls

The positive controls were basically MLR assays. However in some instances, a positive malaria CTL response observed in a concurrent assay acted as a control for any negative CTL assay(s). In Fig. 11, the assay [control] was very consistent, as the percent specific lysis increased with increasing E:T ratio. This was done using PBMC as the effectors.

**Conclusion**

Increasing the effectors increased the specific lysis of the targets.

4.4.2. Depleted-CD4+ and -CD8+ effectors

Due to unavailability of enough cells and Dynabeads, only a few samples [n=11] were run using the depleted-CD4+ and -CD8+ T-cell sub-populations as effectors. However, there was no positive response recorded for these samples. Following the observation that no positive response was observed for PBMC effectors from the same individuals at the same time, it appeared that the depletion process may not have abrogated the responded. This was confirmed by the MLR control assay [Fig. 12] in which it was observed that there was a positive response for the PBMC as well as the depleted-CD4+ and -CD8+ sub-populations. From Fig. 12 it was evident that CD8+ cells were the most important in the CTL as depletion of CD4+ [enrichment of CD8+ cells] resulted in almost the doubling of the specific lysis while depletion of CD8+ [enrichment of CD4+] cells resulted in the halving of the specific lysis.

**Conclusion**

i) Dynabead depletion of CD4+ or CD8+ cells did not abrogate but affected the CTL responses.
Fig. 11

Mixed Lymphocyte Reaction

![Graph showing specific lysis (%) at different E:T ratios (60:1, 30:1, 15:1). The graph indicates higher specific lysis at a ratio of 60:1 compared to 30:1 and 15:1.](attachment:image)
Mixed Lymphocyte Reaction
(E:T Ratio = 40:1)

- Specific Lysis (%)
  - PBL
  - d-CD4
  - d-CD8

Effectors

- PBL
- d-CD4
- d-CD8
ii) The non-responses obtained when these samples were run may have been due to general non-responsiveness being a consequence of non-availability and/or few CTL precursors in their PBMC.

iii) CD8+ cells are the major T cell subpopulations involved in CTL activity.

4.5. Mann-Whitney test

Table 2. Infection indices for the positive and negative responders in CTL assays*

<table>
<thead>
<tr>
<th>Positive responders</th>
<th>Infection index</th>
<th>Negative responders</th>
<th>Infection index</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=10)</td>
<td></td>
<td>(n=13)</td>
<td></td>
</tr>
<tr>
<td>Volunteer 1</td>
<td>2.6</td>
<td>Volunteer 10</td>
<td>2.3</td>
</tr>
<tr>
<td>&quot; 2</td>
<td>1.9</td>
<td>&quot; 11</td>
<td>1.9</td>
</tr>
<tr>
<td>&quot; 3</td>
<td>1.4</td>
<td>&quot; 12</td>
<td>2.3</td>
</tr>
<tr>
<td>&quot; 4</td>
<td>NR</td>
<td>&quot; 13</td>
<td>0.9</td>
</tr>
<tr>
<td>&quot; 5</td>
<td>1.9</td>
<td>&quot; 14</td>
<td>0.7</td>
</tr>
<tr>
<td>&quot; 6</td>
<td>0.4</td>
<td>&quot; 15</td>
<td>2.3</td>
</tr>
<tr>
<td>&quot; 7</td>
<td>0.9</td>
<td>&quot; 16</td>
<td>1.1</td>
</tr>
<tr>
<td>&quot; 8</td>
<td>2.1</td>
<td>&quot; 17</td>
<td>2.4</td>
</tr>
<tr>
<td>&quot; 9</td>
<td>0.9</td>
<td>&quot; 18</td>
<td>0.9</td>
</tr>
<tr>
<td>&quot; 21</td>
<td>0.9</td>
<td>&quot; 19</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; 20</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; 22</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot; 23</td>
<td>1.5</td>
</tr>
</tbody>
</table>
NR: No record of infection index.

*An individual was considered a positive responder when a CTL assay based on his cells had a peptide specific lysis equal to or greater than 10% while an individual having less than 10% peptide specific lysis was considered a negative responders.

When the infection indices of the susceptible and resistant individuals were subjected to Mann-Whitney test, it was observed that there was no significant difference between the positive and negative responders (U=61.5; U_{0.005} (2) 9,13 =89; P > 0.1). This suggests that there was no significant relation between CTL responses and the time of *P. falciparum* re-infection (infection index).
5.0. DISCUSSION

Immunization of animals and humans with irradiated sporozoites protects against sporozoite challenge (Egan et al., 1993). In the murine model, this immunity wanes over a period of months (Nussenzweig and Nussenzweig, 1984), and the protection is effected by CD8+ CTL since treatment of sporozoite-immunized mice with anti-CD8+, but not anti-CD4+ monoclonal antibody completely abrogated immunity (Weiss et al., 1988). Circumsporozoite protein-specific CTL were reported to be present following immunization of mice with irradiated sporozoites of *P. falciparum* (Kumar et al., 1988). The adoptive transfer of CTL clones also conferred protection against sporozoite challenge by *P. yoelii* (Rodrigues et al., 1991) and *P. berghei* (Romero et al., 1989; Weiss et al., 1992).

In humans, immunization with irradiated *P. falciparum* sporozoites conferred sterile immunity lasting 9 months (Edelman et al., 1993) and induced CTL directed against the 368-390 domain of the *P. falciparum* CSP (Malik et al., 1991). It was also reported that individuals endemically exposed to *P. falciparum* infection had naturally acquired CD8+ CTL against the *P. falciparum* CSP (Sedegah et al., 1992). However, Doolan et al. (1991a) observed that only a few Caucasians naturally exposed had CSP-specific CTL in their peripheral blood. Recently, they reported a low CTL responsiveness to the *P. falciparum* CSP in naturally exposed endemic populations (Doolan et al., 1993). In these assays, the standard 51Cr release assay was employed for the CTL responses, however, in this study
thymidine incorporation assays were used in the assessment of CTL responses.

5.1. Thymidine incorporation assays

Most of the CTL reports in malaria research employed the use of the standard Chromium \(^{51}\text{Cr}\) release assay (Malik \textit{et al.}, 1991, 1993; Udhayakumar \textit{et al.}, 1994). In this study, thymidine incorporation assay, the JAM test as described by Matzinger (1991), was employed for the CTL assay. Although a comparison was not made between the two assays, it was however observed that under the prevailing circumstances, the use of the JAM test was ideal. This was so considering that the use of the \(^{51}\text{Cr}\) assay could have been: i). very expensive since a highly organized and efficient means of \(^{51}\text{Cr}\) acquisition could have been required because of its short half-life [27.7 days]; ii). time-wasting and with inconveniences during transportation of the assay materials to Nairobi for reading as there was no \(\gamma\)-counter in Kisumu where the study was carried out; and iii). dangerous due to higher radiation risks during use in the laboratory and transportation to Nairobi \([^{51}\text{Cr} \text{more potent than } ^{3}\text{H}]\). The spontaneous killing observed in this study (less than 30\%) was comparable with that reported by Doolan \textit{et al.} (1993) using \(^{51}\text{Cr}\) although the targets used were PHA blasts and not EBV transformed B cells. However, although it was difficult to maintain the PHA blasts during the 6 days of CTL induction, in Volunteer 3 it was possible to get a positive CTL response using PHA blasts with a spontaneous killing of less than 20\% (except for one target type which had 35.8\%). This was quite similar to the background lysis of less than 33\% reported for \(^{51}\text{Cr}\)-pulsed PHA targets (Doolan \textit{et al.}, 1993). From these observations, it seems possible to conveniently use the JAM test \([^{3}\text{H}\text{J}].\)
incorporation] in malaria CTL instead of the standard $^{51}$Cr assay. The latter also has the disadvantage of having cytoplasmic leakage (Weiss, personal communication) which makes it less sensitive. Lysis by CTL is characterised by a programmed fragmentation of the nuclear DNA, a process known as apoptosis (Fesus, 1992). This property makes the JAM test more sensitive since the DNA from dead cells, having been degraded into small fragments, is washed through the filters, hence, the radioactivity measured corresponds to intact DNA (Matzinger, 1991). This is a reflection of the number of living target cells that remain in each well. On the use of either PHA blasts or EBV transformed B cells as targets, both had the limitation of having to be in the growth phase. However, the EBV lymphoblastoid targets were relatively better than the PHA blasts since individuals usually respond differently to PHA stimulation. Besides, PHA stimulation peaks on day 3 of stimulation, hence pulsing on day 6 finds the cells already exhausted. However, if PHA blasts can be manipulated effectively until day 6, then the use of these targets would be the most ideal since it would dispense with the need to first get PBMC for transformation and maintenance of the transformed B cells as long term cell lines. This manipulation could possibly be effected by the use of rIL-2 and the right batches of PHA (Doolan, personal communication).

5.2 Optimal peptide concentration

Individuals exposed to *P. falciparum* infections naturally acquire CTL against the CSP (Sedegah *et al.*, 1992). These cells may be present at low precursor frequencies, hence the need to expand them *in vitro* before CTL assays can be carried out. In this process, different peptides are used with the assumption that if CTL precursors specific for that particular
peptide are present, then they will be clonally expanded. This process is termed CTL induction. Peptides were also added to the target cells [pulsing] for processing and/or presentation on their surfaces so that they could be recognised by CTL specific for them.

In the CTL induction, peptide concentrations of 10μg/ml (Doolan et al., 1991a, 1991b, 1993) and 25μg/ml (Sedegah et al., 1992) have been used. In this study two peptide concentrations [5μg/ml and 10μg/ml] were tested. Although the comparison was done at two different ratios, there was a small difference between the two concentrations (Fig. 2). Following the observation by Doolan et al. (1993) that it was possible to induce CTL for influenza virus matrix peptide and EBV nuclear antigen 3 [EBNA3] at 5μg/ml in their positive controls, this concentration was used for CTL induction in this study. It was further reported by Doolan et al. (1991b) that the optimal concentration for the induction of cytotoxic activity ranged from 0.1-100μg/ml with a final peptide concentration of 1-5μg/ml being optimal for the generation of influenza virus matrix peptide-specific CTL.

For target pulsing, peptide concentrations of 10μg/ml (Doolan et al., 1993), 25μg/ml (Sedegah et al., 1992) and 100μg/ml (Udhayakumar et al., 1994) have been reported. In this study concentrations 10μg/ml and 30μg/ml were tested (Fig. 2). This study employed the use of 10μg/ml of peptides for pulsing. Although different peptide concentrations have been used for target pulsing, a dose-response study by Kumar et al. (1988) demonstrated significant killing at concentrations as low as 0.2μg/ml for a peptide residue 371-390. When responses to this peptide and the 368-390 residue [7G8 strain] were compared at concentrations of 1μg/ml, 10μg/ml, and 100μg/ml, responses to residue 371-390 were observed to
be dose-dependent, increasing with peptide concentration while responses to the 368-390 residue were dose-independent (Kumar et al., 1988). Although it has been reported that the optimal length of a CTL epitope may be about 8-10 amino-acids (Hill et al., 1992) at the N-terminal of the previously reported 23 amino-acid CTL epitope (Malik et al., 1991), the 371-390 residue lacked the first 4 amino-acids required in this epitope. Therefore the observed dose-dependent response to peptide residue 371-390 could be due to "augmentation" of the missing 4 amino-acids at the N-terminal, with increasing concentrations. All the peptides used in this study, except peptide 127, were 368-390 residues but derived from different *P. falciparum* isolates. Peptide 347 resembled the 368-390 residue used by Kumar et al. (1988).

5.3 **CTL responses to the different variants of the 368-390 residues of the CSP**

The human CTL epitopes were first reported to map within a polymorphic C-terminal domain of the *P. falciparum* CSP spanning the amino-acid residue 351-395 (Doolan et al., 1991a). Studies by Malik et al. (1991) using overlapping 20- or 23-mer peptides spanning the residues 311-400 of the CSP reported that CTL activity was only associated with the 368-390 peptide. This epitope, the TH3R, was reported to be very polymorphic (Yoshida et al., 1990; Shi et al., 1992). For the 368-390 peptides to function as CTL epitopes, they should be able to bind MHC class I molecules (agreptopic binding) and should also contain residues that can fit into the T cell receptor (epitopic binding) (Townsend and Bodmer, 1989). The epitopic binding is associated with the hydrophilic amino acids, while agreptopic binding is associated with the hydrophobic amino acids.
(Good et al., 1987). Following the observation that the variation in the Th2R region is restricted to the putative T cell receptor domain of the amphiphilic complex, Shi et al. (1992) suggested that the parasite was evolving to the Th2R in an epitopic rather than agretopic fashion. They further reported that the polymorphism within the Th3R resulted in the alteration of the amphiphilic balance, with the amino acid substitutions resulting in changes from hydrophobic to hydrophilic or neutral amino acids (Shi et al., 1992). This observation suggests that the Th3R, unlike the Th2R, is evolving both in epitopic as well as agretopic fashion. Udhayakumar et al. (1994) recently reported that natural amino-acid substitutions in the CS protein resulted in the loss of CTL recognition in a mouse model. This loss of recognition could be due to effects on either the epitopic and/or agretopic binding being a consequence of the amino-acid substitutions.

Although positive or significant CTL response was arbitrarily defined as any antigen specific lysis equal to or greater than 10%, this was quite in agreement with a previous definition by Doolan et al. (1991b) in describing a method for the generation and assay of peptide-specific CTL in field conditions. Doolan et al. (1993), also considered any percent peptide-specific lysis value greater than 10.54% as being significant, although this figure represented the 95% confidence interval for all their values.

In this study only 10 individuals [25.0%] showed positive CTL responses when PBMC were used as the effectors. The low number of responders was consistent with previous malaria CTL reports in humans (Doolan et al., 1991a, 1993; Sedegah et al., 1992). Sedegah et al. (1992) suggested that this could be due to a low CTL circulating precursor frequency resulting in CTL not being identifiable each time an assay is run,
even among known responders. According to Doolan et al. (1993), under conditions of natural exposure, CTL immunological responsiveness to the CS protein is very low. Although Malik et al. (1991) reported a high percent of CTL responders [3 out of 4], this was after an artificially high exposure to the bites of 369-1052 irradiated infected mosquitoes over a period of 7-12 months. But even then, the CTL were not consistently detected as they were detected on only 17-67% of occasions. Under natural conditions, even in regions of intense transmission, it is rare for a person to receive more than 100 infective bites/year (Hoffman et al., 1991). However, according to Malik et al. (1991), their high frequency of positive donors could have been due to promiscuity of the peptide for different HLA molecules, similar to those reported for CS peptide and class II HLA molecules (Sinigaglia et al., 1988a), or due to some shared HLA class I molecules among the three CTL positive responders.

From the CTL responses, cross-reactivity was observed in the CTL epitopes. Effector cells stimulated with peptide 347 could lyse target cells pulsed with peptide 347 as well as other peptides. Although cross-reactivity could not be tested for all the peptides in each individual due to shortage of cells, it was observed in Volunteer 2 (Fig. 4), that targets pulsed with peptide P2, P8, and 348 could not be lysed with peptide 347-induced CTL. The common factor among peptides P2, P8, and 348 was that they all had a glutamine [hydrophilic] change at position 5 instead of the glutamic acid [hydrophilic] in the 347 peptide. However, peptide P8 had an additional alanine [hydrophobic] change for the glutamic acid at position 9. This change was reported to be responsible for loss of CTL recognition in mice (Udhayakumar et al., 1994). Peptide 348 also had an additional isoleucine [hydrophobic] change for glutamic acid at position 9. Of interest
was the observation that peptide 351-pulsed targets, which had a glycine [hydrophobic] change at position 7 besides the glutamine and isoleucine changes like peptide 348, did not abrogate CTL recognition. This could possibly be due to some "synergistic" effects afforded by the change from aspartic acid [hydrophobic] to glycine, which acts against the loss effected by the glutamine and isoleucine changes alone, in positions 5 and 9 of the 348 peptide. This observation together with that of Udhayakumar et al. (1994) suggest that substitutions at positions 5 and 9 play an important role in loss of CTL recognition. The changes at position 9 from glutamic acid to alanine [P8] or isoleucine [348 or 351] are essentially changes from hydrophilic amino acids to hydrophobic amino acids. This change alters the amphiphilic balance and thus results in epitopic variation. This variation may have contributed to loss of recognition reported by Udhayakumar et al. (1994) in a mouse model. However, the effect of the change from glutamic acid to glutamine at position 5 is not clear since both amino acids are hydrophilic, hence, no alteration in the amphiphilic balance. Besides, at pH 7.0 the molecular weights of glutamic acid and glutamine are 147 and 146 respectively. Although Udhayakumar et al. (1994) did not observe any loss of CTL recognition by peptides P2 and P7 [both with glutamine change at position 5], the observation in this volunteer that there was no CTL response to peptides P2, P8, and 348, together with the reports by Hill et al. (1992) that the glutamine change at position 5 seems to have deleterious effect in binding to human HLA-B35, suggest that this change may be involved in agreptopic evasion by the parasite. However, the role played by other substitutions should not be ignored as the glycine substitution at position 7 in peptide 351 could possibly have a role to play in CTL recognition. The serine [hydrophilic]
substitution at position 2 did not seem to abrogate CTL recognition since lysis was observed for both peptide P4 and 349 which had the substitution although peptide 349 had a further asparagine [hydrophilic] substitution for aspartic acid at position 4. These observations suggest that the parasite has evolved epitopic and agretopic variation to evade the immune surveillance by the CTL. However, it was observed in volunteer 21 (Fig. 10) that when peptide P8 was used for CTL induction, the resulting effectors lysed targets pulsed with peptides 347, P2, P3, P8, and 351. This observation raises the question of the precise role played by the amino acid substitutions within the CTL epitope. This observation was quite in contrast to the observations in volunteer 2 (Fig. 4) in which 347-induced CTL failed to lyse P2-, P8-, and 348-pulsed targets. Of interest was the observation that all the peptide-pulsed targets lysed by the P8-induced CTL had the glutamine change at position 5 [except peptide 347]. Lysis was observed even for peptide P3, a sequence from an isolate reported only in Papua New Guinea (Shi et al., 1992 ). Besides the glutamine change at position 5, this peptide also had a cysteine [hydrophobic] change for the tyrosine [neutral] amino acid at position 8 as well as a serine [hydrophilic] change for asparagine [hydrophilic] amino acid at position 10. The observation that CTL recognition could be achieved for a sequence from a different geographical area suggests that although amino acid variation within the CTL epitope is geographically restricted (Shi et al., 1992 ; Doolan et al., 1992), CTL recognition may not be geographically restricted. These observations calls for the use of multiple peptides in optimizing for CTL activity, more so following the reports by Doolan et al. (1991b) that the generation of peptide-specific CTL was not impaired by the presence of the other accompanying peptides.
Also of interest was the observation that 347-induced CTL could recognize peptide 351-pulsed targets. This was in contrast to the observation that three amino acid substitutions abrogated CTL recognition (Udhayakumar et al., 1994). In this study, it was also observed that the glutamine change in peptide P2 abrogated CTL recognition, unlike the former observation in which this change had no effect on CTL recognition. These two observations supports the suggestion that the pattern of cross-reactivity in the CTL epitope may not be identical in human and mouse systems (Udhayakumar et al., 1994). It may be generalised from these observations that the glutamine substitution at position 5 and the alanine substitution at position 9 have a major role in the loss of CTL recognition in humans and mice respectively. This difference in CTL response in the two models could possibly be due to differences in their exposure to the different isolates. As reported by Udhayakumar et al. (1994) the B10.BR female mice were exposed only to peptide 347 following immunization with the recombinant vaccinia virus containing the CS gene of the *P. falciparum* [7G8 strain]. In this study, human adult males were naturally exposed to all the isolates reported in Kenya (Shi et al., Unpublished data). Thus, it was likely that following this exposure the human volunteers could have acquired precursor CTL specific for the *P. falciparum* isolate having the sequence represented by peptide 351. This was supported by the observation that in Volunteer 3, CTL recognizing both peptide 347 and 351-pulsed targets could be induced using peptide 351(Fig 7). Although the CTL induced by peptide 347 were more effective than those induced by peptide 351, this could be due to the differences in the prevalence of the respective isolates (Table 1). Although, to my knowledge, no comparative CTL responses on sexes has ever been reported, the fact that female mice
were used in the other study (Udhayakumar et al., 1994) while human males were used in this study could also account for disparity in these observations.

When CTL responses were compared between the 23-mer peptide 347 and the peptide 127 representing the reported optimal length of the CTL epitope (Hill et al., 1992), it was observed that in general there was a small difference between their responses when used for pulsing targets. In Volunteer 4 (Fig. 8), it was even observed that the use of peptide 127 had a greater response than peptide 347. Although peptide 127 had 12 amino acids, this observation concurs with previous reports that the optimal length of the CTL epitope could be only about 8-10 amino acids (Hill et al., 1992). However, when this peptide was used in CTL induction in Volunteer 1 (Fig. 3), there was a much smaller response compared to the use of peptide 347 (Fig. 2). This observation suggests that the short peptide,[127] may only be optimal for CTL recognition but not induction.

The observation in volunteer 5 (Fig. 9) that CTL responses were only detected on the second, and not first week, of peptide stimulation, brings in the question of the optimal duration for CTL stimulation or induction. In this study, a 6-day primary in vitro culture stimulation was used as reported elsewhere (Malik et al., 1991; Sedegah et al., 1992). However, other workers have employed 8-10 days primary in vitro culture followed by a 5 day secondary stimulation (Doolan et al., 1991b, 1993). However, Udhayakumar et al. (1994) had 6 days of primary stimulation followed by 3 days of secondary stimulation. Thus, the observation from this volunteer indicates that CTL activity could not always be stimulated in primary in vitro cultures. This observation supports the suggestion that the CTL memory cells may in general require more than one in vitro antigenic
stimulation for optimal activation (Doolan et al., 1991b). However, although antigen presenting cells [APC] were added in the cultures in the other studies, in this study only rIL-2, anti-CD3 and anti-CD28 monoclonal antibodies were added together with the stimulating peptide.

CTL responses to the CSP peptide residue 368-390 was reported to be peptide-specific and MHC-restricted (Malik et al., 1991; Sedegah et al., 1992). Studies by Hill et al. (1992) showed that there was an association between the HLA class I antigen, HLA-B53, and protection from severe malaria. From this observation, they suggested that this could be mediated by HLA-class I restricted CTL. Although HLA typing was not done in this study, it can generally be concluded that the CTL responses appeared to be MHC-restricted. This follows the observation that when mis-matched [heterologous] targets were used there was little or no response, compared to responses when matched [autologous] targets were used (Fig 4,5,6). However, there is a need for HLA typing on these volunteers to see if their HLA molecules could be identical to those reported for a West African population by Hill et al. (1992), or if differences do occur probably due to geographical location.

The T cells important in CTL responses against the CSP peptide 368-390 have been reported to express the CD8 surface markers as treatment with anti-CD8+, but not anti-CD4+, monoclonal antibodies abrogated the CTL responses (Sedegah et al., 1992). In this study, due to lack of enough Dynabeads and cells, and the desire to determine the effect of polymorphism in the CTL epitope, few samples were run using CD8- and CD4-depleted sub-populations. However, for the samples run using depleted sub-populations there was no positive response observed. Since no positive responses could be observed for PBMC effectors from the same
volunteers during the same time, this suggests that lack of CTL responses may not have been due to effects of the beads used in the depletion process. This was further confirmed by the positive control MLR assay (Fig. 12), in which it was observed that the use of beads for depletion did not abrogate CTL responses. From figure 12 it can also be deduced that CD8+ bearing cells are the most important cells in CTL because the depletion of CD4 bearing cells [enrichment of CD8+ cells] results in doubling of the % specific lysis [38.9%] compared to when PBMC are used as effectors [21.3%]. However, when CD8 bearing cells were depleted [enrichment of CD4+ cells] the % specific lysis was about half [13.3%] that of PBMC effectors. Furthermore, following the observation by Tsuji et al. (1990) that murine CD4+ CTL clones conferred protection against P. berghei sporozoites in naive mice, and that exposure to sporozoites induces class II restricted cytotoxic CS-specific CD4+ T cells in humans (Moreno et al., 1991), it is desirable that a comparative phenotypic CTL responses be undertaken before the role of CTL in naturally exposed individuals can fully be defined.

Although there was no relationship between the CTL responses and susceptibility or resistance to P. falciparum malaria infection (U = 61.5; U0.005 (2) 9,13 = 89; P > 0.1) it is likely that the criteria used in this study to delineate individuals into susceptible or resistant groups may not have fully defined these groups. The use of the infection index, which is basically the time when one is reported to have been positive for P. falciparum parasites for the first time during the 12-week follow-up period, as a measure of resistance or susceptibility may not be definitive. First, this period is probably not long enough to conclusively define resistance to P. falciparum malaria. Secondly, from the immunological
point of view, besides the entomologic and epidemiologic considerations, acquired immunity to malaria is stage- and species-specific (Howard, 1987), developing gradually following repeated exposure (McGregor et al., 1956). This immunity was not absolute since parasitaemia could persist in the absence of a clinical disease (Neva, 1977). Considering that the volunteers in this study were adult-male residents of a malaria holoendemic area, parasitaemia could not fully define resistance or susceptibility. Besides, naturally exposed adults in malaria endemic areas have been known to resolve their parasitaemia without any chemotherapeutic intervention (Lal, personal communication). Thirdly, these assays were run after the follow-up period, and it is not unusual for a previously "resistant" individual to become "susceptible" and vice versa, in a subsequent follow-up (Duffy, personal communication). Fourthly, protection conferred by CTL may be directed at the disease rather than at parasitaemia per se. However, despite these short-comings the individuals referred to in this study as resistant or susceptible, were "resistant" or "susceptible" when their infection indices were compared amongst them. The failure to find any relationship between CTL responses to the CSP Th3R epitope and susceptibility or resistance to *P. falciparum* infection may also imply that CTL against the Th3R may be insufficient alone to confer full protection. This would support the observation by Khusmith et al. (1991) that immunization of mice with PyCSP or PySSP2 vaccines gave only partial protection (50-75%) against malaria while 100% protection was reported when mice were immunized using transfected P815 cells expressing both PyCSP and PySSP2. The recent observation that a CD8+ CTL clone directed against the PySSP2 could provide complete protection against *P. yoelii* sporozoite challenge in the absence of other parasite-
specific immune responses (Khusmith et al., 1994), calls for CTL assays using other reported CTL antigens. That this protection could be achieved even when the CTL clone was administered 3h. after sporozoite inoculation at a time when the sporozoites had entered hepatocytes (Khusmith et al., 1994) suggests that the CTL eliminate infected hepatocytes, hence the need to assay CTL activity using hepatocyte-expressed antigens such as the liver stage antigen-1 [LSA-1]. Similarly, the reports that the protective effect of the CD8+ CTL clones directed against the PySSP2 correlated with their level of IFN-γ production in vitro (Khusmith et al., 1994) calls for cytokine assays in defining fully the role of these cells in immunity against sporozoite challenge.

Finally, in the design and development of an effective preerythrocytic malaria vaccine, it would be desirable to include all the potential candidate immunogens. The observation by Khusmith et al. (1991) that immunization of mice using CSP alone was less protective than when CSP was used together with SSP2, and that a CD8+ CTL clone directed against the SSP2 could protect against P. yoelii in the absence of other parasite-specific immune responses (Khusmith et al., 1994), it is desirable that CTL responses to other antigens like LSA-1 and SSP2 be tested, with the aim of including them in the candidate multivalent vaccine. These results and those from a previous study (Udhayakumar et al., 1994) suggest that the use of mouse models in vaccine related studies may not give a true representation of what really takes place in humans. This calls for a search for models which will mimic closely, what happens in humans in vivo. Since the desired vaccine should protect all individuals in a given population, the CTL studies should be undertaken in all individuals including children and women, and not only adult males as was the case in
this study. From the observations in this study and those by Udhayakumar et al. (1994), it is clear that antigenic polymorphism has an important implication in designing a CSP-based vaccine construct. These findings concur with the suggestion that immune pressure selects and maintains antigenic polymorphism in malaria endemic areas as a parasite strategy to evade host immune responses.
6.0. SUMMARY OF CONCLUSIONS

6.1. Thymidine incorporation assay can be conveniently used for the study of malaria specific CTL responses instead of the commonly used standard chromium release assay.

6.2. The glutamine and alanine amino acid substitutions for glutamic acid at position 5 and 9 respectively play a major role in CTL recognition of peptide-pulsed EBV targets.

6.3. Peptide-specific CTL recognize targets in an MHC-restricted manner.

6.4. The shorter peptide, 127, is an optimal size for CTL recognition.

6.5. CTL induced by a specific peptide have cross-reactivity with other variant peptides.


6.7. CD8+ bearing cells are the major cells involved in CTL activity against EBV targets in MLR assays.
6.8. There was no relationship between the time of *P. falciparum* infection and resistance or susceptibility (\( U = 61.5 ; U_{0.05} (2) = 89; P > 0.1 \)).

6.9. The 12 weeks follow-up period may have been a short time for the definition of resistant and susceptible individuals to *P. falciparum* malaria.
CHAPTER SEVEN

7.0. SUGGESTIONS FOR FUTURE WORK

7.1. A longitudinal study should be carried out to define precisely and accurately resistance and susceptibility to *P. falciparum* malaria in the study area.

7.2. More samples should be analysed so as to enable a more conclusive definition of the role of CTL responses in human malaria.

7.3. The composition of the sample population should be widened to include children and both male and female adults so as to fully define the role of CTL in immunity to human malaria.

7.4. Assays for CTL activity using other reported CTL antigens such as the liver stage antigen-1 [LSA-1] and the sporozoite surface protein 2 [SSP-2] should also be done within the same study area.

7.5. Carry out phenotypic studies so as to understand the role played by specific T cell sub-populations in CTL activity.

7.6. Carry out HLA-typing to determine the particular molecules involved in or associated with resistance or susceptibility.
7.7. Define the plasma or serum profiles of cytokines such as IL-4, IFN-γ, e.t.c. in these individuals.
REFERENCES


Ruangjirachuporn, W.; Afzelius, B.A.; Helmby, H.; Hill, A.V.S.; Greenwood, B.M.; Carlson, J.; Berzins, K.; Perlman, P.; and Wahlgren, M.


Shi, Y.-P.; Alpers, M.P.; Povoa, M.M.; and Lal, A.A. (1992). Diversity in the immunodominant determinants of the circumsporozoite protein of


APPENDIX I

INFORMED CONSENT EXPLANATION 92D
(To be read and questions answered in a language in which the volunteer is fluent).

TITLE OF STUDY. A study correlating infection by falciparum malaria sporozoites with T cell reactivity to the circumsporozoite protein.

INSTITUTIONS. Kenyatta University; Kenya Medical Research Institute, Nairobi; Walter Reed Army Institute of Research, Washington D.C.; Naval Medical Research Institute, Bethesda, Maryland; National Institutes of Health, Bethesda, Maryland; The Torrey Pines Institute for Molecular Studies, San Diego, California; Georgetown University, Washington D.C.; Johns Hopkins University, Baltimore, Maryland; Stockholm University, Stockholm.

PRINCIPAL INVESTIGATOR. Walter Weiss, M.D., M.P.H., Visiting Scientist, Kenya Medical Research Institute, P.O.Box 30137, Nairobi, Telephone 729303.

PARTICIPATION INFORMATION. You (or your child) have been asked to participate in a medical research study. It is very important that you understand the following general principles which apply to all participants in our studies: 1). Participation is entirely voluntary. 2). Persons may withdraw from participation in this study or any part of the study at any time. Refusal to participate will involve no penalty of loss of benefits to which you are otherwise entitled. 3). After you read the explanation, please feel free to ask any question that will allow you to understand clearly the nature of the study.

INTRODUCTION. Malaria is a disease which affects many people throughout the tropics. It is caused by parasites which are normally transmitted by mosquito bites. Malaria may be mild, but can also sometimes be serious or even fatal if not diagnosed and treated promptly. Some people seem to get malaria less easily than others. This study will try to discover why this is so. It may be that if we knew what naturally protected some people...
from malaria, we could make a vaccine which would protect all people from malaria. This is the reason we are asking you to join this study.

PROCEDURES TO BE FOLLOWED. Subjects who volunteer in this study will first receive pills containing two medicines which cure malaria. These medicines are quinine and doxycycline, and are taken two and three times a day for seven days. Before starting the medicines and on the eighth day, a drop of blood will be taken from your (or your child's) finger, to test for malaria infection. Every week for the next 12 weeks a drop of blood will be taken to check for malaria.

After these 12 weeks the first part of the study will finish, and some persons will stop being in the study. If you are selected to continue in the second part of the study, a clinical officer will give you (or your child) a physical examination to see if you are healthy. You will be tested for malaria, and treated if you have it. Volunteers will also be asked for 10 teaspoonfuls (50 ml) of blood for tests to see if you are healthy. If all these tests show that subjects are in good health, they will continue in the study through the next rainy season, approximately 6 more months. They will be checked for malaria by taking a drop of blood from the fingers every 7 to 14 days. At certain times during the study, volunteers may be asked to donate 1 teaspoonful (5 ml), 2 teaspoonfuls (10), or 10 teaspoonfuls (50 ml) of blood. This blood is needed so that we can test the white blood cells which fight malaria infections. Volunteers will not be asked to donate 10 teaspoonfuls (50 ml) of blood more often than every 12 weeks. This gives time for the body to produce more blood. If we find that a volunteer's blood count is getting low, we will not take your blood until it returns to normal.

Volunteers may also be asked to take part in experiments to test how mosquitoes are attracted to bite. In these experiments, you (or your child) will be asked to stay out during part of the night, and a health worker will collect all the mosquitoes that try to bite you.

Subjects who agree to join this study must come to the Walter Reed Project Clinic at the Saradidi Rural Health Project for all their medical care. They must also agree not to take any medicines except those given to you at the clinic.

RISKS. The medicine you (or your child) will be given to cure malaria are safe, and are the same ones that are usually given to cure malaria. Volunteers who cannot take these medicines for any reason can leave the study. Blood will be taken several times during the study. The body makes new blood all the time and the blood we take will be replaced. If you think you have a medical problem please report.
your ( or your child's ) blood count gets lower than normal , no blood will be taken , and health workers will give vitamins to return your blood count to normal .

BENEFITS . At the beginning of the study , you ( or your child ) will be treated to get rid of malaria . Volunteers will also receive medical care at the Walter Reed Clinic at the Saradidi Rural Health Project .

DISCOMFORTS . At the beginning of the study , and again when you get malaria , you will be given medicines , quinine and doxycycline , to cure malaria . These medicines can cause nausea and ringing in the ears in some persons . After the study has begun , a drop of blood will be taken from your finger each week . This blood will be tested for malaria . You will be asked to donate 1 teaspoonful ( 5ml ) , 2 teaspoonfuls ( 10ml ) , or 10 teaspoonfuls ( 50ml ) of blood from your arm at various times of the study .

ASSURANCE OF CONFIDENTIALITY OF VOLUNTEERS IDENTITY . Records relating to your participation as a research subject will remain confidential . The records may be reviewed by representatives of the Kenya Medical Research Institute , the U.S . Army Medical Research and Development Command , as part of their responsibility to oversee research . Your name will not be used in any report resulting from this study . You will receive a copy of this consent form .

CIRCUMSTANCES UNDER WHICH YOUR PARTICIPATION MAY BE TERMINATED WITHOUT YOUR CONSENT .
1. Health conditions under which your participation possibly would be dangerous .
2. Other conditions which might occur that would make your continued participation detrimental to your own health .
3. If you are not selected to be a volunteer in this study , you may ask the doctors why you were not chosen . If this is because of an abnormal test result , you will be counselled as to the importance of this for your general health .

MEDICAL CARE FOR INJURY OR ILLNESS . You will be entitled to medical care at no cost for any injury or illness which occurs as a proximate result of your participation in this research project .

PERSONS AND PLACES FOR ANSWERS IN THE EVENT OF RESEARCH RELATED INJURY . If you think you have a medical problem , please report to the
Walter Reed Clinic, Saradidi Rural Health Project, Siaya. If for some reason this is not possible, contact Dr. Walter Weiss, Walter Reed Project, P.O.Box 54, Kisumu, Kenya. Tel. 43389 or Prof. A.S.S. Orago, Department of Zoology, Kenyatta University P.O.Box 43844, Nairobi, Tel. 810901-19 ext 305.

FOR INFORMATION OR ANSWERS TO QUESTIONS CONCERNING YOUR RIGHTS AS A RESEARCH SUBJECT YOU MAY CONTACT:
The Chairman of the Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute, P. O. Box 54840, Nairobi, Kenya, tel. 722541

IF THERE IS ANY PORTION OF THIS CONSENT EXPLANATION SHEET THAT YOU DO NOT UNDERSTAND, ASK THE INVESTIGATOR BEFORE SIGNING.

I acknowledge receipt of this agreement, to include: the Consent Explanation and the Informed Consent Agreement.

Volunteer's signature __________________________ Date __________
Volunteer's printed name ________________________

Parent's signature ____________________________ Date __________
Parent's printed name _________________________

Witness's signature __________________________ Date __________
Witness's printed name ________________________

---
APPENDIX II

Recipe for the Reagents

1. **C-RPMI-1640**: Complete RPMI-1640 [Roswell Park Memorial Institute-1640] Comprised RPMI-1640 [GIBCO BRL, Life Technologies, Inc, Grand Island NY ,USA.] supplemented with :-
   - 5% heat inactivated human AB+ serum [GIBCO BRL, Life Technologies]
   - 5% heat inactivated Foetal Bovine Serum [GIBCO BRL, Life Technologies]
   - 1% 200mM L-Glutamine [GIBCO BRL , Life Technologies]
   - 100U/ml of Penicillin [GIBCO BRL , Life Technologies]
   - 100μg/ml of Streptomycin [GIBCO BRL , Life Technologies]
   - 1x10⁻⁵/ml of 2-Mecaptoethanol

2. **C-RPMI-1640-10**
   Comprised RPMI-1640 [GIBCO BRL , Life Technologies] supplemented with :-
   - 1% 200mM L-Glutamine [GIBCO BRL , Life Technologies]
   - 10% Foetal Bovine Serum [GIBCO BRL , Life Technologies]
   - 100U/ml of Penicillin [GIBCO BRL , Life Technologies]
   - 100μg/ml of Streptomycin [GIBCO BRL , Life Technologies]
   - 1% Non Essential Amino Acids [Sigma Chemical Co., St. Louis, MO USA]
   - 1% Sodium Pyruvate [Bioproduct Inc., Maryland , USA]
   - 1x10⁻⁵/ml of 2-Mecaptoethanol

3. **Wash Buffer** :
   - 2% Foetal bovine serum in phosphate buffered saline [PBS]

4. **Phosphate buffered saline** :
   - 1g of Phosphate buffer [Polysciences Inc, Warrington PA] in a litre of distilled, deionized water or tissue culture water.

5. **Normal saline** :
8.5g of sodium chloride [Sigma Chemical Co., St. Louis MO, USA] in a litre of distilled, deionized water or tissue culture water.

6. 0.2% Trypan Blue:

2g of trypan blue powder (direct Blue 14) [EM Industries Inc., Cherry Hill, NJ] in 1000ml of normal saline.

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## APPENDIX III

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### APPENDIX IV

#### BIODATA OF ALL VOLUNTEERS

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**Notes:**
- ND: Not determined
- M: Male

**Results:**
- Autologous
- Specificity: T"
# APPENDIX V

## RAW DATA FOR THE POSITIVE RESPONDERS

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3rd. encounter (PHA blasts)

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One week stimulation

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Two weeks stimulation

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VOLUNTEER 6

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<p>| 127(10) | Autologous | 00.0 | 00.0 | 00.0 |</p>
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**VOLUNTEER 8**

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### MIXED LYMPHOCYTE REACTIONS

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