STUDIES ON CELLULAR IMMUNE RESPONSES OF PERIPHERAL BLOOD MONONUCLEAR CELLS FROM INDIVIDUALS IN AN ENDEMIC RURAL COMMUNITY TO A SYNTHETIC *PLASMODIUM FALCIPARUM* MEROZOITE ANTIGEN

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DECLARATION

I certify that this work has not been submitted for a degree at any other University, and I further declare that the work embodied in it is my own.

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DEDICATION

This thesis is dedicated to the glory of God, and to my dear wife Madeleine for her encouragement and sacrifice.

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ABBREVIATIONS

AMA		Apical membrane antigen
APC		Antigen presenting cell
CPM	-	Counts per minute
CSP		Circumsporozoite protein
CTL		Cytotoxic lymphocytes
DMSO	Dimethylsulphoxide	
DTH		Delayed type hypersensitivity
EBA		Erythrocyte binding antigen
ELISA	Enzyme-linked immunosorbent assay	
FCS		Fetal calf serum
GLURP		Glutamine rich protein
gm		Gramme(s)
HLA		Human leucocyte antigen
IFN-γ		Interferon gamma
Ig		Immunoglobulin
IL-		Interleukin-
IU		International units
k		Kilo
kDa		Kilodalton
l		Litre(s)
LPS		Lipopolysaccharide
MHC		Major Histocompatibility Complex
min		Minutes

ml	-	Millitre(s)
mm		millimetre(s)
MRC		Medical Research Council
MSA	-	Merozoite surface antigen
μg		Microgram
μl		Microlitre(s)
MW		Molecular weight
NHS		Normal human serum
NIH		National Institute of Health
PBMC		Peripheral blood mononuclear cells
PBS		Phosphate buffered saline
pH		Negative logarithm base 10 of hydrogen ion concentration
PI		Production index
PMA		Phorbol myristate acetate
PPD		Purified protein derivative of Tuberculin
RAP-1		Rhoptry associated protein 1
RESA		Ring infected erythrocyte surface antigen
RPMI (1640)		Rosewell Parke Memorial Institute Medium 1640
SERP		Serine rich protein
SI		Significant index
sIL-2R		Soluble interleukin 2 receptor
Spag		Soluble purified *P. falciparum* antigen
SPOD		Peroxidase labelled streptavidin

TDR	Tropical Disease Research
Th1	T helper 1
Th2	T helper 2
TNF-α	Tumour necrosis factor alpha
TT	Tetanus toxoid
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research

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SUMMARY

Several *Plasmodium spp.* antigens have been found to be associated with protection against malaria and a few have been tested in the field in human volunteers. However, these efforts have achieved limited success, and as a result the search for additional more potent malaria vaccine components continues.

The work reported in this thesis was executed with the objective of determining the suitability of three peptides obtained from the merozoite surface antigen, MSA-2, as putative sub-unit malaria vaccine components.

Previous studies have suggested that a malaria vaccine capable of inducing long-lasting protective immune responses would most likely require incorporation of antigens expressing both B-and T-cell epitopes. In this respect the MSA-2 molecule which expresses both B- and T-cell epitopes has been shown to induce immune responses that eliminates mortality due to *P. chabaudi* malaria and significantly reduces parasitaemia in mice. It is, however, necessary to determine the protective ability of MSA-2 in man, since immunity induced in the animal model is not necessarily identical to the situation in humans. Investigations of this nature in man requires the use of peripheral blood mononuclear cells (PBMC) for *in vitro* assays.

Clinical examination of the subjects included in this study, namely Ghanaian children and Ghanaian adults from a malaria endemic area, and unexposed control Danish adults, revealed no signs of clinical malaria at the time of blood sampling. However, *P. falciparum* parasites were found in blood samples of some of the Ghanaians with a significantly higher prevalence in the children (49%) than in adults (12%).

Initial experiments with fresh and cryo-preserved PBMC showed no significant differences ($P<0.005$) in the ability to proliferate and to produce interferon gama (IFN-γ) upon stimulation with tetanus toxoid (TT) as antigen. Cryo-preserved PBMC were therefore used in the experiments.

In vitro lymphoproliferative assays using three MSA-2 peptides, namely G1, G2C and G4 which contain known T-cell epitopes showed significantly higher responses ($P<0.03$) in the exposed Ghanaian adults compared with the children and Danish adults. Thus, approximately 30% of cultures from Ghanaian adults responded to each of the peptides, and one each of the cultures from the Ghanaian children (5%) and Danish adults (6%) responded to a single peptide. However, similar experiments using the control antigens, purified protein derivative of Tuberculin (PPD) and TT revealed no significant differences in lymphoproliferative responses between the three groups ($P>0.05$). This implies that the observed differences in the response to the MSA-2 peptides may be due to differences in immunological memory related to the level of prior exposure to malaria.

Interferon-gamma (IFN-γ) was detected in 32%, 36% and 29% of PBMC cultures from the Ghanaian adults following stimulation with the G1, G2C and G4 peptides, respectively. In all, 61% of these cultures produced IFN-γ in response to at least one of the peptides. None of the cultures from Danish adults produced IFN-γ. The difference was statistically significant ($P<0.001$), even though using the control antigens, PPD and TT, PBMC cultures from both groups produced similar amounts of IFN-γ.

Interleukin-4 production was also detected in response to each of the peptides in PMBC cultures of only the Ghanaian adults. However, the number of

responding cultures was lower, and there was no statistical difference between the two groups. Similar to the results obtained for IFN-γ production, there was no statistical difference between the Ghanaian and Danish adults with respect to IL-4 production in responses to control antigens. With one exception, PMBC cultures from Ghanaian adults produced only IFN-γ or IL-4 in response to the MSA-2 peptides.

In all, 82% of PMBC cultures from the exposed Ghanaian adults responded to MSA-2 peptides by proliferation or cytokine production. In contrast, only 14% of PMBC cultures from the unexposed Danish adults responded. The ability of the MSA-2 peptides to selectively stimulate PBMC of exposed Ghanaian adults seems to support the immunogenicity of the T-cell epitopes within the MSA-2 peptides in some individuals following natural exposure to malaria parasites. Furthermore, the association of these responses with the expected immunity of Ghanaian adults may suggest that MSA-2 T-cell epitopes could play a role in protective immunity against malaria.

CHAPTER 1.

INTRODUCTION

1.1 Introduction

Malaria is arguably the most important tropical parasitic disease (TDR, 1985). It is a significant cause of abortion, still birth, child mortality, and death in pregnant women. It also causes impaired growth in children and loss of productive activity in adults (TDR, 1987).

Extrapolations from epidemiological studies conducted in Africa indicate that the disease is responsible for not less than 1.0 to 2.8 million deaths annually, mainly among children below the age of five years (WHO, 1993). The enormous number of lives and labour lost together with the cost of treatment of patients, exerts a negative impact on development and thereby make malaria a major social and economic burden (TDR, 1993).

Malaria is caused by protozoan parasites of the genus *Plasmodium* of which four species, *P. falciparum*, *P. vivax*, *P. malariae* and *P ovale* are responsible for the disease in man. These parasites are transmitted by *Anopheles* mosquitoes which act as vectors of the disease. *Vivax* malaria covers the widest geographic area, including temperate, tropical and subtropical zones. However it does not occur in large areas of tropical Africa. On the other hand *ovale* malaria is found mainly in tropical Africa, and *malariae* malaria is widely distributed, but it is not as common as *vivax* malaria (TDR, 1987). *Falciparum* malaria is the most lethal and frequently occurring form of the disease throughout the tropics and subtropics. In all about 300 million people are believed to be infected worldwide with malaria parasites, with 90% of them living in tropical Africa. Of these, about 120 million develop clinical disease which is mostly caused by *P. falciparum*. This parasite is also responsible for over one third of the infections in the rest of the

world. According to TDR (1993), nearly 40% of the world's population, over two billion people, are exposed to the risk of malaria infection.

Human malarial infection begins with the bite of an infected female *Anopheles* mosquito. Injected sporozoites migrate through the bloodstream to the liver where they invade hepatocytes, and undergo a phase of maturation and asexual reproduction. Numerous asexual progeny, known as merozoites leave ruptured liver cells, enter the bloodstream and invade circulating erythrocytes. This invasion initiates the erythrocytic phase of the life cycle of the parasite. Parasites in the red cells multiply in species-characteristic fashion and break out of the host cells synchronously. Successive broods of merozoites appear at 48-hour intervals with *P vivax,* P. ovale, and *P falciparum* or every 72 hours with *P malariae* to initiate a new intra-erythrocytic cycle.

During the erythrocytic cycles, some parasites differentiate into male or female gametocytes. This initiates the sexual cycle which can only continue with the sporogonic phase in the vector (Bruce-Chwatt, 1985).

During a typical malaria infection, the various stages of the parasite that occur in the mammalian host present a wide variety of antigens to the host's immune system. However, only a small proportion of the antigens are believed to be capable of stimulating protective immune responses. Some of the other antigens are either irrelevant to protection or may even induce undesirable host responses (TDR, 1985). Furthermore, stage-specific as well as strain-specific differences in the immunity to *Plasmodium* infections have been reported (Howard, 1987; Riley *et al.*, 1993).

Both antibody-dependent and antibody-independent immune effector mechanisms appear to be involved in naturally acquired protective immunity to malaria. As early as the 1950's, Colbourne (1955) reported that this immunity appears to depend upon maintenance of immunological memory in the presence of low level parasitaemia, inhibiting new infections or maintaining the infections at a low level without clinical symptoms (Bruce-Chwatt, 1963). The ability of antibodies to give protection against *Plasmodium* is evident from the protection conferred to neonates and infants by maternally derived antibodies and from clinical treatment trials with immune serum or purified immunoglobulins (Cohen *et al.*, 1961; McGregor *et al.*, 1963). The total level of *Plasmodium* specific antibodies in individuals residing in areas of high malaria endemicity has been found to be age dependent. It increases during childhood and reaches maximum levels in early adult life (McGregor *et al.*, 1970). It has been observed by several workers that the antibodies produced are directed against all the stages of the parasite that occur in the mammalian host, namely the sporozoites, blood stages and gametocytes (Nardin *et al.*, 1979; Cohen and Butcher, 1971; Mendis *et al.*, 1987). However, no significant correlation has been found between total anti-plasmodium antibody levels and malaria infection or clinical disease in individuals studied over long time periods. Thus, in general antibody levels have been found to be more indicative of previous infection than of functional acquired immunity (Voller, 1971; McGregor, 1986; Marsh *et al.*, 1989). Consequently, Weidanz and Long (1988) hypothesized that the development of protective immunity depends on the acquisition of a critical number of T-cells specific for malaria antigens which subsequently control the immune response through regulation of macrophages, B-

cells, production of substances toxic to the infecting parasites and direct cytotoxicity (Ockenhouse and Shear, 1983, 1984; Kabilan *et al.*, 1987; Sinigaglia *et al.*, 1987; Hoffman *et al.*, 1989a; Schofield *et al.*, 1987a,b).

Two subsets of helper T-cells (CD4 positive T-cells), Th1 and Th2 have been characterized based on the pattern of their cytokine production (Mossman *et al.*, 1986). Th1 cells produce IFN-γ, IL-2, and lymphotoxin among other soluble mediators, whereas Th2 cells secrete IL-4, IL-5, IL-6, and IL-10. Functionally, Th1 cells have mainly been associated with cellular immune responses such as delayed type hypersensitivity (DTH) reactions. On the other hand, Th2 cells have been related to B-cell help leading to antibody production (Mossman and Coffman, 1989). Recently both Th1 and Th2 cells have been found to play a protective role during *P. chabaudi chabaudi* infection in mice (Taylor-Robinson *et al.*, 1993).

Epidemiological studies in areas with stable endemic *P. falciparum* malaria have shown that parasitaemia is most common in young children and that the incidence of parasitaemia declines with age. Christophers (1924) suggested that this trend provides evidence of gradual acquisition of specific immune responses which inhibit parasite growth and eliminate blood stage parasites. However, McGregor (1960) and Gilles (1961), reported that individuals living in malaria endemic areas may pass through 5 stages before immunity is acquired. According to them, stage 1 involves 0 2 month old infants. These children who are generally protected against malaria by maternal antibodies, have low incidence of parasitaemia which is normally below 10%. Stage 2 involves 2 6 months old infants. The first

clinical attacks of malaria which are normally mild are encountered in this group which have a high incidence of parasitaemia (Gilles, 1961). After this age stage 3 is reached during which repeated malaria attacks associated with severe clinical illness disrupt the normal pattern of growth. Profound anaemia and high mortality rates have also been reported among members of this group (McGregor *et al.*, 1956). Stage 4 includes older children among whom there is a rapid decline in severity and frequency of clinical episodes, despite the persistence of relatively high parasitaemia (Ziemann, 1924). This stage represents the first phase of clinical immunity (or antitoxic immunity). The fifth and final stage comprises adolescents and adults who experience fewer and milder clinical attacks. The immunity in this group is not sterile as adults living in holoendemic areas will often harbour low parasite levels even though they exhibit no clinical symptoms (Bruce-Chwatt, 1963). The age at which an individual passes through each stage is dependent upon the endemicity of malaria in the area. Based on these epidemiological findings, individuals living in areas of high malaria endemicity may be categorized into, "non-immune" young children, "semi-immune" older children and "immune" adults.

The rapid emergence of parasite strains which are resistant to several anti-malarial drugs and failure of most malaria control programs utilizing the present repertoire of available tools has prompted the call for a vaccine against malaria (TDR, 1993). There has been considerable progress in defining malaria antigens, a few of which seem to play a role in protective immunity. Furthermore, genes that control the expression of some of the identified protective antigens have been characterized (TDR, 1993). However, in the attempt to develop vaccines against

malaria, researchers have concentrated on aspects of humoral immunity, thereby neglecting cell-mediated immunity. With this observation in mind, the British Medical Council and the Malaria Immunology Scientific Working Group of WHO-TDR have emphasized the importance of investigating the role of T-cells in development and acquisition of immunity (MRC, 1989; TDR, 1985). In addition, several workers including Troye-Blomberg and Perlmann (1988) and Weidanz and Long (1988) argued that although B-cells are the source of antibody producing plasma cells, B-cell differentiation and antibody production is regulated by T-cells. Hence, to induce efficient and long-lasting antibody responses which can be boosted by natural infections, the parasite antigens used in vaccines must contain epitopes recognized by regulatory T-cells as well as B-cells (TDR, 1987). Subsequently, the importance of cell mediated immunity in protection against malaria has been established by studies with rodents in which T-cell mediated immunity to merozoites was shown to play an important role in protection (Jayawardena *et al.*, 1982).

T-cell responses to *P. falciparum* merozoite surface antigens in mice and rhesus monkeys have been shown to be under genetic control, that is MHC-restriction (TDR, 1987). Studies in man, although not conclusive, also appear to suggest MHC-restriction of T-cell responses to *P. falciparum* antigens (Quakyi *et al.*, 1989). These findings may suggest that future malaria vaccines should contain several T-cell epitopes, if the majority of vaccinated subjects are to respond favorably (TDR, 1987). This would mean that there is the need for thorough understanding of the nature and function of responses induced by malaria specific immunodominant T-cell epitopes. Furthermore, the rational development of malaria

vaccines requires an understanding of the mechanisms underlying both protective immunity and the immunopathological complications associated with the disease (TDR, 1985). The present study, therefore, aimed at investigating *in vitro* T-cell responses to one of the putative malaria vaccine components, the merozoite surface antigen 2 (MSA-2) in Ghanaians exposed to the risk of malaria. Responses to three peptides from different regions of the molecule all containing aminoacid sequences (epitopes) which can stimulate T cells from immune individuals (Rzepczyk *et al.*, 1989) were used for this purpose.

1.2 Objectives of the Study

1) To study *in vitro* lymphoproliferative T-cell responses to MSA-2 peptides G1, G2C and G4, in peripheral blood mononuclear cells (PBMC) of children and adults from a malaria endemic Ghanaian community.

2) To compare *in-vitro* lymphoproliferative T-cell responses to MSA-2 in PBMC of subjects from a malaria endemic area of Ghana with those of unexposed Danish controls.

3) To examine the nature of the T-cell response to the MSA-2 peptides in Ghanaian adults with respect to IFN-γ and IL-4 production.

Justification

The study of cellular immune responses to the MSA-2 antigen in Ghanaian children who are highly susceptible to malaria and Ghanaian adults who are less susceptible to the disease, would provide useful information needed to determine the suitability of MSA-2 as a vaccine candidate antigen.

CHAPTER 2.

LITERATURE REVIEW

2.1 Classification and identification of *Plasmodium* species

Based on morphological and biological criteria malaria parasites are classified under the phylum Protozoa, the subphylum Sporozoa, the class Telosporea, the subclass Coccidia, the order Eucoccidia, suborder Haemosporina, family Plasmodiidae, and genus *Plasmodium* (Honigeberg *et al.*, 1964; Levine, 1973). More than 100 *Plasmodium* species are known to infect a wide range of hosts including reptiles, birds, rodents and primates, with each parasite species exhibiting a narrow host specificity. Four species, namely, *P. falciparum*, *P.vivax*, *P. ovale* and *P. malariae*, are responsible for the disease in man. However, malaria caused by *P. falciparum* is the most lethal, resulting with the highest morbidity and mortality.

These human infective *Plasmodia* are easily distinguished by their morphological appearances using ordinary light microscopy of Giemsa or Field stained blood films.

2.2 Life cycle of the malaria parasite

Plasmodium spp. undergo complex life cycles, alternating between a vertebrate host and an arthropod vector.

Malaria parasites are transmitted to humans through the bite of infective female mosquitoes belonging to the genus *Anopheles*. In this process, infective sporozoites are introduced into the skin of a susceptible host through the saliva of the vector. In the host, the injected sporozoites migrate through the bloodstream to the liver where they invade liver cells within half an hour after transmission to begin the exoerythrocytic hepatic stage of the life cycle. In the liver cells the

parasites undergo asexual multiplication by which they develop through different stages that culminates in the formation of thousands of merozoites. With falciparum malaria, about 30,000 merozoites may be released from an infected liver cell 7-12 days after invasion.

The released merozoites in turn invade erythrocytes to initiate the asexual erythrocytic stage of the life cycle. In the erythrocyte the parasite develops through ring, trophozoite and schizont stages with species distinguishing characteristics (that can be used in differential diagnoses). The intra-erythrocytic parasite is surrounded by a parasitophorous vacuole membrane. A great deal of parasite protein synthesis takes place inside infected erythrocytes especially during the trophozoite stage of development. During this development, the parasite modifies host cells in several ways so as to enhance its own survival. In the red cell, asexual division within the schizont stage results with about 8-24 daughter merozoites which are finally released following red cell rupture to initiate subsequent intra-erythrocytic cycles. Generally, the intra-erythrocytic cycle tends to become synchronous after a few cycles. In the case of *P. falciparum*, *P.vivax* and *P. ovale*, the generation time has been determined to be 48 hours whilst that of *P. malariae* is 72 hours. The release of parasites into the blood stream is normally accompanied by fever which therefore becomes periodic because of the synchronous nature of merozoite release. In a susceptible host this asexual erythrocytic cycle may be repeated many times, during which a few invading parasites differentiate into sexual forms, (gametocytes) which alone can infect the insect vector.

Plasmodium species infect the mosquito following ingestion of infective gametocytes in the blood of an infected vertebrate. In the gut of the mosquito

gametocytes are released from infected erythrocytes to begin the sexual stage of the life cycle. First, the gametocytes differentiate into male and female gametes and the male microgamete fertilizes the female macrogamate resulting in the formation of a zygote. Shortly after fertilization, meiosis takes place leading to genetic recombination, thus creating new haploid parasite variants. Indeed, apart from the zygote all the parasite stages that occur in the vector and the vertebrate hosts are haploid. The zygote develops into a mobile ookinete which penetrates the peritrophic membrane surrounding the bloodmeal and the gut endothelium, to which it attaches. The ookinete then transforms into an oocyst which attaches to the outside of the mosquito gut wall suspended in the haemocoele. Inside the oocyst a massive multiplication of parasites takes place resulting in the formation of hundreds of thousands sporozoites. The sporozoites invade the mosquito salivary glands and mature into infective forms which alone can initiate infections in the vertebrate host. It takes about 10-11 days at 25°C for gametocytes to develop into infective sporozoites (reviwed by Theander, 1992; Riley *et al.*, 1993).

2.3 Human malaria

The clinical manifestations of human *P. falciparum* infections include chills, fever, headache, nausea, vomiting and diarrhoea. A prominent feature of the febrile response is its tendency of periodicity.

In areas with stable malaria transmission clinical symptoms of malaria are normally confined to children and pregnant women (Theander, 1992). Greenwood (1987; 1991) observed that the incidence of clinical malaria in rural areas of the Gambia is in the range of 1-5 annual attacks per child, with each clinical attack

having a mortality rate of about 0.5% (Greenwood,1990). Nevertheless, cerebral malaria and severe anaemia with mortality rates of 15-30% are responsible for most deaths resulting from malaria in tropical Africa. Severe anaemia is especially prevalent in the younger children whereas cerebral malaria is more frequent in slightly older children (Greenwood, 1991, Brewster, 1990). It has been proposed that severe malaria may partly be due to exposure to more virulent parasite strains with additional properties including the expression of variant antigens that are new to the hosts immune system (Clark, 1989). However, the immune response of the host may also contribute to the pathogenesis of severe malaria, since the symptoms including, fever, nausea, vomiting, rigor, headache and thrombocytopenia, are similar to the physiological effects of certain cytokines including TNF-α (Clark, 1991).

Immigrants and tourists as well as children and adults living in areas of unstable malaria transmission are all susceptible to clinical attacks. In such immunologically naive individuals malaria parasitism is nearly always associated with disease (Marsh, 1992). In non-endemic areas, the diagnosis of clinical malaria may thus be made on the basis of fever and a positive blood film.

In malaria endemic areas, a case definition is extremely difficult even though clinical malaria is often defined as fever with temperatures greater than 37.5°C associated with more than 2500 parasites per μl blood. The definition has limitations since there appears to be no strict correlation between parasite burden and clinical symptoms. Thus children with very low parasitaemia sometimes have symptoms while other children with much higher parasitaemia have none (Marsh,

1992). To assume that a child needs to present with both fever and reasonably high parasitaemia to be declared ill from malaria may therefore exclude some true cases (Schellenberg *et al.*, 1994). Nevertheless, in the presence of asymptomatic *P. falciparum* infections, fever could be due to other disease conditions like pneumonia and influenza. Hence one needs to apply strict exclusion criteria to rule out other disease conditions presenting with fever when diagnosing clinical malaria in endemic areas.

2.4 Genetic resistance to malaria

Some red cell abnormalities including the sickle cell trait, alpha thalassemia, beta thalassemia, ovalocytosis, and glucose-6-phosphate-dehydrogenase deficiency have been associated with various degrees of resistance to malaria (Weatherall, 1987). This is partly because high prevalence rates of these abnormalities are found in areas where malaria is endemic (Allison, 1954; ; Flint *et al.*, 1986). It is so far established that the sickle cell trait confers protection against clinical malaria and especially against severe malaria but not against infection as such (Hill, 1991). HLA antigens have also been linked with protection against severe clinical malaria in Gambian children. In this respect, the class I antigen, HLA-Bw53 was reported to be associated with protection against both cerebral malaria and severe anaemia while the class II antigen HLA DRw13 was associated with protection against severe anaemia (Hill, 1991).

2.5 Development of protective immunity to malaria

Studies in malaria endemic populations on the acquisition of immunity to malaria have shown that the decline in the levels of parasitaemia is usually preceded by a decline in morbidity and mortality from malaria. In a study conducted in the Gambia where *P. falciparum* transmission was seasonal but relatively stable from year to year, malaria parasite rates were found to decline only after the age of 10 to 12 years (Riley *et al.*, 1990a). In the same study clinical disease (fever associated with parasitaemia) was found to peak at 6 years of age. However peak mortality was found to occur in children aged 4 years (Greenwood *et al.*, 1987). Hence the incidence of severe disease and death from malaria declines rapidly in young children at a time when they still harbour considerable parasite loads. In agreement, Playfair *et al.* (1990) and indeed Sinton (1939) proposed that clinical or anti-disease immunity may be distinct from anti-parasite immunity. It is likely that different protective mechanisms are involved in these two separate machanisms of protective immunity and different antigens may be involved in their induction (Playfair *et al.*, 1990).

2.6 Anti-disease Immunity

Some of the soluble exoantigens released into circulation during rupture of schizont infected erythrocytes have been shown to share chemical and biological characteristics with bacterial endotoxins such as lipopolysaccharide (LPS) (Jakobsen *et el.*, 1987). These exoantigens directly trigger monocytes and macrophages to release endogenous pyrogens like interleukin-1 (IL-1) and tumour necrosis factor (TNF) (Bate *et al.*, 1989; Taverne *et al.*, 1990; Jakobsen *et al.*, 1991), which have

been implicated in the pathogenesis of malaria (Clark and Cowden, 1991). It has been shown in mice that antibodies against these exoantigens can block the induction of pyrogenic cytokines and reduce severity of clinical symptoms (Clark and Cowden, 1991). Playfair *et al.* (1990) proposed that a similar situation may exist in humans. Thus, anti-disease immunity is believed to exist in humans with relatively high parasitaemia without any clinical disease.

2.7 Anti-parasite Immunity

Immunity against malaria also depends on the ability to control parasite multiplication. This has been shown to involve both antibody dependent and antibody independent effector mechanisms (Troye-Blomberg and Perlmann, 1988; Weidanz and Long, 1988). For example, antibody against the circumsporozoite protein (CSP) can inhibit the entry of sporozoites into liver cells (Hollingdale, 1984), and the prevalence of such antibodies has been shown to increase with age in endemic populations (Hoffman *et al.*, 1986). Also, sporozoite and liver stage antigen specific cytotoxic T-lymphocytes (CTL) have been demonstrated *in vitro* in mice (Kumar *et al.*, 1988; Hoffman *et al.*, 1989b). However, the importance of cytotoxic responses against infected hepatocytes in the development of immunity in humans have not been established. Also Interferon gamma (IFN-γ) released by immune T-cells, have been shown to mediate killing of intrahepatic parasites *in vivo* (Ferreira *et al.*, 1986).

It is also known that antibody against merozoites can block their invasion into erythrocytes *in vitro* (Wahlin *et al.*, 1984) and cytophilic antibodies can opsonize parasites for phagocytosis (Lunel and Druilhe, 1989). Furthermore,

agglutinating antibodies can immobilize free merozoites (Green *et al.*, 1981). Antibodies can also block the adherence of mature parasitized erythrocytes to capillary endothelium and thereby facilitate their clearance from circulation and reduce the risk of cerebral malaria (David *et al.*, 1983). In these processes, T-cells are believed to provide help for antibody production through release of cytokines such as IL-4, IL-5 and IFN-γ (Weidanz and Long, 1988). Cytokines such as IFN-γ, activate macrophages to phagocytose infected erythrocytes and thereby kill malaria parasites (Brown and Kreier, 1986).

A different aspect of anti-parasite immunity is the finding that antibodies to gamete surface antigens (Carter *et al.*, 1985) and gamete-specific T-cells can block transmission of malaria parasites to mosquitoes (Harte *et al.*, 1985; Naotunne *et al.*, 1991).

2.8 Parasite evasion of the immune system

Malaria parasites are intracellular during most of their life cycle. As a result, they are exposed to the extra cellular environment for only brief periods of time. This situation helps them to evade much of the host's defence mechanisms. Also, antigenic variation involving antigens which may be crucial to parasite survival, and inhibition of immune responses as well as variation of parasite proteins on the surface of infected cells such as erythrocytes (Hommel, 1983), are important evasive strategies employed by the parasite to slow down the development of immunity against malaria. Roberts (1992) observed that parasite antigens expressed on the surface membranes of infected erythrocytes may be linked with cytoadherence properties. Also, Marsh and Howard (1986) argued that

the expression of parasite antigens on red cell membrane may slow down development of immunity against different strains of the malaria parasite.

2.9 T-cell activation and the major histocompatibility complex

Antigen specific T-lymphocytes recognize degraded antigen on antigen presenting cells (APC), in the form of complexes with polymorphic cell surface proteins encoded by the major histocompatibility complex (MHC) (Male *et al.*, 1991). In humans, the MHC is referred to as Human Leukocyte Antigens (HLA) of which two major types are involved in antigen presentation. These are the Class I and Class II antigens. The HLA antigens involved in antigen presentation determines the type of T-cells that recognize the antigen and become activated. This is because antigens presented in association with MHC Class I molecules are recognized by $CD8^+$ T-cells, whilst antigens presented in association with MHC Class II molecules are recognized by $CD4^+$ T-cells. The expression of CD4 and CD8 on mature T-cells is mutually exclusive. It has been determined that the $CD4^+$ T-cell subset roughly corresponds to the functionally defined helper/inducer subset whereas $CD8^+$ T-cells are mainly cytotoxic (Imboden and Weiss, 1988; Haas *et al.*, 1990).

The parts of a peptide recognized by T-cells are reffered to as T-cell epitopes. T-cell epitopes normally contain a linear row of 9-15 amino acids (Roit, 1991). Following activation some T-cells eventually differentiate into memory T-cells with a characteristic pattern of cell-surface molecules (or markers) (Sanders *et al.*, 1988). On subsequent encounter with antigen, memory T-cells proliferate, releasing different cytokines which effect various biological functions (Sanders, *et*

al., 1988). These include help to B-cells for antibody production and stimulation of natural killer cells and monocytes (Sinigaglia *et al.*, 1987; Ockenhouse and Shear, 1983).

2.10 Regulatory T-cell subsets

Analysis of murine T-cell clones has revealed that $CD4^+$ cells can be divided into two subpopulations, based on their repertoire of cytokine production (Mossman *et al.*, 1986). Upon activation, $CD4^+$ cells of the T-helper 1 subset (Th1) produce IL-2 and IFN-γ among other cytokines, while cells of the T-helper 2 subset (Th2) produce IL-4 and IL-5 (Mossman and Coffman, 1989). Although the relationship between these cell types is not clear, it appears that Th1 cells mediate certain antibody independent responses and Th2 cells provide help for specific antibody production (Taylor-Robinson *et al.*, 1993). In the murine malaria model using *P. chabaudi*, Th1 cells have been shown to be important in the early phase of infection, whereas the final clearance of the parasite load, coincides with the appearance of malaria-antigen specific antibody mediated effector mechanisms (Langhorne *et al.*, 1990).

2.11 The relative roles of B- and T-cells in malaria

Experiments with laboratory rodents have contributed greatly to our understanding of the relative importance of the protective roles of T- and B-cells (Jayawardena, 1981, Weidanz and Long, 1988; Del Giudice *et al.*, 1988). These experiments have shown that there is a large variation in T- and B-cell requirements, depending on the strain and species of the infecting *Plasmodium*

parasites and the genetic constitution of the mice (Jayawardena, 1981; Long, 1988).

Correlation between total anti-malaria antibodies and protection is poor, indicating that many of the formed antibodies do not have any protective value. However, passive transfer of protection with IgG from immune sera has been demonstrated in both humans (Cohen *et al.*, 1961; McGregor *et al.*, 1963), and in different animal models (Weidanz and Long, 1988). In addition, several parasite specific monoclonal antibodies have been shown to either protect or block transmission of rodent malaria (Hollingdale *et al.*, 1984; Miller, *et al.*, 1984). However, even in those experimental models in which the protective importance of antibodies is well established, it is agreed that maintenance of immunity is under the control of T-cells (Weidanz and Long, 1988). In general, anti-malaria antibodies are of T-dependent isotypes (Weidanz and Long, 1988; Troye-Blomberg and Perlmann, 1988), and T-cells also control the antibody independent effector systems involved in parasite clearance such as help for generation of antigen specific cytolytic cells (Hoffman *et al.*, 1989a), production of cytokines (Schofield *et al.*, 1987a,b; Sinigaglia et. al, 1987), and activation of non-lymphoid cells leading to intracellular killing (Ockenhouse and Shear, 1983, 1984).

2.12 T-cell responses to malaria antigens

There is good evidence from experimental animal models that cellular immune responses are involved in protective immunity to malaria. In some models, thymectomized animals failed to become immune whilst intact animals developed a long lasting immunity (Brown *et al.*, 1968; Weinbaum *et al.*, 1976). In the case of *P. yoelii* infections in mice, maximum protection depended on the cooperation

between T and B lymphocytes (Mogil, 1987), whereas immunity to *P. chabaudi adami* appears to be independent of antibody (Grun and Weidanz, 1981). Although the extent to which animal models parallel the human response to malaria infection is not clear, accumulating evidence indicate that T-cells play an important role in the human response (Troye-Blomberg and Perlmann, 1988; Weidanz and Long, 1988). Thus, for example, stage specific malaria antigens have been shown to be recognized *in vitro* by T cells of individuals from malaria endemic regions (Hviid *et al.*, 1992).

2.12.1 Responses to pre-erythrocytic *Plasmodium* stages

Donors from malaria endemic areas often possess sporozoite antigen-reactive T-cells, directed against the circumsporozoite (CSP) antigen, a major surface protein of sporozoites (Dame *et al.*, 1984; Good *et al.*, 1988). In mice control of sporozoite infection is partly effected by $CD8^+$ CSP-specific CTL response against infected hepatocytes (Schofield *et al.*, 1987b; Hoffman *et al.*, 1989b). However, the importance of cytotoxic responses against infected hepatocytes in the development of immunity in man is not known. As reported by Schofield (1987a), and Mellouk (1987), the best established human response against infected liver cell involves the cytokine IFN-γ.

2.12.2 Responses to erythrocytic *Plasmodium* stages

Most malaria parasite antigens exposed to the immune system are from asexual blood stages. These include the ring infected erythrocyte surface antigen, Pf155/RESA, which is a polypeptide originating from merozoites (Perlmann *et al.*, 1984; Coppel *et al.*, 1984). This antigen is deposited into the erythrocyte surface membrane during parasite invasion. Pf 155/RESA has been shown to induce specific T-cell response in malaria exposed individuals. This response includes proliferation, cytokine production and T-cell dependent antibody production (Kabilan *et al.*, 1988). Different human T-cell response types to Pf155/RESA have been characterized (Kabilan *et al.*, 1988), suggesting the existence of functionally distinct human T-cell subsets similar to Th1 and Th2 type T-cell described in mice (Troye-Blomberg *et al.*, 1990).

Several T-cell epitopes have been identified in the *P. falciparum* major merozoite surface protein (MSA-1), using synthetic or recombinant peptides and T-cells from people of malaria endemic areas (Hviid *et al.*, 1992). Responses to variable regions of MSA-1 by T-cells from Gambian donors were found to increase with age, whereas responses to conserved regions decreased (Hviid *et al.*, 1992). Moreover both proliferation and IFN-γ production in response to the C-terminal part of the molecule appeared to be associated with resistance to clinical disease and high parasitaemia.

Another *P falciparum* merozoite surface antigen (MSA-2), has been shown to contain T-cell epitopes both in the constant and variant regions of the antigen (Rzepczyk *et al.*, 1989, 1990), and to induce lymphoproliferation in malaria exposed donors in an age dependent way by both proliferation and IFN-γ

production.

Other asexual blood stage antigens which contain T-cell epitopes that induce specific proliferation and cytokine production from T-cells of malaria exposed donors include soluble purified *P. falciparum* antigen (Spag), purified from culture supernatant (Jepsen and Andersen, 1981; Jakobsen *et al.*, 1990, 1991; Hviid *et al.*, 1990); glutamine rich protein (GLURP) and serine rich protein (SERP), which are soluble proteins stored in the parasitophorous vacuole and released during schizont rupture (Borre *et al.*, 1991; Roussilhon, *et al.*, 1990).

No evidence of T-cell effector mechanism against parasitized erythrocytes has been found, which is not surprising considering the scarcity of MHC class I antigens on erythrocytes. Cytokines such as IFN-γ do not have direct inhibitory effect on blood stage *P. falciparum* parasites (Hviid *et al.*, 1988; Ferreira *et al.*, 1986), but might be involved in the immune responses through activation of monocytes (Ockenhouse and Shear, 1984).

T-cells from malaria exposed individuals from the Gambia have been found to respond to crude gametocyte antigen, whilst unexposed individuals responded weakly (Riley *et al.*, 1990). T-cells from 40% of the same exposed Gambian donors responded to affinity purified gametocyte antigen Pfs 48/45, whilst non-exposed donors were completely unresponsive (Riley *et al.*1990)

2.13 The Impact of Malaria Infection on the Immune System

There is much evidence to suggest that acute malaria leads to a temporary state of reduced immunocompetence (Houba, 1988; Goodnewardene *et al.*, 1990; Hviid *et al.*, 1992; Riley *et al.*, 1993). Acute malaria is associated with increased

susceptibility to salmonellosis (Bennett and Hook, 1959; Mabey *et al.*, 1987), and other bacterial diseases (Greenwood, 1974), as well as reactivation of chronic or latent viral infections such as those caused by *Herpes zoster* (Cook, 1985), *H. simplex* (Scott, 1944), and Epstein-Barr virus (Whittle *et al.*, 1984, 1990). Children with malaria parasites also tend to respond less well to some vaccines, such as those against *Clostridium tetani*, *Salmonella typhi*, and group C meningococci, compared to uninfected children (Greenwood *et al.*, 1972; Williamson and Greenwood, 1978). However, Smedman *et al.* (1986) found the responses to live attenuated measles vaccine to be higher in malaria infected children. This observation was suggested to be due to prolonged survival of the virus within immunocompromised *Plasmodium* infected children (Smedman, *et al.*, 1986).

Also, Greenwood *et al.* (1988) found that children living in highly endemic areas who were protected from infection by chemoprophylaxis were less susceptible to other infectious diseases than unprotected children. Investigations with such protected children have revealed superior cellular proliferative and IFN-γ responses to malaria antigens (Otoo *et al.*, 1989). Nevertheless, the reduction in overall childhood mortality in children protected by chemoprophylaxis was found to be considerably greater than the expectation from prevention of deaths due to malaria alone (Greenwood *et al.*, 1988). This may suggest that malaria infection predisposes to death from other diseases (Greenwood *et al.*, 1988). Moreover, children protected by chemoprophylaxis against malaria responded better than unprotected children to routine childhood vaccinations (McGregor and Barr, 1962). It is, therefore, generally accepted that malaria patients are more susceptible to other infectious diseases because of reduced ability to mount effective immune

responses. Interestingly, however, *P. falciparum* infection has been found to result in low or absent *in vitro* lymphoproliferative responses in some apparently immune individuals (Troye-Blomberg *et al.*, 1983a, 1984, Bygbjerg *et al.*, 1986; Ho *et al.*, 1986, Webster *et al.*, 1988, Hviid *et al.*, 1991). This lack of responsiveness appears to be associated with the mere presence of parasites in the blood, since some individuals with asymptomatic parasitaemia have also been found to be unresponsive (Theander *et al.*, 1986b; Hviid *et al.*, 1990). This kind of observation led Riley *et al.* (1988) to conclude that infection does not affect only proliferative responses but also lymphokine (IL-2 and IFN-γ) secretion. Nevertheless, some malaria-specific T-cells are present in the peripheral circulation during acute disease and can be activated. This may explain the success of Sinigaglia *et al.* (1985) and Pink *et al.* (1987) in isolating T-cell clones specific for defined *P. falciparum* antigens from the blood of malaria patients.

The lack of *in vitro* response of PBMC to malaria antigens on the other hand contrasts with the high levels of soluble IL-2 receptor (sIL-2R) and soluble CD8 antigen found in sera of malaria patients (Riley *et al.*, 1993; Hviid *et al.*, 1991a; Hviid *et al.*, 1991b; Josimovic-Alasevic *et al.*, 1988; Kremsner *et al.*, 1989; Nguyen-Dinh and Greenberg, 1988; Deloron *et al.*, 1989). These markers suggest that cellular activation does take place and hence the reduced responses in PBMC may be due to reallocation of specific T-cells to other sites. Evidence from studies in mice show that activated T-cells and antigen presenting cells (APC) migrated to the spleen and liver during acute malaria (Dockrell *et al.*, 1980; Playfair and De Sousa, 1982; Kumararatne *et al.*, 1987). More recently, Langhorne and Simon-Haarhus (1991), clearly demonstrated the presence of

malaria specific T-cells in the spleen but not in the peripheral blood of mice during acute infection with *P. chabaudi*. However, responding cells could be found in both spleen and peripheral blood after treatment. It is likely that a similar phenomenon may occur in *P. falciparum* infected humans. This is likely to be true since lack of T-cell responsiveness is most pronounced in patients with enlarged spleens (Wyler, 1976; Greenwood *et al.*, 1977). Furthermore, it has been reported that T-cells with high expression of the surface antigen LFA-1, which is known to be involved in cellular adhesion, are transiently lost from circulation in such patients (Hviid *et al.*, 1991b) although the molecule is upregulated on *P. falciparum* stimulated T-cells *in vitro* (Hviid *et al.*, 1993b). However, the extent of decrease in circulating T-lymphocytes during acute malaria appears to suggest that the loss cannot be attributed solely to depletion of malaria-specific T-cells. Some of the re-allocated cells may recognize epitopes that cross-react with malaria antigens or may be activated in a nonspecific fashion by T-cell derived cytokines. Mueller *et al.* (1989) have argued that cells activated in such a nonspecific manner may be induced to express activation markers and to sequester in the spleen.

Loss of PBMC responses in malaria patients may also be partly due to activation of "suppressor" $CD8^+$ cells. Activation of suppressor T-cells has been reported in mice infected with *P. berghei* (Lelchuk *et al.*, 1981), *P vinckei* (Chilbert *et al.*, 1981), and *P. chabaudi* (Russo and Weidanz, 1988). The ratio of $CD8^+$ to $CD4^+$ T-cells in the peripheral blood of patients with acute *P. falciparum* malaria is higher than normal (Theander *et al.*, 1986; Troye-Blomberg *et al.*, 1983), and high levels of soluble CD8 antigen are found in the sera of malaria patients (Kremsner and Bienzle, 1989; Elhassan *et al.*, 1994). $CD8^+$ T-cells

obtained from immune individuals during the malaria transmission season has been shown to suppress proliferative responses to *Plasmodium* antigens *in vitro*, further supporting the role of $CD8^+$ suppressor cells (Theander *et al.*, 1993). A low molecular mass glycoprotein isolated from *P. berghei* infected erythrocytes can suppress primary antibody responses to T-dependent but not T-independent antigens *in vivo* (Khansari *et al.*, 1981; Srour *et al.*, 1988), and *P. falciparum* schizont extracts can suppress *in vitro* lymphoproliferative responses to purified malaria antigens and other soluble antigens (Riley *et al.*, 1989). The precise parasite components that induce these suppressive effects, and the manner in which the suppression is mediated have not as yet been adequately characterized.

2.14 *In vitro* analysis of cellular immune responses in malaria

T-cell responses to *Plasmodium* antigens in humans have been demonstrated *in vitro* by lymphocyte proliferation as well as by induction of cytokines such as IFN-γ and IL-4 in peripheral blood mononuclear cells (PBMC) of adults from malaria endemic areas and in individuals recovering from clinical malaria (Ho *et al.*, 1990; Riley *et al.*, 1988; Troye-Blomberg *et al.*, 1990). These responses have been shown to be long lasting, being detectable years after initial exposure (Bygbjerg *et al.*, 1985; Ho and Webster, 1989).

Hoffman *et al.* (1989a) found a positive correlation between *in vitro* proliferative T-cell responses to a specific epitope and resistance to *P. falciparum* malaria.

It has been reported by several authors that *in vitro* exposure of T-cells from *P. falciparum* primed donors to crude or defined parasite antigens result in

the release of IFN-γ mainly by $CD4^+$ cells (Chizzolini *et al.*, 1990; Troye-Blomberg *et al.*, 1990; Chougnet *et al.*, 1990; Mshana *et al.*, 1990). IFN-γ is an important T-cell derived regulatory lymphokine known to increase the expression of MHC class II antigens and to activate macrophages. It is one of the factors believed to be important for the induction of cell mediated immunity to the parasite (Shear *et al.*, 1989; Stevenson *et al.*, 1990). IFN-γ has been demonstrated in sera of patients with recent acute malaria (Rhodes-Feuillette *et al.*, 1985). Recently, serum IFN-γ was shown to be higher in non-parasitaemic donors than in donors with parasitaemia, suggesting a protective effect of that cytokine *in vivo* (Deloron *et al.*, 1991). In vitro studies have shown that the target for IFN-γ mediated inhibition appears to be the infected hepatocyte (Ferreira *et al.*, 1986, Maheshwari *et al.*, 1986). In addition, IFN-γ may also act through activation of macrophages and other effector cells (Meis and Verhave, 1988).

Another cytokine produced during *Plasmodium* infections is IL-4, which has been reported to be important in T-cell help for antibody production (Howard *et al.*, 1983). However, Liew *et al.*, (1991) reported that IL-4 and IL-10 may inhibit the effect of IFN-γ on macrophage activation.

The proliferative response to malaria antigens *in vitro* is usually limited to the $CD4^+$ T-cell subset (Mshana *et al.*, 1991) although proliferating $CD8^+$ T-cell clones recognizing malaria antigens have been obtained (Sinigaglia *et al.*, 1987). Recent evidence suggest that different T-cell subsets may respond to malaria antigen stimulation by either proliferation, lymphokine production or both (Troye-Blomberg *et al.*, 1990). This has been shown by the lack of association between these parameters (Troye-Blomberg *et al.*, 1985, 1990; Kabilan *et al.*,1988, 1990).

It is important, therefore, not to measure only proliferation or lymphokine production in the effort to determine antigen specific T-cell responses, but to measure both and also determine the functional characteristics of the activated cells in relation to the type of cytokine released. This is of particular importance in selection of vaccine immunogens.

2.15 Asexual malaria vaccine candidate antigens

Since the clinical manifestations of malaria are caused mainly by the asexual erythrocytic stages several authors have proposed the use of antigens from these stages in the development of a sub-unit vaccine against malaria (Howard and Pasloske, 1993). Such vaccines could induce a response against the extracellular parasite stages exposed to the host immune defence (Bruce-Chwatt, 1985).

Many asexual malaria antigens are polymorphic with multiple alternative antigenic forms, often characterized by sequences of tandem amino acid repeats (Howard and Pasloske, 1993; Roberts *et al.*, 1993). A range of antigenically conserved portions of the peptides will probably have to be included in a vaccine, in view of the capacity of malaria parasites for antigenic variation and the potential problem of MHC restriction (Howard and Pasloske, 1993). This will help minimize parasite avoidance of vaccine elicited immune responses and hopefully ensure protective responses in all individuals despite HLA differences. To date several proteins expressed by merozoites, including merozoite protein 1 (MSA-1), merozoite protein 2 (MSA-2), apical membrane antigen (AMA), and the erythrocyte binding antigen (EBA), have been identified as putative malaria vaccine components (reviewed by Romero, 1992). Of these, the merozoite surface antigens

1 and 2 are considered to be among the best vaccine candidate antigens for human trials (Howard and Pasloske, 1993).

2.15.1 Merozoite Surface Antigen 1

The MSA-1 is a high molecular weight protein that is synthesized during schizogony (Holder, 1988). It is a proteolytic fragment with an approximate molecular weight of 200 kDa, that is processed into fragments of 83, 42, 38, 28-30 and 19 kDa *in vivo*. The fragments can be demonstrated on the surface of mature merozoites (Holder, 1988; McBride and Heidrich, 1987). With the exception of the 19 kDa C-terminal fragment which is carried through into newly invaded erythrocytes, most of the other fragments are shed before or during red cell invasion (Blackman *et al.*, 1990). The amino acid sequence of MSA-1 varies between isolates and can be divided into three regions, namely a variable region, an isolate specific region and a highly conserved dimorphic region (Tanabe *et al.*, 1987; Peterson *et al.*, 1988). Riley and colleagues (1992) found that antibodies to the dimorphic regions of MSA 1 are prevalent in the sera of individuals exposed to *P. falciparum* infection. Also, using synthetic peptides and T-cells from donors of malaria endemic areas or MSA-1 specific T-cell clones, several workers including Crisanti *et al.* (1990) identified T-cell epitopes in the dimorphic regions of the molecule. T-cell proliferative responses and antibodies to the conserved regions of MSA-1 have been shown to correlate with clinical immunity (Riley *et al.*, 1992). The possibility of using MSA-1 as vaccine antigen is supported also by the finding that a conserved 42 kDa C terminal fragment induced antibodies that inhibited growth of *P. falciparum in vitro* (Chang *et al.*, 1992)

2.15.2 Merozoite Surface Antigen 2

Another merozoite surface antigen is MSA-2 which is a glycosylated and myristilated protein (Ramasamy, 1987). It has a strain dependent molecular weight ranging from 35 to 56 kDa (Smythe *et al.*, 1988; Clark *et al.*, 1989; Ramasamy, 1987; Fenton *et al.*, 1989).

MSA-2 has been shown to be dimorphic (Fenton *et al.*, 1991). Sequence analysis shows the C- and N- terminal regions to be highly conserved, whereas a large central region is variable (Thomas *et al.*, 1990; Marshall *et al.*, 1992). Anders *et al.* (1993) reported the presence of both repetitive and non repetitive sequences in the variable regions of this molecule, and antibodies to both repetitive and nonrepetitive regions of MSA-2 were prevalent in sera of people living in malaria endemic areas. MSA-2 was one of the antigens identified in immune complexes formed at the surface of merozoites when antibodies in immune serum were used to inhibit merozoite dispersal (Lyon *et al.*, 1986). A monoclonal antibody against an epitope within a repeat region of MSA-2 was found to block red cell invasion by merozoites *in vitro* (Saul *et al.*, 1989).

MSA-2 has been shown to contain T-cell epitopes which are clustered in the variant parts of the antigen. Subsequently, proliferation of T-cells from individuals of malaria endemic regions have been found to be induced by MSA-2 repetitive and non-repetitive sequences (Rzepczyk *et al.*, 1990, 1992). However, it is not known whether naturally acquired immune responses to repetitive and non-repetitive regions of MSA-2 could protect against infection in man (Anders *et al.*, 1993). Saul *et al.* (1992) demonstrated in mice that peptides from the conserved regions of MSA-2 can protect against *P. chabaudi* infections.

2.16 Subunit malaria vaccines and MHC restriction

Immunity to *P. falciparum* malaria is complex, and the mechanisms of protective immunity are not fully understood (Hviid *et al.*, 1990). It is, however, believed that a suitable vaccine against malaria would most certainly have to be a subunit vaccine, with components expressing both T-cell epitopes and B-cell epitopes essential for eliciting long lasting immune responses against natural infections (Riley *et al.*, 1991). Moreover, the immune response should preferable occur in all individuals and be directed against epitopes that do not vary between different parasite strains (Riley *et al.*, 1991). However, several studies in mice (Del Giudice *et al.*, 1986; Good *et al.*, 1986, 1988; Lew *et al.*, 1989) and in human populations (Good *et al.*, 1988b, Chizzolini *et al.*, 1988; Carter *et al.*, 1989; Quakyi *et al.*, 1989; Zevering *et al.*, 1990) revealed a wide spread non-responsiveness to sporozoite, merozoite and gamete vaccine candidate antigens. These differences in immune responses in a population may be due to MHC class II restriction (Good *et al.*, 1988a). It could thus be assumed that only a limited number of MHC haplotypes would be able to present a particular epitope to the T-cells. This would seriously compromise the effectiveness of subunit malaria vaccines.

MHC Class II molecules are encoded in the human leukocyte antigen D (HLA D) region and include at least three families of gene products; HLA-DR, DQ and -DP. These are polymorphic proteins capable of interacting with only discrete number of peptides (Babbitt *et al.*, 1986, Buus *et al.*, 1987). MHC genes exhibit an unusually high degree of allelic variation and unlike laboratory mice, human populations are outbred and frequently heterozygous at each genetic locus.

On the other hand, it is argued that due to the complex polymorphism of the human HLA complex and the finding of "promiscuous" peptides able to bind a wide range of class II molecules (Sinigaglia *et al.*, 1988, 1990; Ho *et al.*, 1990), unresponsiveness in humans caused by MHC restriction might not be a major constraint for subunit vaccine development. Recent reports also suggest that peptides binding to a particular class II molecule share a common structure (Sette, 1989; O'Sullivan *et al.*, 1991) and it might therefore be possible to select multi-determinant peptides with the capacity to bind many MHC types.

2.17 Previous and ongoing vaccine trials

The first clinical trials for the safety and immunogenicity of anti-*P. falciparum* sporozoite vaccines were undertaken in human volunteers in the United States by the Walter Reed Army Institute of Research (WRAIR) and the National Institute of Health (NIH), Bethesda. Results of the WRAIR/NIH trial indicated that the first prototype vaccine was well tolerated but less immunogenic in humans than in mice and rabbits (TDR, 1987), and it failed to demonstrate vaccine efficacies greater than 20-30% (TDR, 1993). The failure of this vaccine was probably due to the inclusion of only B-cell epitopes for antibody production (Ballou *et al.*, 1987). Since then the importance of T-cell epitopes has been recognized for B-cell help and memory (Good *et al.*, 1988c).

One synthetic candidate vaccine, the SPf66 against *P falciparum* malaria, was developed by the scientific group led by Dr. M.E. Patarroyo in Bogota, Colombia. The SPf66 has been field tested in more than 20,000 people in Latin America (Sempertegui *et al.*, 1994). The vaccine appeared to be safe and could

stimulate antibody responses in a majority of individuals immunized. Reports from one randomized, placebo-controlled double-blind trial carried out in Colombia suggest that the vaccine had an efficacy of about 40-60% in certain selected age groups (Patarroyo *et al.*, 1992; TDR, 1993).

Recently additional trials of this vaccine have been carried out in Tanzania (Alonso *et al.*, 1994a; TDR, 1993). The results of this trial showed the efficacy of the vaccine to be about 31% (Alonso *et al.*, 1994b). Other field trials of "SPf66" are in progress in the Gambia, Thailand and Colombia (TDR, 1994).

In addition to SPf66, at least five parasite antigens with promising vaccine potential are being prepared for use in human trials over the next few years. These promising antigens are the merozoite surface antigen 1 (MSA-1), the apical membrane antigen (AMA-1), the serine rich antigen (SERA), the erythrocyte binding antigen (EBA), and Pfs 25, a molecule that could induce immune response capable of blocking infection in mosquitoes and thus put a break on malaria transmission (TDR, 1994).

CHAPTER 3.

MATERIALS AND METHODS

3.1 Study area

The study was conducted with human subjects from Gomoa Onyadze, a village in the coastal region of Ghana, about 80km west of Accra, Ghana. This coastal area has a stable all year round malaria transmission. In a previous study, Afari et al. (1993) estimated malaria parasite prevalence rates as high as 40-60% in children and considerably lower in adolescents and adults.

3.2 Target population

Blood samples were collected from 57 healthy adults, aged 16 years and above, and 21 children between the ages of 5 and 16 years from the study area. Control blood samples were obtained from 18 healthy Danish adults who had never lived in any malaria endemic area. Blood sampling was done after informed consent had been obtained.

3.3 Blood sampling, isolation and storage of peripheral blood mononuclear cells

Twenty milliliters of venous blood from each donor was drawn aseptically into heparinized vacutainer tubes (Becton-Dickinson Ltd, Rutherford, NJ, USA) containing 400IU of heparin, and transported immediately to the laboratory. Blood processing was also done under sterile conditions. Sixteen milliliters of Ficoll-Paque (Lymphoprep, Nyegaard, Oslo, Norway), a density centrifugation medium, was transferred into 50ml Leucosep tubes (Greiner and Sohne, Germany), and centrifuged briefly to allow the Lymphoprep to go through its separating disc. The heparinized blood was then poured into the Leucosep tube and centrifuged at

800×*g* for 15 min, resulting in separation of a top plasma layer; a second layer of peripheral blood mononuclear cells (PBMC); a third layer of Lymphoprep above and below the separating disc; and a bottom layer of red blood cells and granulocytes.

The plasma was removed and stored at -20°C, whilst the mononuclear cells were transferred into a 50ml centrifuge tube and washed twice in washing medium consisting of RPMI 1640 supplemented with 5% heat inactivated foetal calf serum (FCS), (Gibco, Grand Island, N.Y, USA) by centrifugation at 250×*g* for 10 min. The supernatant was discarded and the cell pellet resuspended in 1.5ml of enriched washing medium containing 10% FCS. The number of mononuclear cells was determined by counting in an improved Neubauer Counting Chamber using methyl violet stain, which is used in assesing nulear morhology when counting fresh cells (Hviid *et al.*, 1993a). The cell density was adjusted to between 8 and 12 million cells per ml with enriched washing medium. Seven hundred and fifty microliter of the cell suspension was added to cryo-preservation tubes and an equal volume of freezing medium (composed of 55% RPMI 1640, 25% FCS and 20% dimethyl sulfoxide (DMSO)), added to each tube just before freezing with a computer controlled cryo-freezing device (Hviid *et al.*, 1993a). It took 60 min for the samples to cool to -140°C, after which they were transferred directly into liquid nitrogen at -196°C until use.

3.4 Microscopy

Thick and thin blood films were prepared on glass slides from the blood samples collected. These were stained with Giemsa stain and screened for malaria

parasites, using an Olympus microscope under oil immersion at ×1000 magnification. The different *Plasmodium* species were identified by their morphological characteristics as observed under the microscope.

3.5 Reagents

3.5.1 MSA-2 peptides

Peptides of the *P. falciparum* merozoite surface antigen (MSA-2) were supplied by Dr. C. Rzepczyk of the Queensland Institute of Medical Research, Brisbane, Australia. The peptides were synthesized by the simultaneous multiple peptide synthesis technique, using derivatized amino acids (Omni Biochemicals, National City, CA, USA) on benzhydrylamine resin (Multiple Peptide Systems, Solanos Beach, U.S.A) as described by Houghten (1985). Synthesized peptides were purified by reverse phase high performance liquid chromatography, using acetonitrile gradient with 0.1% trifluoroacetic acid as counter-ion (Jones, 1991). The MSA-2 peptides used in the study were the G1, G2C, and G4 as shown in Table 1a. Each peptide was used for stimulation of T-cell proliferation at final concentrations of 0.3μg/ml, 3μg/ml, 6μg/ml and 12μg/ml.

3.5.2 Control antigens

Purified protein derivative of tuberculin (PPD) and tetanus toxoid (TT) were used in this study as positive control recall antigens. These antigens were obtained from the Statens Serum Institute, Copenhagen, Denmark and used at final concentrations of 12μg/ml purified PPD and 4μg/ml purified TT, respectively.

3.5.3 Cell activators

Antigen specific production of IL-4 was measured by a method employing an amplification step using phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Corporation, MO, USA) and Ca-ionophore (ionomycin, Calbiochem, CA, USA) at final concentrations of 50ng/ml and 1mM, respectively (Kurtzhals *et al.*, 1992).

TABLE 1a

THE MSA2 PROTEIN OBTAINED FROM FC27 STRAIN OF *PLASMODIUM FALCIPARUM*

G1	REPEAT	REPEAT	G2 G3	G4
Conserved N-terminus	Variant region			Conserved C-terminus

TABLE 1b

THE MSA-2 PEPTIDES USED FOR THE STUDY

Peptide	Sequence	Region in MSA2
G1	NESKYSNTFINNAYNMSIR	Conserved N-terminus
G2C	TAADTPATESISPSPPC*	Variant region
G4	RNNHPQNTSDSQKECTDGNK	Conserved C-terminus

*This cystein is not part of the MSA2 sequence

3.6 Proliferation assay and generation of IFN-γ containing supernatants

Frozen PBMC were retrieved from liquid nitrogen and quickly thawed in a waterbath at 37°C. The cells were immediately washed twice by centrifugation (250*xg* for 10 min) in a washing medium made of RPMI 1640 supplemented with 5% heat inactivated pooled normal human serum (NHS) from non-immune donors, 58.4μg/ml of L-glutamine, 20IU/ml of penicillin and 20μg/ml of streptomycin. Cell viability after storage was found to be greater than 90% as determined by trypan blue exclusion.

The PBMC were cultured in the presence of MSA-2 peptides using culture medium consisting of RPMI 1640 supplemented with 15% NHS, 58.4μg/ml L-glutamine, 20IU/ml penicillin and 20μg/ml streptomycin. The cultures were initiated with 100,000 PBMC in 150μl of culture medium in each well of 96-well roundbottomed microtiter plates (Nunc, Roskilde, Denmark). Twenty microliter of each antigen diluted in culture medium was added to triplicate wells for each PBMC sample to give the appropriate concentrations. Control unstimulated cultures received 20μl of culture medium without antigen. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 7 days. Twenty-four hours prior to termination of cultures, the cells were pulsed with 185 mBq/ml of ^{3}H-thymidine (New England Nuclear, Boston, MA, USA), added in 20μl of culture medium per well. Culture supernatants from the triplicate wells were pooled and stored at -20°C for later determination of IFN-γ. The cells were harvested onto glassfibre filters and the incorporation of ^{3}H-thymidine into DNA determined by liquid scintillation spectrometry as measured in counts per minute (CPM). For each concentration of the peptides the median CPM was calculated. The CPM and cytokine production recorded were from the triplicates which gave the highest proliferative response. Control unstimulated cells were set up in four replicates consisting of triplicate wells. The highest background incorporation of ^{3}H-

thymidine and the highest IFN-γ concentration produced by a triplicate set of unstimulated wells were recorded and used in estimation of stimulation index.

In order to define a positive proliferative response to an antigen, two equations involving a stimulation index (SI) and an increment value (δ), were used:

$$S.I. = \frac{CPM_{(antigenstim.)}}{CPM_{(un\text{-}stim.)}}$$

$$\delta = CPM_{(antigenstim.)} - CPM_{(un\text{-}stim.)}$$

A proliferative response was considered positive when SI was greater than 2 and δ was greater than 1000CPM.

3.7 Generation of interleukin-4 containing supernatants

To measure IL-4 production in antigen-stimulated cultures, an experimental set similar to the one described for IFN-γ production was used, except that the cells in each well were pulsed with 20μl mixture of 10μM ionomycin and 500ng/ml PMA in the last 24 hours before culture supernatants were harvested (Kurtzhals *et al.*, 1992).

3.8 Determination of the effects of freezing and storage method on functional characteristics of PBMC

In order to correctly identify the effect of the MSA-2 peptides on the PBMC of Ghanaian and Danish donors, experiments were first conducted to determine the effect of freezing on immunological functional characteristics of PBMC.

In these experiments, PBMC obtained from healthy Danes were each divided into

two, and one half frozen and stored for at least 2 hours using the procedure described earlier (see, section 3.3). The frozen cells were retrieved and together with the unfrozen batch prepared for proliferative and cytokine analysis. The stimulating antigen used was TT and the cytokine measured was IFN-γ. T-cell proliferation and IFN-γ production of the previously frozen and unfrozen PBMC were then compared.

3.9 Preparation of reagents for cytokine ELISA

3.9.1 Preparation of Protein A sepharose column

Polyclonal rabbit IgG antibodies against human IFN-γ and IL-4 were purified by Protein A affinity chromatography. Protein A sepharose gel (Pharmacia, Uppsala, Sweden) was treated as recommended. Briefly, the gel was added to PBS, pH 7.4, mixed and allowed to settle. Half of the supernatant was decanted away and the gel resuspended before adding it carefully to a column (PD-10; bed volume:9.1 ml, bed height:5 cm) (Pharmacia, Uppsala, Sweden). The packed gel was then rinsed with PBS after which 0.1M glycine, pH 2.4 was added. The column was then again rinsed with PBS before application of 2% (w/v) bovine serum albumin in PBS. The gel was kept soaked in this solution while the column was left to stabilize for an hour at room temperature.

3.9.2 Purification of rabbit IgG

Polyclonal anti-cytokine antibodies had been induced in rabbits by immunization with either recombinant IFN-γ or IL-4. The anti-cytokine containing rabbit sera were a generous gift from Dr. K. Bendtzen, University of Copenhagen. After washing of the columns sera were added and recirculated for 2 hours in PBS to allow binding of IgG to the protein A. The gel was then washed carefully with PBS until all the colour of the rabbit

serum had completely disappeared, indicating that unbound protein had been removed.

Five hundred microliter of 0.1M glycine, pH 2.4 was then added to the column and collected in a fraction tube containing TRIS buffer. Similarly, eleven other fractions were collected, each with 500μl of glycine.

The protein concentrations in the fractions were measured using a Biorad protein detection reagent (Biorad, Munich, Germany) to identify fractions containing the eluted IgG. The protein containing fractions were pooled and divided into two equal portions. One half was dialyzed against PBS, pH 7.4 and the other against 0.1M $NaHCO_3$, pH,8.4 at 4°C overnight. The protein fraction dialyzed against PBS, was preserved in 0.1% (w/v) sodium azide and stored at 4°C and used for coating of micro ELISA plates.

3.9.3 Biotinylation of polyclonal rabbit anti-IFN-γ or IL-4 IgG antibody

Rabbit anti-IFN-γ or anti-IL-4 IgG dialyzed against $NaHCO_3$ were conjugated to biotin using a stock biotin solution consisting of 50mg/ml of hydroxysuccinimidobiotin in DMSO. The concentration (C) of biotin required for conjugating the antibody was determined from the equation: $C_{biotin} = C_{antibody} \times 1.821$ and the appropriate concentrations were obtained by dilution of the stock solution with 0.01M PBS, pH 7.2. The volume (V) of this biotin solution required to conjugate the antibody was determined from the equation: $V_{biotin} = (V_{antibody}/20)$. This volume of biotin solution was added slowly in drops to the antibody solution whilst shaking, after which the mixture was incubated at room temperature for 4 hours in the dark, with continuous shaking. The biotinylated antibody was then dialyzed against excess PBS for 5 days with daily changes of the buffer. The prepared conjugate was added with an equal volume of

glycerol and stored at -20°C.

3.10 Cytokine ELISA

IFN-γ and IL-4 were measured in the generated culture supernatants by double sandwich-ELISA using rabbit anti-human IFN-γ or IL-4 described by Kemp *et al.* (1992) with biotin-avidin amplification. Due to shortage of reagents, the cytokine assays were carried out on supernatants generated from MSA-2 stimulated PBMC of 28 of the Ghanaian adult donors and 7 Danish adults. Supernatants generated from PBMC stimulated with control peptides (PPD and TT) of 18 adult Danes were analyzed for the two cytokines.

3.10.1 Procedure

Maxisorb micro ELISA plates (Nunc, Rockilde, Denmark) were coated with anti-cytokine antibody. The antibodies were diluted in PBS, pH 7.4 to give 3.7 μg/ml of anti-IFN-γ or 4.0 μg/ml anti-IL-4, and 100μl volumes dispensed into each well. To coat, the plates were sealed and incubated at 4°C for 3 days, after which they were washed 3 times with PBS-tween (washing buffer) and stored for at least two weeks at 4°C. The concentrations of coating and biotinylated antibodies used in the ELISA were arrived at by checker board titration of coating and biotinylated antibodies against standard IFN-γ and IL-4. Selected concentrations were those that gave the best distinction between low cytokine concentrations and blank wells, and which required the lowest amount of antibody. When ready for analysis, plates were washed thrice with washing buffer and then incubated with 150μl/well blocking buffer consisting of 2% (w/v) human serum albumin in PBS, pH 7.4 at 37°C for 2 hours. Again, the plates were washed 3 times with washing buffer. Samples were diluted 1:1 with incubation buffer (consisting of 650μl of rabbit serum, 0.2mM

polyethylene glycol, 6.8mM NaCl, 1 drop of Tween 20 in 15.5 ml PBS; pH 7.4) and incubated 100μl/well in duplicates at 4°Covernight. For estimation of cytokine concentrations in the samples, serial dilutions of known concentrations of purified standardized native cytokines generated from cultures of human PBMC were made in incubation buffer, diluted 1:1 in culture medium and incubated as described for the test samples (thus ensuring equivalent amounts of culture medium and buffer in each well). The plates were then washed 4 times to remove excess unbound protein and incubated with 100μl/well of biotinylated anti-cytokine antibody diluted 1:1000 for IFN-γ or 1:1400 for IL-4 in incubation buffer. The plates were once again washed 4 times with washing buffer to remove excess unbound antibody conjugate and further incubated with 100μl/well of peroxidase labelled streptavidin (SPOD) diluted 1:2000 with incubation buffer at room temperature on a shaker for 30 mins. This was followed by another 4 times wash after which substrate solution consisting of 66.7% 1,2-phenylenediamine dihydrochloride, 0.1M citric acid-phosphate buffer, pH 5.0 and 0.0125% H_2O_2, was added 100μl/well. The plates were incubated in the dark for 10-15 min. during which the reaction (colour development) was monitored and stopped with 100μl/well of 2.5M H_2SO_4 when there was visible difference between the most diluted standard and blank wells. The optical densities were recorded at 492nm using an automated ELISA plate reader. Standard curves of absorbance against standard cytokine concentrations on a semi-log graph were used to estimate the concentration of cytokines in test samples.

In order to define a positive cytokine response a cytokine production index (PI) was calculated as:

$$P.I. = \frac{Cytokine_{(antigenstim.)} - Cytokine_{(un\text{-}stim.)}}{Cytokine_{(un\text{-}stim.)}}$$

Cytokine production was considered positive when the production index was greater than 2.

The assays were calibrated to detect IL-4 within the range of 63-10,000pg/ml, and IFN-γ within the range of 1-64 IU/ml (specific activity of the reference protein being $2x10^8$IU/mg). The ELISA did not cross-react with IL-1, IL-2, TNF, GM-CSF, or IL-6. The IFN-γ ELISA did not cross-react with IL-4, and the IL-4 ELISA did not cross react with IFN-γ (Kurtzhals *et al.*, 1992).

3.11 Statistics

The Chi-square test for un-paired data was used for comparison of proportions. Ratios between proportions were approximated by odds ratios due to the cross-sectional study design. Binomial confidence intervals were calculated when applicable. Paired data were compared using rank correlation analysis. Calculations were done using Sigmastat 1.02 (Jandel Scientific Corporation, 1986-1992) and EpiInfo 5.1 (U.S.D, Inc. Stone Mountain, G.A., U.S.A.) computer programmes. Two-tailed P-values <0.05 were considered significant.

CHAPTER 4.

RESULTS

4.1 Prevalence of malaria parasites among the subjects

None of the individuals examined had any signs of clinical malaria at the time of blood sampling. Nevertheless, as shown in Table 2, *P. falciparum* was frequently found in the blood samples of the Ghanaians. The prevalence of asymptomatic parasitaemia was significantly higher ($P<0.001$) in the children than in the adults. No other *Plasmodium* species were encountered.

4.2 Lymphoproliferative responses

The proliferative responses to the MSA-2 peptides (G1, G2C and G4) and positive control antigens (PPD and TT) are summarized in Figures 1-5 and Table 3a. As indicated, PBMC from Ghanaian adults gave higher responses to all the MSA-2 peptides than those from Ghanaian children and Danish adults (Figures 1-3). On the other hand the lymphoproliferative responses to the control antigens were high in PBMC of all the three groups of donors studied (Figures 4 and 5). Using the criteria for a positive proliferative response as defined in Materials and Methods the frequency of responding cultures was found to be significantly higher ($P < 0.03$) in Ghanaian adults compared to Ghanaian children or Danish adults for all the MSA-2 peptides tested (Tables 3a and 3b). Approximately 30% of PBMC cultures from Ghanaian adults responded to each of the peptides while only 1 of 21 samples from Ghanaian children and 1 of 18 from Danish adults responded to G1 and G2C, respectively (Table 3a). The proportion of cultures responding to PPD and TT did not differ significantly ($P = 0.2$) between Ghanaian adults, Ghanaian children, and Danish adults (Table 3a).

TABLE 2

PREVALENCE OF *P. FALCIPARUM* PARASITAEMIA AMONG GHANAIAN ADULTS AND CHILDREN

	Ghanaian adults	Ghanaian children	Prevalence ratio (95% CI)[+]	P value[*]
Prevalence of parasitaemia	8/69 (11.6%)	21/43 (48.8%)	0.14 (0.05-0.59)	0.00001

[*] Prevalences of *P. falciparum* parasitaemia were compared by Chi-square for unpaired data.

[+] Prevalence ratio (95% confidence interval) was calculated as odds ratio for prevalence in adults vs. children.

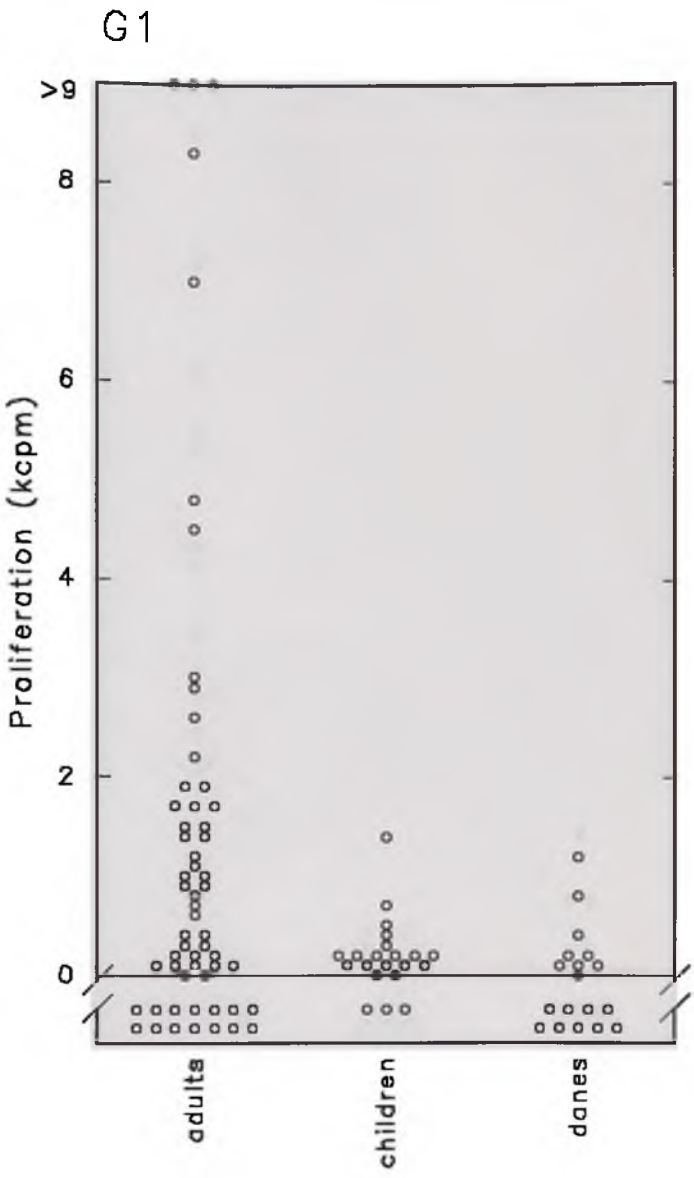

Figure 1. Proliferative response (increment value) to MSA-2 peptide G1 in peripheral blood mononuclear cells (PBMC) from Ghanaian adults and children and non-exposed Danish adults. Each data point represents the median of triplicate cultures from one individual.

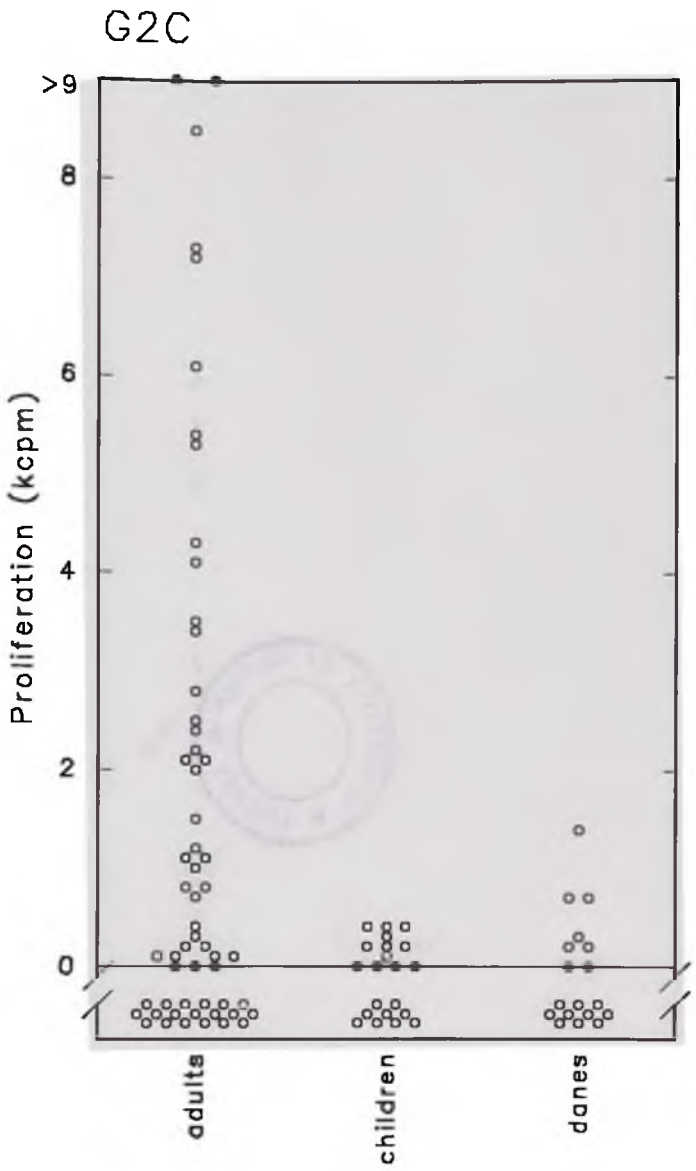

Figure 2. Proliferative response (increment value) to MSA-2 peptide G2C in PBMC from Ghanaian adults and children and non-exposed Danish adults.

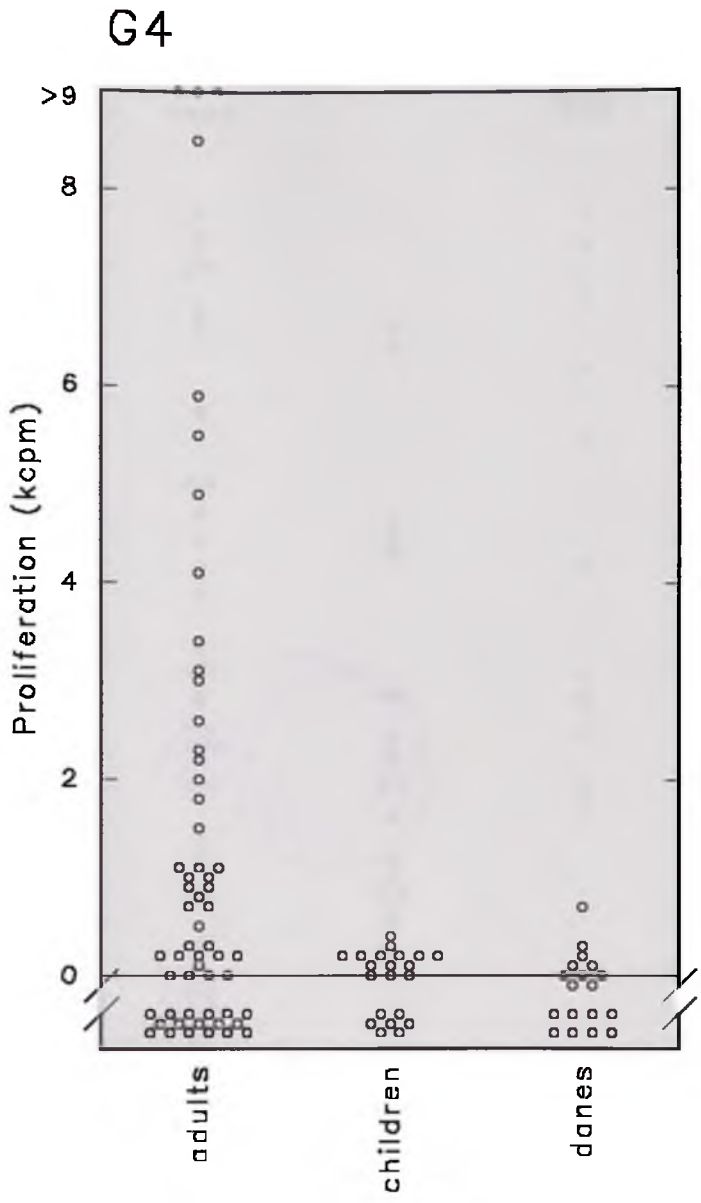

Figure 3. Proliferative response (increment value) to MSA-2 peptide G4 in PBMC from Ghanaian adults and children and non-exposed Danish adults.

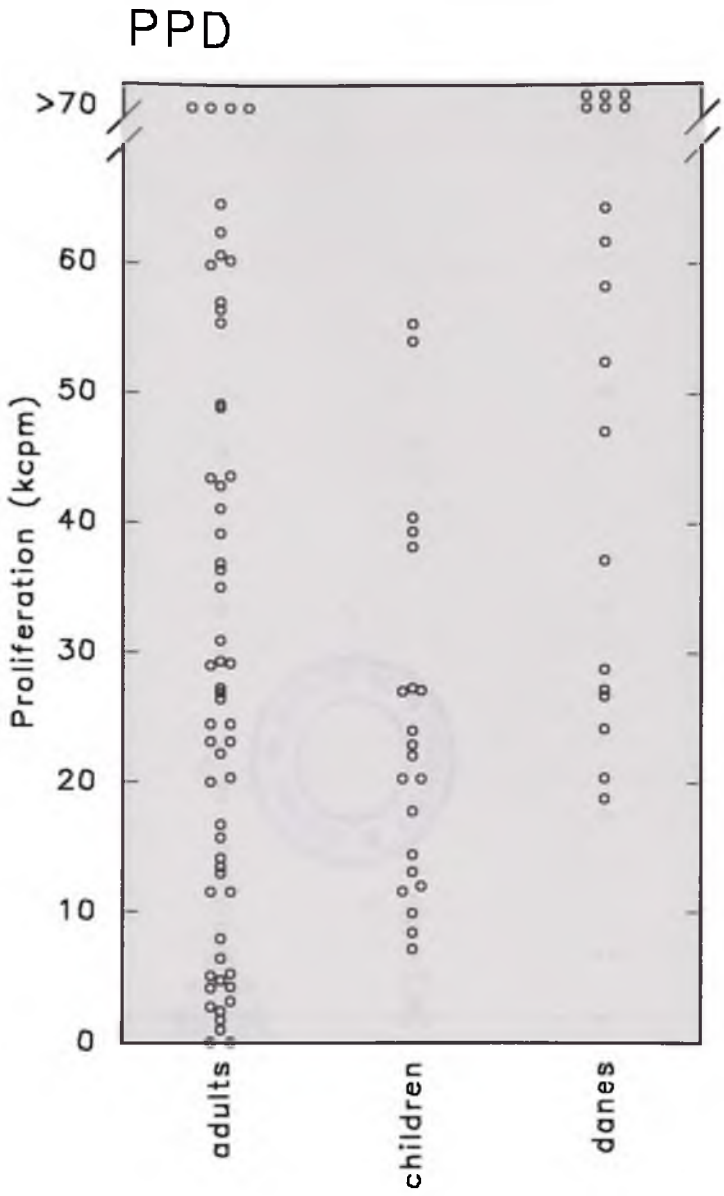

Figure 4. Proliferative response (increment value) to purified protein derivative of tuberculing (PPD) in PBMC from Ghanaian adults and children and Danish adults.

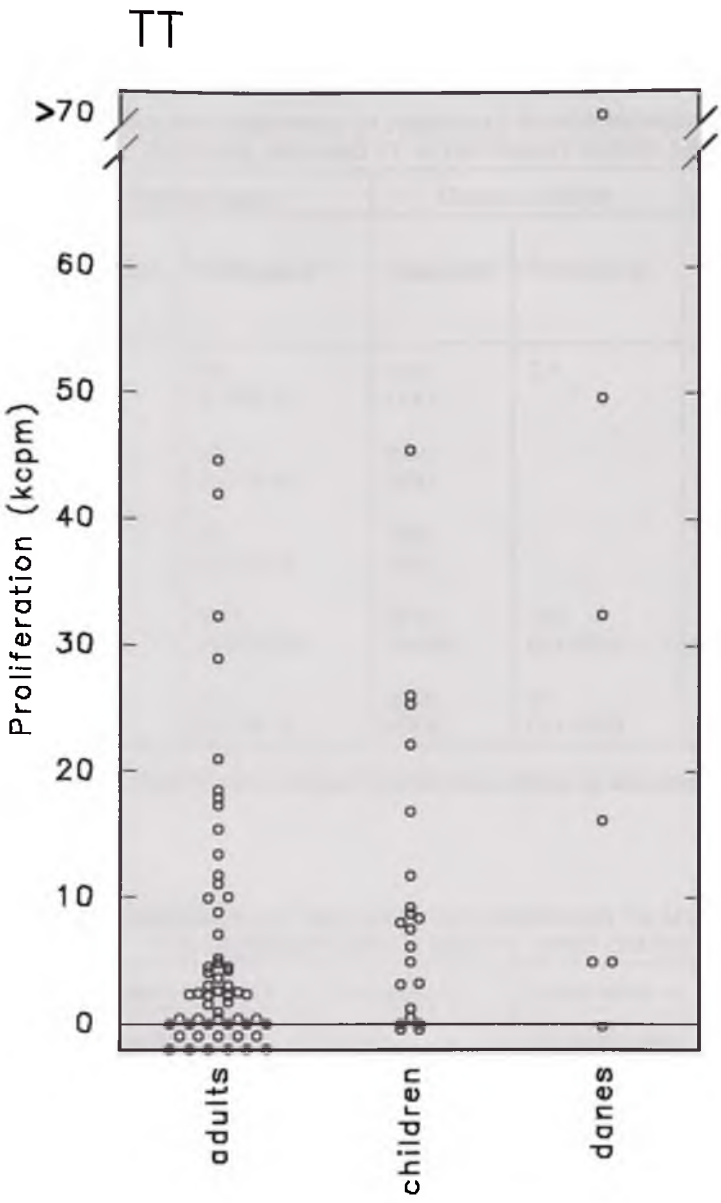

Figure 5. Proliferative response (increment value) to tetanus toxoid (TT) by PBMC from Ghanaian adults and children and Danish adults.

TABLE 3a

LYMPHOPROLIFERATIVE RESPONSES IN PERIFERAL BLOOD MONONUCLEAR CELLS TO MSA-2 PEPTIDES, PPD AND TT IN DIFFERENT DONOR GROUPS

Antigen	Ghanaian adults		Ghanaian children		Danish adults	
	Responders	Proliferation*	Responders	Proliferation	Responders	Proliferation
G1	20/57 (35%)	3.5 (1.9-27.8)	1/21 (5%)	2.5	0/18 0%	
G2C	20/57 (35%)	4.3 (1.7-16.9)	0/21 (0%)		1/18 (6%)	1.4
G4	17/57 (30%)	5.1 (1.7-16.3)	0/21 (0%)		0/18 0%	
PPD	53/57 (93%)	26.4 (4.7-141.2)	21/21 (100%)	22.1 (7.1-55.4)	18/18 (100%)	55.4 (18.8-125.6)
TT	34/57 (60%)	2.3 (3.6-44.7)	16/21 (76%)	7.5 (3.1-45.5)	6/7 (86%)	16.1 (4.9-126.1)

*Median and (range) of proliferative response in responding cultures as measured in kilo counts per minute.

TABLE 3b

STATISTICAL COMPARISON OF PROLIFERATIVE RESPONSES TO MSA-2 PEPTIDES BETWEEN THE DIFFERENT DONOR GROUPS USING CHI-SQUAURE

Peptides	Ghanaian children vs. Ghanaian adults		Danish adults vs. Ghanaian adults	
	Prevalence ratio+ (95% CI)	P value	Prevalence ratio (95% CI)	P value
G1	0.1 (0.0-0.7)	0.02	0.0 (0.0-0.6)	0.001
G2C	0.0 (0.0-0.5)	0.004	0.1 (0.0-0.8)	0.03
G4	0.0 (0.0-0.6)	0.004	0.0 (0.0-0.74)	0.008

+Prevalence ratio was calculated as the ratio between the proportion of responding cultures in the two groups (odds ratio) with 95% confidence interval.

There was no significant differences ($P > 0.05$) between the three groups for responses to either PPD or TT.

4.3 Cytokine production

Nine (32%), 8 (29%), and 10 (36%) of the cultures from 28 Ghanaian adults produced IFN-γ in response to G1, G2C and G4, respectively, whereas none of the cultures from 7 Danish adults did (Figure 6). This difference was not statistically significant ($P > 0.05$). However, PBMC from a very significantly higher ($P < 0.001$) proportion of Ghanaians (61%) than Danes (0%) produced IFN-γ in response to at least one of the peptides .

After re-stimulation with ionomycin and PMA IL-4 was detectable in MSA-2 peptide stimulated cultures of both Ghanaians and Danes (Figure 7). However, using the criteria for a positive IL-4 response, 3 (10.7%), 1 (3.6%) and 1 (3.6%) of the samples from 28 Ghanaian adults but none of those from the 7 Danes were positive for IL-4 in response to G1, G2C and G4, respectively. None of the PBMC samples from Ghanaian adults responded to more than one peptide antigen.

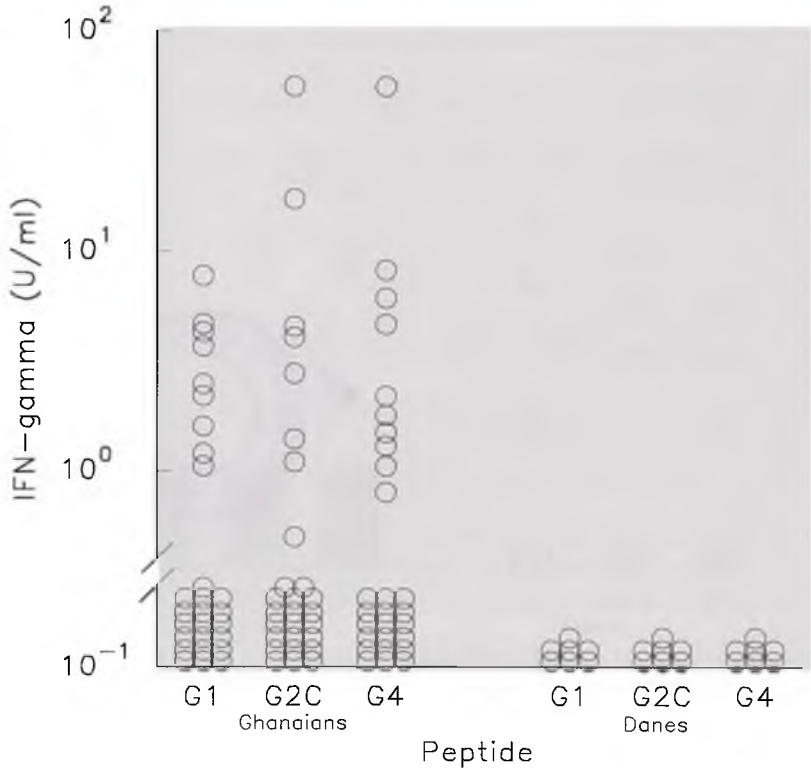

Figure 6. Interferon-γ production (increment value) induced by MSA-2 peptides G1, G2C and G4 in PBMC from Ghanaian adults and non-exposed Danish adults. Supernatants were pooled from triplicate cultures and the mean of two measurements recorded.

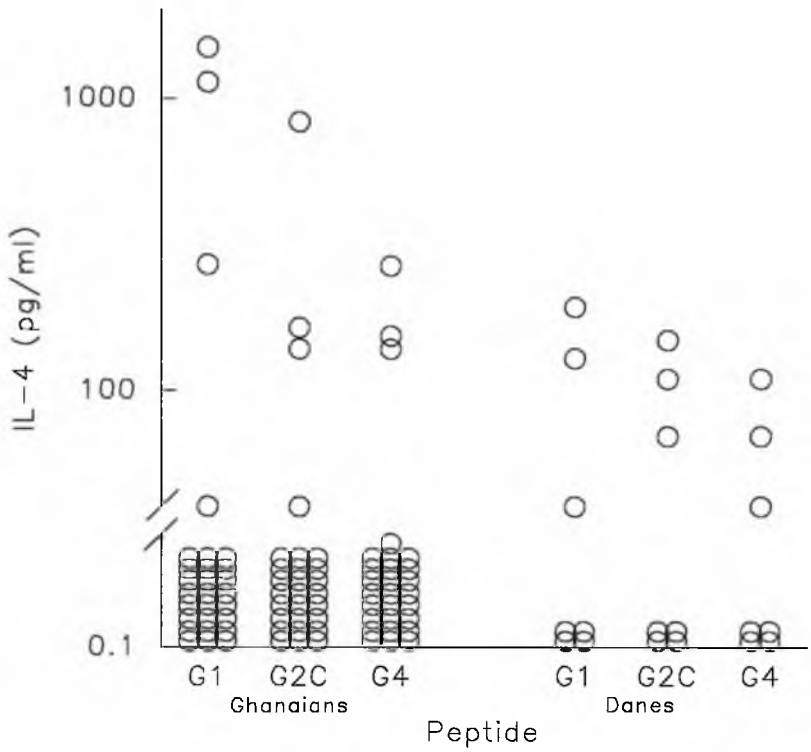

Figure 7. Interleukin-4 production (increment value) induced by MSA-2 peptides G1, G2C, and G4 in PBMC from Ghanaian adults and non-exposed Danish adults. IL-4 was measured in antigen stimulated cultures boosted with ionomycin and PMA for the last 24 h of culture (see text).

Using the control antigens PPD and TT it is shown in Tables 4 and 5 that PBMC from both Ghanaian and Danish adults could be stimulated to produce IFN-γ and/or IL-4. As shown, there were no statistical differences ($P > 0.15$) between the proportions of PBMC cultures producing IFN-γ or IL-4 in response to either of the antigens.

4.3.1 Pattern of IFN-γ and IL-4 production

Figure 8 shows corresponding measurements of IFN-γ and IL-4 in PBMC from Ghanaian adults stimulated with G1, G2C, or G4. Only one of the responding cultures produced both IFN-γ and IL-4.

4.4 Overall response of PBMC to any of the MSA-2 peptides

Eighty two percent of PBMC cultures from Ghanaian adults and 14% of the cultures from Danes responded to MSA-2 peptides in at least one T-cell assay (Table 6). This overall response to the peptides was significantly higher ($P = 0.002$) for the Ghanaian adults. Similarly, the responses to only the G1 and G4 peptides were each significantly higher for the Ghanaians. The responses to G2C were, however, not significantly different ($P = 0.1$) between the Ghanaians and the Danes.

4.5 Determination of the effects of freezing and storage method on proliferation and IFN-γ assays

Figure 9 and 10 show corresponding measurements of proliferation and IFN-γ production in response to TT stimulation in fresh and frozen PBMC from Danes. A strong positive correlation was found between the fresh and frozen

samples in both the proliferation assay (rank correlation coefficient (R_s) = 0.937, $P < 0.005$) and cytokine production (R_s = 0.760, P = 0.005).

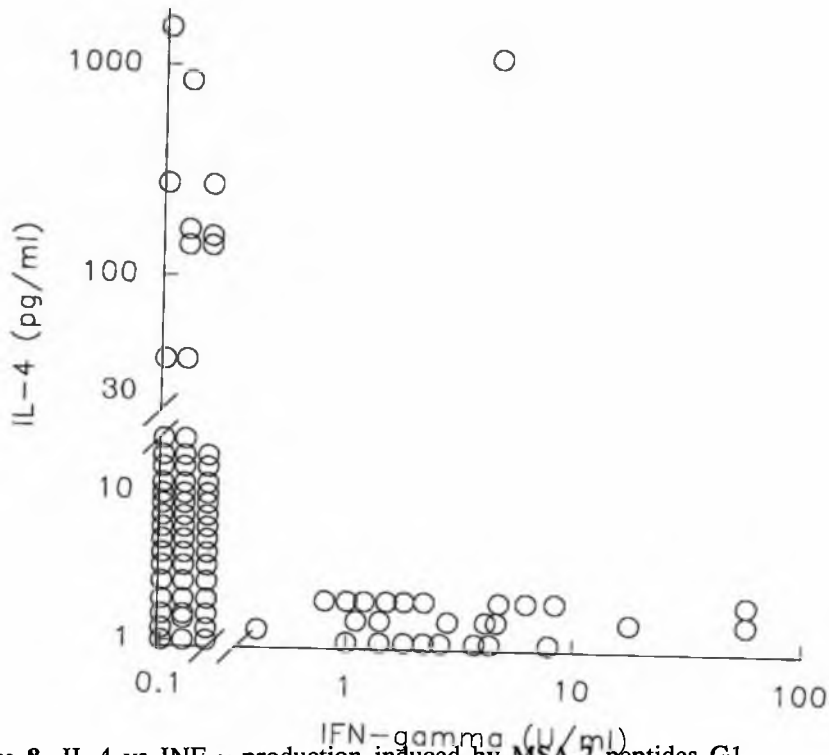

Figure 8. IL-4 vs INF-γ production induced by MSA-2 peptides G1, G2C and G4 in PBMC from Ghanaian adults. The cytokines were measured in parallel cultures with (IL-4) and without (IFN-γ) boosting with ionomycin and PMA (see text). For every donor corresponding values of IFN-γ and IL-4 production has been shown for each of the peptides.

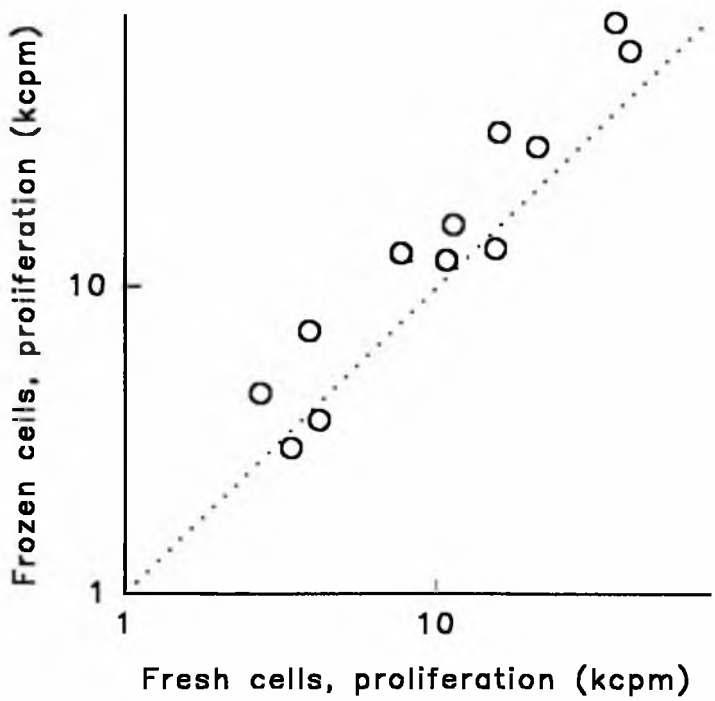

Figure 9. Proliferative response (increment value) induced by tetanus toxoid in frozen vs. fresh PBMC from Danish adults. The dotted line indicates the identity line.

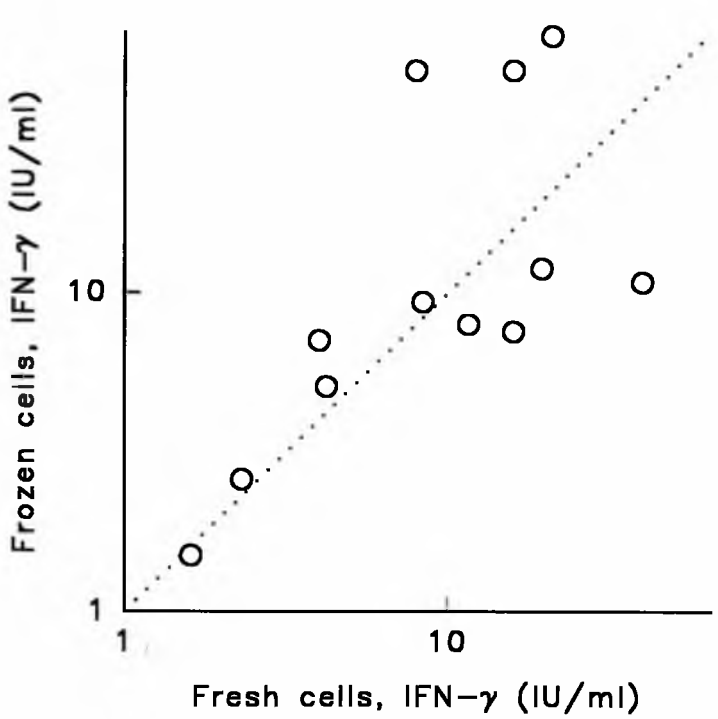

Figure 10. IFN-γ production (increment value) induced by TT in frozen vs fresh PBMC from Danish adults. The dotted line indidcates the identity line.

TABLE 4

INTERFERON-γ PRODUCTION IN PPD AND TT STIMULATED PBMC ISOLATED FROM GHANAIAN AND DANISH DONORS

Antigen	Ghanaian adults		Danes		Pvalue
	Responders	Level of IFN-γ	Responders	Level of IFN-γ	
PPD	27/28 (96%)	34.7 (3.0-66)	19/18 (94%)	26.3 (2.4-61)	1.0
TT	19/28 (68%)	9.9 (5.4-33)	16/18 (89%)	12.7 (1.5-33)	0.16

TABLE 5

INTERLEUKIN-4 RELEASE FROM PPD AND TT STIMULATED PBMC ISOLATED FROM GHANAIAN AND DANISH DONORS FOLLOWING IONOMYCIN AND PHORBOL MYRISTATE ACCETATE AMPLIFICATION

Antigen	Ghanaian adults		Danes		P value
	Responders	Level of IL-4	Responders	Level of IL-4	
PPD	6/28 (21%)	450 (150-640)	4/18 (22%)	86 (53-200)	1.0
TT	10/28 (36%)	1260 (530-2200)	8/18 (44%)	1720 (112-3410)	0.15

TABLE 6

RESPONSES OF PBMC TO ANY OF THE MSA-2 PEPTIDES USING T-CELL PROLIFERATION, IFN-γ PRODUCTION, AND IL-4 PRODUCTION)

Peptides	Ghanaian adults	Danes	Prevalence ratio (95%CI)	P-value
	Responders*	Responders		
G1	17/28 (61%)	0/7 (0%)	0.0 (0.0-0.6)	0.008
G2C	15/28 (54%)	1/7 (14%)	0.1 (0.0-1.5)	0.1
G4	15/28 (54%)	0/7 (0%)	0.0 (0.0-0.8)	0.01
All peptides	23/28 (82%)	1/7 (14%)	0.04 (0.0-0.4)	0.002

*Proportion of individuals whose PBMC reacted to at least one MSA-2 peptide in any T-cell assay.

CHAPTER 5.

DISCUSSION

5.1 Discussion

Plasmodium parasites infecting the definitive host are mainly intracellular with only brief extracellular phases. Thus, during the major part of the life cycle the parasites are sheltered against the effect of the humoral immune response (reviewed by Theander, 1992). Furthermore, the lack of MHC antigens on the erythrocyte surface limits the role of cytotoxic effector T-cells in the elimination of infected red blood cells (Theander, 1992). In contrast, the extracellular sporozoites and merozoites are exposed to both humoral and cellular immune mechanisms, thereby making them more suitable as targets for vaccine induced protection. It is partly for this reason that the merozoite surface antigens are considered in the development of subunit vaccines against malaria.

The merozoite surface antigen (MSA-2) has been shown to be important for merozoite invasion of erythrocytes (Smythe *et al.*, 1988; Clark *et al.*, 1989; Ramasamy, 1987; Fenton *et al.*, 1989). Indeed, Saul *et al.* (1992) showed that mice immunized with MSA-2 peptides, were protected against *P. chabaudi* mortality with reduced parasitaemia that correlated with serum concentrations of anti-peptide antibodies. Saul *et al.* (1992), therefore, argued that effective immune response directed against MSA-2 in man may result in antibody blockade of merozoite invasion of erythrocytes. However, Ballou *et al.* (1987) and Good *et al.* (1988c) reported that sustained and protective antibody responses against malaria requires T-cell help.

The work reported in this thesis has shown that a significant proportion of Ghanaian adults with reduced incidence of clinical malaria (Afari *et al.*, 1991) living in a malaria endemic area had developed cellular immune responses to peptides of the MSA-2 molecule. By contrast PBMC from Danish adults without prior exposure to *P. falciparum*

generally did not respond to the MSA-2 peptides although cells from these donors gave high responses to the recall antigens PPD and TT. These results suggest that the observed response in the Ghanaian adults was due to T-cell memory induced by exposure to *P. falciparum* infection. Interestingly, however, a single sample from the Danish donors responded to the G2C variable region peptide. This result was not surprising, because, reactivity to purified malaria antigens including MSA-2 in PBMC of unexposed individuals have been found in a number of studies (Rzepczyk *et al.*, 1989, 1990; Jakobsen *et al.*, 1993). Greenwood *et al.* (1975), and Gabrielsen and Jensen (1982) showed that responses to crude malaria antigen preparations may be due to mitogenic activity, but it is likely that responses to purified antigens in unexposed individuals originate from previous exposure to cross-reacting antigens, such as could originate from other infectious agents (Hviid *et al.*, 1992; Jakobsen *et al.*, 1993).

Lymphoproliferative responses to both constant and variable regions of the MSA-2 molecule were higher in adults than in children of the Ghanaian study population. This may indicate that acquisition of memory T-cells to MSA-2 peptides is gradual, depending on the duration of exposure to malaria parasites. In the case of the variable region peptide, G2C, this could be due to exposure to an increasing number of parasite strains with age. On the other hand, the slow acquisition of a cellular response to constant regions, G1 and G4 peptides, could be due to diversion of the immune system during early infections by highly immunogenic or cross reacting variable regions or by malaria induced immunosuppression (Theander, 1992). It is however interesting to note that T cell responses to other purified malaria antigens show the opposite pattern, i.e., responses are higher in children than in adults (Jakobsen *et al.*, 1993; Boudin *et al.*, 1994). Indeed, using another merozoite

antigen, the rhoptry associated protein 1 (RAP-1), higher proliferative responses were found in children than in adults of the same Ghanaian study population as used for the present study (Jakobsen *et al.*, 1993). It, therefore, appears that whereas the T-cell response to certain epitopes increase with increasing time of exposure, the response to other epitopes occur early in life and may be down-regulated later in life. In the absence of malaria symptoms, *P. falciparum* parasitaemia was significantly more frequent in the exposed Ghanaian children than in the adults. This observation was in concordance with previous findings of different patterns of protective immunity at different ages, namely, anti-disease immunity in children and anti-parasite immunity in adults (Playfair *et al.* 1990). The present data therefore suggest that the MSA-2 response could be linked to anti-parasite immunity whilst the RAP-1 response is linked to anti-disease immunity. However, this will have to be investigated in a longitudinal study recording both parasitaemia and clinical data.

The low proportion of PBMC samples from Ghanaian adults responding to each of the MSA-2 peptides may be due to MHC restriction which may be of importance in both humoral (Quakyi *et al.*, 1989) and cellular (Zevering *et al.*, 1990) responses to a number of malaria antigens. This may in part explain the data obtained with this study, in that, the inclusion of three T-cell epitopes from the MSA-2 molecule increased the proportion of PBMC from individuals responding in the T-cell assays. Thus agreeing with a report from TDR (1987), Troye-Blomberg *et al.* (1990) and Howard and Pasloske (1993) have proposed that a malaria subunit vaccine needs to be incorporated with a large number of T-cell epitopes, preferably from the different *Plasmodium* stages that occur in the human host. In support of this view the number of responding cultures increased markedly when the response to all three MSA-2 peptides were considered. Yet still, the responder frequencies

to the MSA-2 peptides by PBMC of both the exposed Ghanaian adults and children seemed disappointingly low, suggesting that the MSA-2 peptides used for the study, would be less promising as subunit vaccine candidate antigens. However, at least two mechanisms could have led to an underestimation of the true responder frequencies. First, the donors could have been immuno-suppressed as a result of ongoing malaria transmission, and second, responding T-cells could have been sequestered outside the peripheral circulation.

In support of immuno-suppression, Theander *et al.* (1993) found that $CD8^+$ T-cells obtained during the wet season but not during the dry season could inhibit malaria antigen stimulated lymhoproliferation in PBMC of individuals from a malaria endemic region of the Sudan. This immunosuppression was independent of the presence or absence of parasitaemia. Similar findings have been made in other malaria endemic areas (Petersen *et al.*, 1989; Riley *et al.*, 1989; Mshana *et al.*, 1991).

With respect to sequestration it has been observed that acute *P. falciparum* malaria is associated with transient inability of PBMC to respond to antigen stimulation *in vitro* (Theander *et al.*, 1986; Hviid *et al.*, 1991). Hviid *et al.*, (1991) and Elhassan *et al.* (1994) have suggested that this lack of responsiveness is due to disease induced reallocation of peripheral lymphocytes by adhesion to inflamed endothelium. Indeed, they found that T-cells expressing high density of the LFA-1 adhesion molecule were temporarily lost from peripheral circulation during acute malaria (Hviid *et al.*, 1991), whereas the soluble form of its ligand, ICAM-1 was increased in the plasma of the patients (Elhassan *et al.*, 1994). Also, in studies of infected mice, Playfair and De Souza (1982) and Kumararatne (1987) demonstrated the presence of malaria specific T-cells in the spleen, liver and lymph nodes even though such cells were not present in peripheral circulation. Furthermore, Hviid *et al* (1991) observed that this phenomenon of sequestration may not be limited to acute disease.

It is, therefore, likely that even in the absence of ongoing clinical disease, recent malaria attacks among the subjects studied could have led to the expression of adhesion molecules on malaria specific T-cells, leading to their sequestration.

Approximately 30% and 10% of the PBMC cultures from Ghanaian adults which proliferated in response to the MSA-2 peptides produced IFN-γ and IL-4, respectively, while none of the cultures from Danish adults produced any of the cytokines. This ability to produce IFN-γ and IL-4 was not attributable to the potential for cytokine production since the responses to the control antigens were similar in the two donor groups. The inability of the majority of cultures responding to the MSA-2 peptides by proliferation to produce measurable amounts of cytokines may, therefore, be attributed to a low frequency of T-cells with reactivity to the single epitopes tested. This could explain in part the much higher cytokine levels found in response to the control antigens containing numerous epitopes. Similarly, Butcher, (1990), Chizzolini *et al.* (1990) and Riley *et al.* (1988) found that *in vitro* lymphoproliferation and cytokine production (IL-2 and IFN-γ) correlated better upon stimulation with crude antigens as compared to purified antigens and peptides.

It has been reported that IFN-γ may play a role in protective immunity through activation of macrophages (Brown and Kreier, 1986) and possibly through regulation of B-cell function (Mossman and Coffman, 1989). Nevertheless, being a potent inducer of TNF-α, IFN-γ may also contribute to the pathology of malaria (Grau *et al.*, 1989; Kwiatkowsky *et al.*, 1990).

IL-4 has been found to be an important B-cell activator that plays a role in determining the immunoglobulin subclass produced by B-cells (Lundgren *et al.* 1989). In a previous study, Troye-Blomberg *et al.* (1990) found that pf155/RESA-specific IL-4

producing PBMC originated from individuals with serum antibodies to that antigen. Since serum antibodies have been shown to block merozoite invasion of erythrocytes in mice (Smythe *et al.*, 1988; Clark *et al.*, 1989; Ramasamy, 1987; Fenton *et al.*, 1989) it seems that induction of an IL-4 response may be important to achieve a protective immune response. Only a small proportion (10%) of MSA-2 peptide stimulated PBMC secreted IL-4. However, it is likely that with the inclusion of more MSA-2 T-cell epitopes, the proportion of IL-4 producing PBMC from the exposed Ghanaian adults could be higher. There is, therefore, a need to conduct further studies to determine the ability of MSA-2 peptides to stimulate IL-4 production. Furtheremore, the exact role of this cytokine in protection against malaria must be better characterized, because high levels of IL-4 induced anti-*P. falciparum* IgE antibodies have been associated with the risk of developing cerebral malaria (Perlmann *et al.*, 1994).

The work reported in this thesis showed that IFN-γ and IL-4 production were poorly correlated in PBMC cultures stimulated by the MSA-2 peptides. Indeed, only one PBMC culture produced both cytokines. Similar findings were made with in Pf155/RESA stimulated PBMC from malaria immune individuals (Troye-Blomberg *et al.*, 1990). Two subsets of $CD4^+$ T helper cells, T helper 1 (Th1) and T helper 2 (Th2) cells have been defined in mice based on differences in their cytokine production (Mossman and Coffman, 1989; Salgame *et al.*, 1991). For example, Th1 cells produce IFN-γ, whilst Th2 cells produce IL-4. The pattern of cytokine production observed in this study, therefore, may suggest that MSA-2 peptide specific Th1- or Th2- like T-helper cells might have been expanded in different individuals possibly leading to different outcomes of the disease. The ability of a PBMC culture to produce both IFN-γ and IL-4 could either be the result of antigen stimulated Th1- and Th2-like cells or Th0 cells capable of producing several

cytokines (Paliard *et al.*, 1988). Also, the use of unseparated mononuclear cell preparation in this work does not exclude the possibility of IFN-γ production by natural killer cells. These findings underscore the importance of further investigating the significance of Th1- and Th2-subsets in protection against malaria.

Hviid and colleagues (1993a) reported that PBMC frozen using a computer controlled gradient cryo-freezer retained both their phenotypes and immune function upon retrieval. In this work PBMC frozen by the same method Hviid *et al.*, (1993a) were found to be similar to fresh cells in their ability to proliferate and produce IFN-γ upon stimulation with TT. The method thus seems suitable for longitudinal studies in which cells obtained from the same individual at different times should be run together in proliferation and cytokine production assays.

CHAPTER 6.

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, T-cell responses to MSA-2 peptides from the conserved and variant regions of the antigen were found in individuals from a malaria endemic area of Ghana. The T-cell responses were significantly higher in the "immune" Ghanaian adults who had low parasite prevalence than in "semi-immune" Ghanaian children with higher parasite prevalence, even in the absence of clinical symptoms. Furthermore the responses to MSA-2 peptides were significantly higher in the Ghanaian adults than in non-exposed Danish adults. It thus seems that T-cell reactivity to MSA-2 peptides coincides with acquisition of anti-parasite malaria immunity.

The study supports the consideration of MSA-2 as a promising malaria vaccine candidate antigen as previously reported in other *in vitro* and *in vivo* studies in humans as well as mice.

Th1- and Th2-like cytokine responses to the MSA-2 peptides may be induced in different individuals but this finding awaits further confirmation.

6.2 Recommendations

T-cell responses to more epitopes of the MSA-2 molecule and other malaria antigens should be tested in a longitudinal study of the major age groups characteristic for the acquisition of different stages of immunity in endemic populations. In a different longitudinal study involving individuals with different presentations of malaria such as uncomplicated and complicated malaria, (eg. cerebral malaria and severe anaemia) responses involved in the pathogenesis of the disease manifestations could be studied. In such studies, clinical and parasitological parameters need to be linked to immunological ones, so as to enable the elucidation of host and parasite factors associated with protection

or immuno-pathology, leading to a better understanding of the immune mechanisms involved in the interactions between malaria parasites and the human host.

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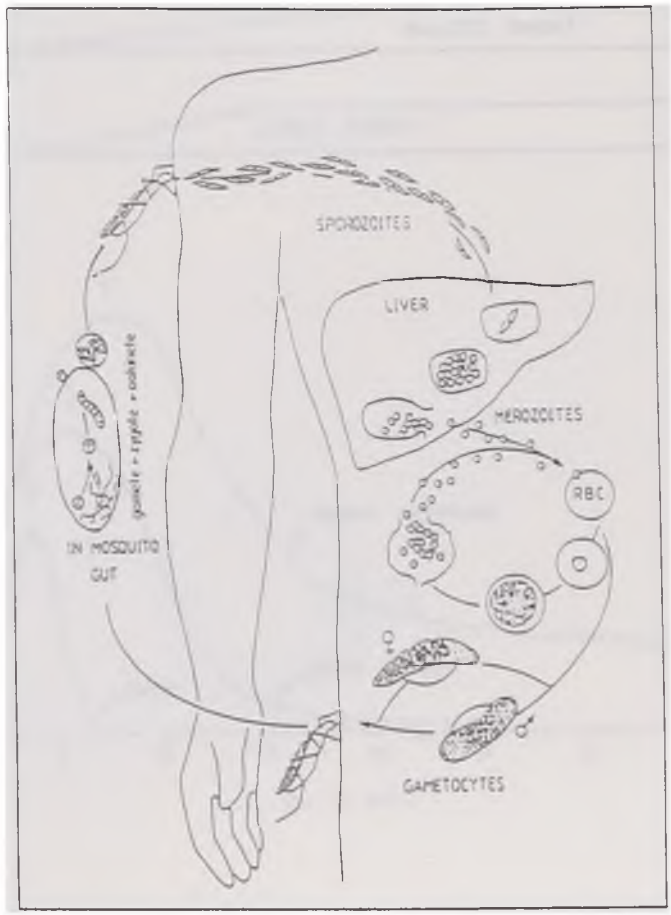
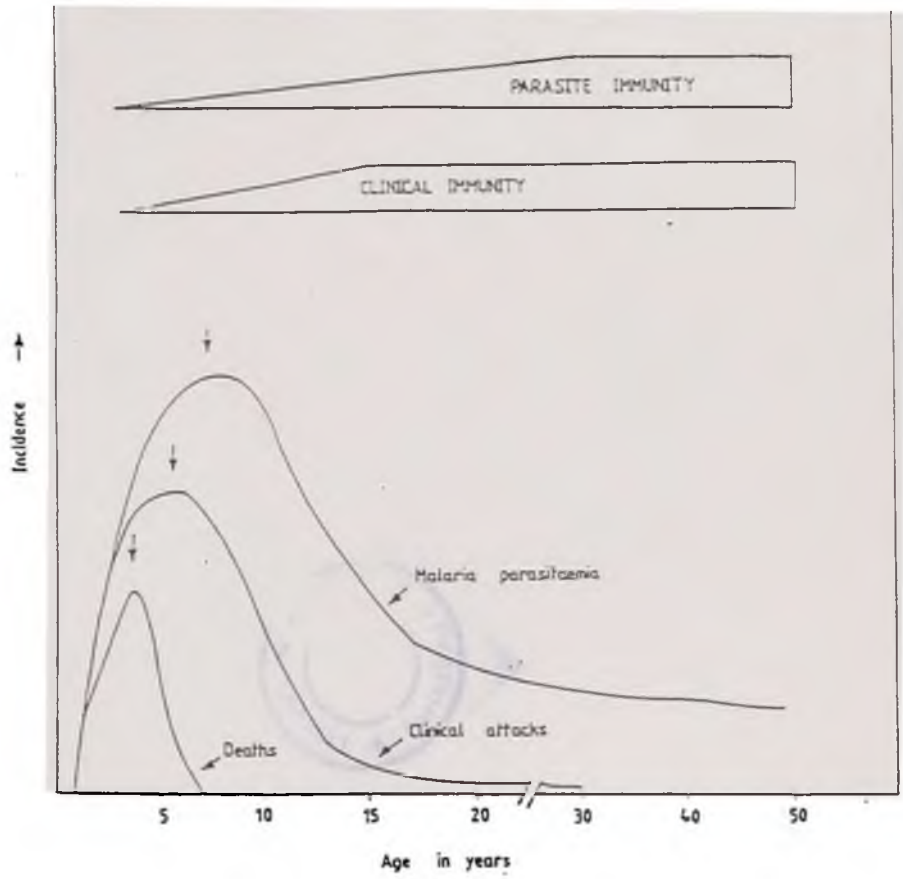
Appendix 1. The life cycle of human malaria parasites (after Good *et al.*, 1988).





Appendix 2. Schematic representation of the development of clinical and anti-parasite immunity in hyperendemic areas. The three curves represent the incidence of deaths from malaria, clinical attacks of malaria, and malaria parasitaemia, respectively. (after Greenwood, 1988).