COMPLEMENT-MEDIATED DESTRUCTION OF RED BLOOD CELLS IN CHILDREN WITH *PLASMODIUM FALCIPARUM* MALARIA

BY

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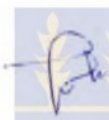
DECLARATION

THE EXPERIMENTAL WORK DESCRIBED IN THIS THESIS WAS DONE BY ME, AT THE IMMUNOLOGY UNIT, NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH, UNIVERSITY OF GHANA AND AT THE CENTRE FOR MEDICAL PARASITOLOGY (CMP) AT THE DEPARTMENT OF INFECTIOUS DISEASE, COPENHAGEN UNIVERSITY HOSPITAL, UNIVERSITY OF COPENHAGEN, DENMARK, UNDER THE SUPERVISION OF DR. B. D. AKANMORI (HEAD, IMMUNOLOGY UNIT), DR. J. A .L KURTZHALS (UNIVERSITY OF COPENHAGEN, DENMARK) AND PROF. F. N. GYANG (DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF GHANA). REFERENCES CITED IN THIS WORK HAVE BEEN FULLY ACKNOWLEDGED.



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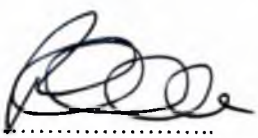

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DEDICATION

TO ALMIGHTY GOD FOR HIS GUIDANCE AND PROTECTION; MY FAMILY; MRS. EUGENIA ABLA KUMMASSAH AND SETOR EYRAM AKU LOTSU FOR THEIR ENCOURAGEMENT AND PRAYERS.

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AGS	Anti-human globulin serum
AI	Auto immune
C1INH	Complement 1 fragment inhibitor
C3d	Complement fragment 3d
CC	Healthy Control
CD	Cluster of Differentiation
CM	Cerebral malaria
CR1	Complement receptor type 1
CSA	Chondroitin sulfate A
DAF	Decay Accelerating Factor
DAT	Direct Antiglobulin Test
DCH	Department of Child health
DNA	Deoxyribonucleic Acid
EPO	Erythropoietin
FITC	Fluoroscein isothiocyanate
G-6PD	Glucose-6-phosphate dehydrogenase
GPI	Glycosyl-phosphatidylinositol
HbS	Haemoglobin type S
HRF 20	Homologous restriction factor 20
ICAM-1	Intercellular adhesion molecule-1
IFNγ	Interferon gamma
Ig	Immunoglobin
IL	Interleukin
IVH	Intravascular haemolysis

LDL	Low density lipoprotein
MACIF	Membrane-Attack-Complex-Inhibitor Factor
MASP-1, 2	Mannose activated surface protein 1,2
MBL	Membrane Binding Lectin
MCP	Membrane Cell protein
MIRL	Membrane inhibitor of reactive lysis
NMIMR	Noguchi Memorial Institute for Medical Research
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PE	Pre-erythrocytic
PfEMP1	*Plasmodium falciparum* Membrane Protein 1
PRBCs	Parasitized red blood cells
RBCs	Red blood cells
RPMI 1640	Rose Park Memorial Institute 1640
SA	Severe anaemia
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIRPα	Signal regulatory protein alpha
TDR	Tropical Disease Research
Th1	T-helper cell 1
TNF	Tumour necrosis factor
TSP	Thrombospondin
UM	Uncomplicated malaria

ABSTRACT

A total of 484 children aged between 1-10 years who reported at the emergency unit of the Department of Child Health, Korle Bu Teaching Hospital with clinical malaria were recruited for this study. The children were categorized into four main groups of clinical malaria namely, severe malaria anaemia (SA), cerebral malaria (CM), uncomplicated malaria (UM) and intravascular haemolysis, or bloody urine (IVH). The aim of the study was to determine the role of complement activation in the pathogenesis of malaria anaemia, including intravascular haemolysis.

The Coombs test or Direct antiglobulin Test (DAT) was used to detect the binding of C3d alone, IgG alone or both to erythrocytes of *P. falciparum* malaria patients as well as healthy or asymptomatically infected children (CC). Of the 484 samples tested, 131(27%) were positive for DAT. Out of the 131 DAT positive samples, 115/131 (87%) were positive for C3d alone whiles a small proportion was positive for either IgG alone or both ($p<0.05$). The results showed that 25/52 (48.1%) of the SA (mean Hb= 4.3g/dl), 23/31 (74.2%) of the IVH (mean Hb=6.1g/dl), 7/25 (28.0%) of the UM (mean Hb=9.0g/dl) and 15/35 (42.8%) of the CM patients (mean Hb=7.3g/dl) were DAT positive. The differences between SA and IVH on one hand and UM on the other for C3d binding were highly significant ($p<0.05$) suggesting a role for C3d binding and the consequential elimination or destruction of erythrocytes as a cause of the anaemia associated with falciparum malaria. Interestingly, 90% of the DAT positive samples were from children below the ages of five years with mean ages of 3.3 and 3.9 years for SA and IVH cases respectively, confirming that younger children rely on complement activation in response to malaria.

Evaluation of DAT against a flow cytometric assay showed good correlation (r^2=0.53). Using flow cytometry, binding of C3d, IgG, IgM, C3bαβ and levels of complement regulatory proteins like complement receptor type 1 or CR1 (CD35), decay accelerating factor or DAF (CD55), membrane attack complex inhibitor or MACI (CD59), and CD47, an immunological marker of self, on RBCs surfaces were quantified. By flow cytometry, higher levels of C3d was measured in the SA and IVH cases (anaemic group) compared to the UM and CM patients. The high level of CD59 in the malarial cases and controls (CC) suggests C3d binding to erythrocytes results in removal by phagocytosis rather than by direct lysis. Expression of CD47 by patients and controls alike will seem to suggest that autoimmunity is not involved in the pathogenesis of the anaemia.

In summary, these results therefore suggest that infection of younger children with *P. falciparum* leads to complement activation, resulting in elimination of erythrocytes by haemolysis as well as phagocytosis, thereby leading to life threatening severe anaemia in some children.

CHAPTER ONE

INTRODUCTION

1.0 INTRODUCTION

Together with AIDS and tuberculosis, malaria is at the top of the list of diseases, which constitute a significant public health problem, causing disease and death in most developing countries. Worldwide, malaria infects about 400 million people and kills an estimated 3 million every year with at least one million being children (Miller, 1999; WHO, 1994). However, the frequency of the disease is greatly reduced in individuals older than 5 years inspite of persistent infection (Le Hesran, 2000). This protection is known as clinical immunity, and it is not seen in low-endemic or sporadically exposed areas.

The disease has created a major public awareness in Ghana in recent times. In spite of its importance in the country, control programmes against the disease were inadequately promoted (Makoto *et al.*, 1986). Studies have shown that children in malaria-endemic areas have lower haemoglobin levels than children in areas without malaria transmission (Bruce-Tagoe *et al.*, 1997) and malaria control programmes that reduce transmission also reduce the incidence of severe anaemia. A recent study also showed that the reduced haemoglobin levels in malaria endemic areas are a direct effect of asymptomatic infections (Kurtzhals *et al.*, 1999). In collaboration with Roll Back Malaria (RBM), the Ministry of Health (MOH) has been able to minimize the impact of the malaria disease in Ghana (TDR News, 2000). Despite this, severe forms of the disease continue to claim the lives of children.

Three serious complications of malaria are thought to have an immunological basis: anaemia, glomerulonephritis and cerebral malaria. The present study focuses on malaria anaemia. Unfortunately little is known about the pathogenesis of malaria

anaemia despite its high prevalence. Possible mechanisms that have been suggested are defective production or an excessive rate of destruction of red cells or a combination of these processes (Abdalla and Weatherall, 1982; Menendez *et al.*, 2000). In most forms of anaemia it is possible to obtain reasonably accurate measurements of both these mechanisms in a relatively steady state. Unfortunately, this is rarely possible during the course of an infective illness. Furthermore, transient bone-marrow suppression seen during the most severe *P. falciparum* infections in both children and adults tend to limit parasite proliferation (Abdalla and Weatherall, 1982).

It has been suspected for some time that anaemia in malaria may not wholly result from destruction of parasitized RBCs but from unparastized RBCs as well (Rosenberg *et al.*, 1973), for in some other protozoan infections such as kala-azar, anaemia develops although no intra-erythrocytic growth of the parasite occurs (Woodruff *et al.*, 1973). While some are of the view that this depletion of RBCs is immune-mediated others dispute this fact. Others like Zuckermann (1966) suggested that at least part of the haemolytic component of the anaemia of *P. falciparum* malaria results from immune mediated destruction of RBCs. The main stimulus to this notion has come through reports of the finding of a positive Coombs' Direct Antiglobulin Test (DAT) in patients with anaemia (Facer *et al.*, 1979). Until recently, there were conflicting reports in the literature regarding the incidence of a positive DAT in patients with acute *P. falciparum* malaria. This inconsistency is perhaps due to the relative poor sensitivity and to the subjectivity of the Coombs' test. Still others like Bruce-Tagoe *et al.* (1997) implicate the destruction of non-parasitized RBCs as well as parasitized RBC (in the causation of anaemia) and attributed this to

autoantibody. Greenwood *et al.* (1978) in a review concluded that with *P. falciparum* infection, hyperactive phagocytosis was mainly to blame. Both enhanced phagocytosis and anaemia were shown in mice with *P. yoelii,* a rodent malaria parasite, to be T-cell dependent (Robert and Weidanz, 1978). It has also been reported that there have been changes in surface glycoproteins of both parasitized and non-parasitized RBC in two murine malarias, and it seems possible that macrophages might recognize and destroy such cells (Howard *et al.*, 1980).

It has been observed that binding of complement factor C3d to RBC is an important contributor to the anaemia of malaria, whereas IgG binding to RBC is rare in children with malaria (Abdalla, 1986). Complement activation may take place by way of the classical pathway, the alternative pathway or the mannose-binding pathway and may be associated with binding of immune complexes or dead merozoites to the RBC surface (Perlmann *et al.*, 1997). C3d may bind to surface complement receptor type 1 or CR1 (CD35) of the RBC, and individuals who are high expressors for CR1 may, thus be at higher risk than lower level expressors (Rowe *et al.*, 1997). In addition to CR1, other plasma proteins such as decay accelerating factor (DAF, CD55) and membrane attack complex inhibitor factor (MACIF; CD59) play a role in regulating haemolysis due to deposition of immune complexes on the surface of the RBCs (Waitumbi *et al.*, 2000).

Infected erythrocytes bind to endothelial cells, and *P. falciparum* antigens known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) inserted into the infected erythrocyte surface mediate this interaction. It has been argued that these antigens are recognized by a group of IgG and by complement fragment such as C3d, which are

degraded to C3b. As a result of this, monocytes, which have C3b receptors on their surfaces, are activated to phagocytose the infected RBCs. In a sense, complement does not seem to be able to kill parasites directly, but could play a role as an opsonin for neutrophils and macrophages (Salmon *et al.,* 1986).

It seems that the involvement of immune-mediated haemolysis in malaria anaemia is controversial and still an open question. Thus, this study was aimed at determining the role of immune-mediated mechanisms in the pathogenesis of malaria anaemia by specifically testing the hypothesis that;

Severe malarial anaemia is caused by complement-mediated elimination of parasite-infected and uninfected RBCs

The following specific objectives were addressed:

- To characterize binding of complement C3d and IgG to RBCs in different categories of malaria patients as well as in healthy children using classical Coombs' test or Direct Antiglobulin Test (DAT).
- To compare DAT or Coombs' test with flow cytometric quantification of C3d and IgG on RBCs.
- Quantify levels of complement regulatory proteins CR1 (CD35), DAF (CD55) and MACIF (CD59) expressed on RBCs by means of flow cytometry.
- To assess complement activation in the pathogenesis of severe malaria anaemia

CHAPTER TWO

LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 The history of malaria

Malaria is a very old disease and prehistoric man is thought to have suffered from it. It probably originated in Africa and accompanied human migration to the Mediterranean shores, India and South East Asia. In the past it used to be common in the marshy areas around Rome and the name is derived from the Italian, (mal-aria) or "bad air"; it was also known as Roman fever. It is caused by parasites of the genus *Plasmodium.*

2.2 The malaria parasite

The *Plasmodium* parasite undergoes a complex life cycle, alternating between vertebrate and arthropod hosts. More than 100 species infecting a wide range of hosts including reptiles, birds, and primates have been identified. Each parasite species exhibits narrow host specificity. Four species, *P. falciparum, P. vivax, P. ovale* and *P. malariae*, cause disease in man – the first being by far the most lethal (TDR News, 1987).

2.3 Biology of the Parasite

The *Plasmodium* species, with the exception of *P.malariae* are exclusively parasites of man. The mosquito is always the vector, and is always an anopheline mosquito, although, out of the 380 species of anopheline mosquitoes, only 60 can transmit malaria. Only female mosquitoes are involved, as the males do not feed on blood. The basic life cycle of the parasite is as shown in **Fig. 2.0**. The infective stages, ***sporozoites*** from the mosquito salivary gland are injected into the human host as the mosquito injects anticoagulant saliva to ensure an even flowing blood meal. Once in

the human bloodstream, the sporozoites invade the liver within 30 minutes and penetrate hepatocytes, where they remain for 9-16 days, multiplying within these cells. In liver cells, *P falciparum* parasites grow, multiply and develop directly into schizonts referred to as pre-erythrocytic (PE) schizonts. PE schizonts of *P. falciparum* take 5-7days to develop. When matured, the schizont and liver cell rupture and the parasites known as ***merozoites*** enter the blood stream. On release, the survival of the parasites is affected by their ability to return to the blood and penetrate RBCs within a few minutes of being released from the schizont and most of them do so through the sinusoid of the liver.

Entry of the parasites into the RBCs starts a cycle in the blood (erythrocytic cycle), which for *P. falciparum* takes 36-48 hours to complete. The merozoites then develop into a trophozoite within a vacuole formed by the internal membrane of the host RBCs. The trophozoite feeds on haemoglobin by ingesting small amounts of RBC cytoplasm, which leads to formation of malaria pigment (haemozoin) as an end product of haemoglobin breakdown. Haemozoin accumulates as brown-black granule in the trophozoite. When the trophozoite is fully developed it divides to form a schizont containing about 8-32 merozoites and the malaria pigment. *P falciparum* erythrocytic schizogony takes place in the capillaries of the internal organ of the body. Mature schizonts rupture from the RBCs releasing merozoites, malaria pigment and toxin into the plasma. The entry of toxic metabolites into the blood circulation of the host causes fever and a 'malaria attack'. The incubation time for *P. falciparum* from infection to an attack is 9-14 days. Those merozoites, which are not

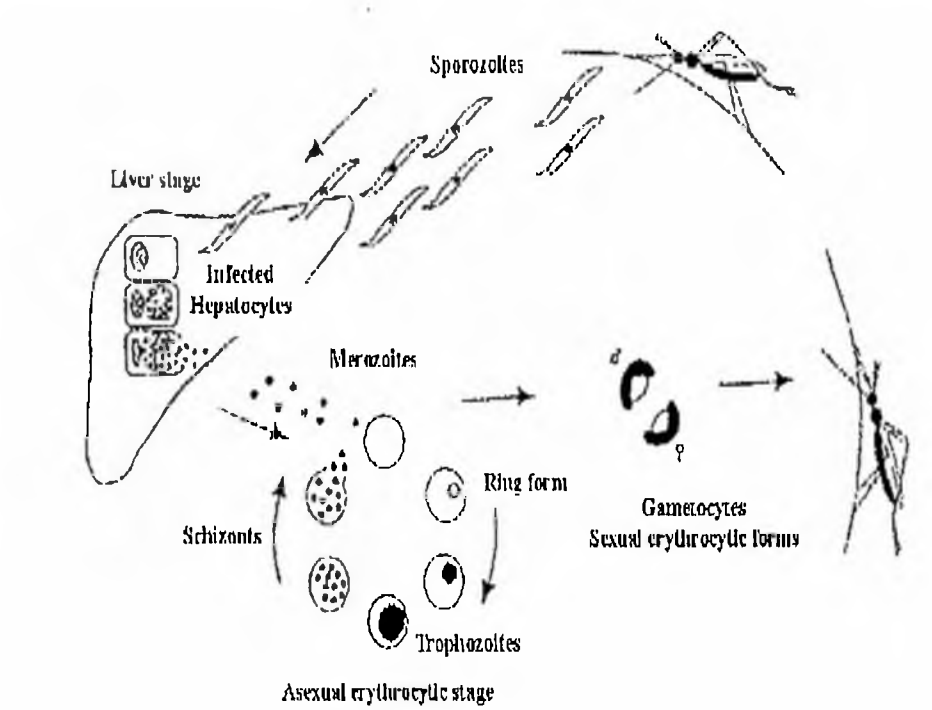


Figure 2.0: The life cycle of *Plasmodium falciparum* in the human host

destroyed by the host's immune system, invade new RBCs, develop into trophozoites and schizonts and cause further RBCs to be destroyed. After several erythrocytic cycles, some of the merozoites enter the RBCs and instead of developing into schizonts follow a sexual development in which they produce ***micro*** and ***macrogametocytes***, which have no further activity within the human host. Another mosquito arriving to feed on the blood may suck up these gametocytes into its gut, where exflagellation of microgametocytes occurs, and the macrogametocytes are fertilized. The resulting **ookinete** penetrates the wall of a cell in the midgut, where it develops into an **oocyst**. Sporogeny within the oocyst produces many sporozoites and, when the oocyst ruptures, the sporozoites migrate to the salivary gland, for injection into another host.

2.4 Clinical presentation of malaria and treatment

Fever is a natural host response to malaria. The classical feature of malaria fever is that it is not sustained, but rather takes the form of intense paroxysms which tend to recur with remarkable periodicity, typically every two or three days coinciding with rupture of RBCs and release of merozoites. The fundamental discovery in malaria pathogenesis was that these classical fever paroxysms, which are described in ancient Chinese writings and in the works of Hippocrates, are caused by erythrocytic schizonts rupturing to release their progeny (Golgi, 1889 as cited by Brewster *et al.*, 1990).

In addition to fever, malaria can exhibit certain life-threatening presentation in a variety of ways, including profound anaemia, cerebral malaria, hypoglycemia, lactic acidosis, jaundice, renal failure, pulmonary oedema and disseminated intravascular

coagulation. For reasons that remain unclear, but are undoubtedly scientifically important, the clinical spectrum of severe malaria shows marked geographic variation. All complications already mentioned might occur in non-immune adults with severe malaria in South East Asia (Warrell *et al.,* 1990). In contrast severe malaria in Africa is largely confined to children, who are liable to suffer cerebral or severe anaemia but rarely develop jaundice, renal failure or pulmonary oedema (Molyneaux *et al.,* 1989; Brewster *et al.,* 1990). Although it has been suggested that such clinical differences might be age-related this is evidently not the whole explanation: for example, jaundice is a common feature of severe malaria in Vietnamese children but not in Gambian children of the same age. Even within Africa there appears to be marked regional differences in the clinical spectrum of severe malaria, with growing evidence that anaemia is a particular problem in areas of very high malaria transmission, while cerebral malaria appears to be more prominent in areas of lower and more seasonal transmission (Snow *et al.*, 1997).

Traditionally, chemoprophylaxis relied on chloroquine, which has been one of the most successful of antimalarial agents produced by mankind. Its beneficial effects on public health have been enormous, and since 1950 it has remained the drug of choice for treatment of an attack of falciparum malaria in West Africa and has been used for prophylaxis (Neequaye *et al.*, 1988). Though chloroquine has been and still is the drug of first choice its massive use over the years has resulted in the appearance of chloroquine-resistant *P. falciparum* in nearly every region afflicted by malaria, particularly South East Asia, South America and Africa countries south of the Sahara (Neequaye *et al.*, 1988). In view of this, the loss of chloroquine as a safe, cheap and

effective remedy for malaria already threatens to overwhelm the beleaguered medical services of many countries including Ghana.

2.5 Malaria vaccine

Due to the heavy toll malaria has on its poor victims and current upsurge of resistance to the known and trusted antimalarials, current researches in malaria is directed towards the development of a vaccine (Miller, 1999). Experiments done on mice showed that DNA vaccines are a promising new approach in that they circumvent the problem of genetic restriction and provide additive protection in mice (Weiner and Kennedy, 1999). This could be exploited and tested in humans. However, recent data from two non-human primate vaccine studies using DNA and/or recombinant protein vaccines based on *P. falciparum* blood stage antigens (use by the parasite for cell invasion and disease progression) show a possible increased risk of development of anaemia in vaccinated animals after challenge with blood stage parasites (Egan *et al.*, 2000; Jones *et al.*, 2001). This raises the possibility that some specific blood stage antigens of the malaria parasites included in malaria vaccines could induce antibodies, which will bind to the surfaces of infected and uninfected erythrocytes and activate complement, facilitating their removal. Thus, some vaccines may reduce parasitaemia by a mechanism, which could contribute to anaemia. Conversely, vaccine delivery systems and adjuvants could be designed to induce immune responses that protect against anaemia. It is therefore essential to provide a better understanding of the mechanisms of anaemia so as to improve vaccine development.

2.6 The morphological changes of the infected erythrocytes

2.6.1 The infected erythrocyte surface

Infection by *P. falciparum* causes changes within the RBC and on the RBC surfaces. Antigens are expressed on the RBC surface as *P. falciparum* mature within it. These antigens include parasite ligand(s), which assist the adhesion of RBCs to capillary endothelium, thus avoiding splenic clearance. Mature stages of *P. falciparum* are thus removed from peripheral circulation due to sequestration in capillaries and venules (Langreth and Peterson, 1985).

Parasite ligands adhere to host receptors such as the leukocyte differentiation antigen CD36, thrombospondin (TSP), intercellular adhesion molecule 1 (ICAM-1) and chondroitin sulfate A (CsA) (Deitsch and Wellems, 1996). Surface radio-iodination of trophozoites led to the identification of a polymorphic, high-molecular-weight trypsin-sensitive protein (Leech *et al.*, 1984) called PfEMP-1, which was recognized by immune serum. It was proposed that this molecule was the parasite ligand for adherence to capillary endothelium. PfEMP-1 is encoded by a multigene family designated *var* (Baruch *et al.*, 1995). More recently, PfEMP-1 has been postulated to be the ligand mediating rosetting, i.e., the binding of uninfected erythrocytes to infected RBCs (Rowe *et al.*, 1997).

Other parasite-derived and parasite-induced molecules are expressed at or near the surface of the trophozoite. As a result of these surface modifications, infected erythrocytes are rendered immunogenic and are subsequently targeted by both humoral and cellular components of the immune system (Doolan and Hoffman, 1997). The immune response to the infected erythrocytes includes, amongst others,

the activation of cytokine-releasing $CD4^+$ cells and the production of antibodies to *P. falciparum*-encoded antigens (Doolan and Hoffman, 1997). Furthermore, it has been shown that complement is activated (Cooper and Fogel, 1966).

2.6.2 Alterations in the complement regulatory proteins

It has been observed that there are alterations of the expression of complement regulatory proteins CR1, CD55 and CD59 (**Fig. 2.1**) on the RBC surface of patients with severe anaemia (Waitumbi *et al.*, 2000). The role of these proteins is to protect the surface of host cells from attack by complement (Holguin *et al.*, 1992). The regulation of the complement system is discussed in more detail.

2.7 Immunity to malaria

From birth till 6 months of age children have immunity to *P. falciparum* infection due to maternal antibodies they obtained at birth. The children rarely get clinical malaria. Between the age of 6 months and 5 years children are prone to clinical attacks, which result in high morbidity and mortality. Immunity to malaria usually requires repeated exposure to the parasite. Thus in areas of stable malaria, children of 5 years and above develop clinical immunity. One reason for the delay is the capacity of the parasite to vary antigens, which are major targets for protective antibodies.

Much remains to be known about the mechanism involved in protective immunity against malaria and the way it is acquired (Troye-Blomberg *et al.*, 1999). This is probably the reason why in spite of so much progress, it has not yet been possible to develop anti-malaria vaccine able to induce parasite specific antibodies and/or T-cells. It was considered in the early 1980s that the induction of efficient protection

against the blood stage forms of *P. falciparum* would not be possible without simultaneously eliciting an auto immune (AI) response against erythrocytes at the risk of inducing an AI pathology (Daniel-Ribeiro, 2000). Despite the description of the reciprocal relationship, i.e. the protective effect of malaria on the development of AI diseases, demonstrated since 1970, no effort has been made to verify the possible involvement of the AI response in protection against malaria. Immunity to malaria has been found to be of two types. These are ***acquired*** and ***innate.***

2.7.1 Acquired immunity

This type of immunity develops during repeated exposure to the malaria parasite. Thus those in the endemic areas mostly have this type of immunity. This has been well established (Troye-Blomberg *et al.*, 1999), and T-cells are the major regulators. The existence of functionally distinct *P falciparum* specific $CD4^+$ T-cells subsets in humans has been observed in several studies. However, there are no definitive links between the activation of various T cells and the course of human *P. falciparum* blood-stage infection in contrast to what is the case in murine models.

2.7.2.Innate immunity

This is defined by the basic resistance that the host possesses. The fact that some individuals are susceptible and others not, mean the host is responsible. This innate immunity is brought about by genetic factors especially those that affect the red blood cells directly, the main site of development of the parasite. These include β-thalassaemia-which affects the rate of haemoglobin synthesis, sickle cell trait (HbS)-the carrier state of haemoglobin type S, and G-6PD (glucose-6-phosphate dehydrogenase) deficiency- a key red blood cell metabolic enzyme, all of which offer

clinical protection against malaria. Friedman (1978) has shown that parasite growth is significantly inhibited by HbS carrier red blood cells and not in homozygous individuals. In another study by Akanmori *et al.* (1991), it was also shown that IgG and C3 titers are lower in the serum of homozygous individuals as compared to heterozygous individuals.

2.7.3 Humoral immunity

In residents of highly endemic malarious areas, immunoglobulin levels are strongly elevated and the level of total anti-malarial antibodies increases with age. Also, passive transfer of immune IgG has been shown to confer some protection. However, only few human studies found correlation between anti-malarial antibody levels and protective immunity, indicating that most of the anti-parasite antibodies have no protective effect (Bolad and Berzins, 2000). Protective antibody responses to the asexual blood stage of the parasites must be strain specific directed against variant antigenic determinants exposed on the surface of free merozoites or on the infected erythrocytes. It has been shown that children in malaria endemic areas largely do not have antibodies specific for the acute malaria attacks (Bull *et al.*, 1998; Giha *et al.*, 2000). The antibodies produced as a result of the infection may specifically bind to antigens on the merozoites surface thereby blocking invasion or to antigens on the surface of infected erythrocyte leading to destruction or elimination of parasitized cells by phagocytosis (Jakobsen *et al.*, 1996; Jakobsen *et al.*, 1997). The antibodies may also prevent cytoadherence of infected RBC to endothelial surfaces and prevent rosetting of infected RBC to non-infected RBC thereby preventing severe illness.

Recently, plasma antibodies from malaria exposed multiparous women were shown to interfere with binding of *P. falciparum* to chondroitin sulfate (CSA) *in vitro*. Thus,

acquisition of antibodies interfering with CSA specific parasite sequestration was shown to be a critical element in resistance to pregnancy associated malaria (Fried *et al.*, 1998; Ricke *et al.*, 2000). However, it has been observed that for an efficient production of protective anti-malarial antibodies an intact and functioning T cell system is required (Troye-Blomberg *et al.*, 1994; Chougnet *et al.*, 1991).

2.7.4 Cellular immunity

Although antibodies are seen to clearly play a role in the control of the asexual blood stage, acquired resistance has been demonstrated in B cell deficient and depleted mice infected with certain Plasmodia species. Indeed $CD4^+$ deletion prevents clearance. Both $CD4^+$ cells and a T helper 1 (Th1) $CD4^+$ clone adoptively transfer immunity in different murine malaria models (Troye-Blomberg *et al.*, 1994). Cytotoxic $CD8^+$ T cells appear mainly to protect against the pre-erythrocytic stage directed against infected hepatocytes (Hockmeyer and Ballou, 1988). In view of these, Rzepczyk *et al.* (1997) have proposed that $\gamma\delta^+$T cells play a protective role against blood stage infection.

2.7.4.1 T-cell control of blood stage infection

Available evidence points to a major role of $CD4^+$T cell in controlling blood stage infection. Immunity to plasmodia, as seen in *P. chabaudi*, is probably a two step process. These are:

(i) $CD4^+$T cells, which are activated during early acute phase of the infection, appear to be of Th1 type, producing predominantly IFNγ and IL2. Later, when infection was controlled, the main antigen specific T cells were Th2 cells for antibody.

(ii) Malaria specific Th1 cells are assumed to activate mononuclear phagocytic cells which kill or inhibit the parasite intra or extracellularly by a variety of mechanisms (Deloron *et al.*, 1991). The presence of a spleen and an intact splenic architecture is also essential for immunity (Troye-Blomberg *et al.*, 1994).

2.7.4.2 Gamma Delta ($\gamma\delta^+$) T cells

During acute infection $\gamma\delta^+$T cells have been shown to account for up to 30-40 % of the peripheral T cells (Ho *et al.*, 1994). A highly significant increase in both the proportion and absolute number of $\gamma\delta^+$T cells has been observed in non immune *P. vivax* patients during clinical paroxysms (Perera *et al.*, 1994). Moreover, $\gamma\delta^+$T cells are increased in the spleen of humans who died of cerebral malaria and in the spleen of monkeys severely infected with *P. coateneyi* (Bordessoule *et al.*, 1990; van der Heyde *et al.*, 1993).

A protective role for $\gamma\delta^+$T cells in malaria has been suggested from animal studies. In mice infected with the avirulent strain *Plasmodium chabaudi adami* the degree of parasitaemia was related to the number of $\gamma\delta^+$T cells in the spleen. Increases in parasitaemia were followed by increase in $\gamma\delta^+$T cells number followed by subsequent lowering of the parasitaemia (Langhorne, 1996).

The $\gamma\delta^+$T cells could also play a role in pathogenesis of malaria. The cytokine profile (IFN-γ, TNF-α, TNF-β) of human peripheral blood $V\gamma9^+$ $\gamma\delta^+$T cells is compartible with a pro-inflammatory response and macrophage activation (Goodier *et al.*, 1995). These cytokines have been implicated in severe and cerebral malaria in humans and in experimental animal models.

2.7.5 Role of autoimmunity

Specific sensitisation of either a part or the entire erythron during infection by antibodies with either antiparasitic or autoantigenic specificities has been widely proposed as the cause of RBC destruction (Zuckerman, 1974). Autoantibodies, primarily of the IgM class (Kreiner *et al.*, 1966) but also of the IgG and IgA classes (Kano *et al.*, 1968; Rosenberg *et al.*, 1973; Voller, 1974) with specificity toward uninfected and infected RBCs and toward trypsinized RBCs have been detected either free in the plasma or cell bound during the acute anaemia phase of malarial infection. The predominance of IgM as the haemaglutinin would explain one of the puzzling and troublesome observations which has plagued proponents of the autoimmune theory, that is, that the DAT reaction which detects cytophilic IgG, is generally negative (Adner *et al.*, 1968) rather than positive during the phases of the disease during which there is disproportionate RBCs loss (Zuckerman, 1974). Several additional autoantigens have been implicated in the autoimmune disorders occurring during malaria, including modified antigen-antibody complexes (Topley *et al.*, 1973). Opsonic antibodies have been eluted from antigen-antibody complexes obtained from washed RBCs of malarious hosts (Cox, 1973). Surface-adherent antigen-antibody complexes appear to fix complement and induce a prehaemolytic or a haemolytic condition. The entire immune complex may be autoimmune responses leading to elimination of RBCs.

Recent data has shown that certain markers are low in concentration on the RBC surface leading to haemolysis that may not be due to *P. falciparum* infection. One of these markers is CD47 (integrin-associated protein) that functions as a marker of self on murine red blood cells. Oldenborg *et al.* (2000) observed that red cells that lacked

CD47 were rapidly cleared from the bloodstream by splenic red pulp macrophages. They therefore argued that CD47 on normal red blood cells prevented this elimination by binding to the inhibitory receptor signal regulatory protein alpha (SIRP α). Thus, macrophages may use a number of non-specific activating receptors and rely on the presence or absence of CD47 to distinguish self from foreign. CD47-SIRPα may represent a potential pathway for the control of haemolytic anaemia. Oldenborg *et al.* (2000) also came out with interesting findings that complement (C3) are not involved in the elimination of the erythrocytes. But the presence of monocytes and macrophages were significant in that they cause the removal of erythrocytes that lacked CD47 on the erythrocytes. However, the role of CD47 in the pathogenesis of severe malarial anaemia has not yet been clarified.

2.8 The Complement system

2.8.1 General outline of the complement system

The human complement system is comprised of about 20 plasma proteins and 10 regulators or receptors on cell membranes, which are important in providing immune protection. Three pathways are involved when the complement is activated (**Fig. 2.1**). These are:

(i) the classical pathways, which is activated by the antigen and antibody interaction,

(ii) the alternative pathway which is activated by polysaccharides and some viral proteins on the cell surface and

(iii) the mannose binding lectin (MBL) pathway, which is activated by certain sugar molecules with high mannose content.

Briefly, activation of complement begins with C1q, C1r and C1s in the classical pathway and C3b in the alternative pathway. C4 and C2 initiate that of the MBL

pathway (**Fig. 2.1**). The central molecule is C3 and it is very crucial in determining the fate of the pathogen. Thus the pathogen may be opsonized followed by erythrophagocytosis or by direct lysis initiated by the complex structure of C5, C6, C7, C8, and C9. This complex structure forms a pore in the cell membrane of the pathogen in question resulting in loss of fluids leading to lysis of the pathogen.

The primary function of complement is to defend the host against microbial infections. The antimicrobial activity of complement involves three different mechanisms (**Fig. 2.2**). First, during activation of complement small bioactive fragments are released into the fluid phase leading to induction of histamine release from mast cells, chemotaxis and leukocyte activation. Secondly, fragments of complement proteins bind to the activating agents (opsonization) and help phagocytic cells to handle them. Thirdly, complement can directly destroy the invader organism by forming pores on the target cell membrane. Complement anaphylatoxins C3a, C4a and C5a induce smooth muscle contraction and increase vascular permeability. The large C4b and C3b fragments bind covalently to the target structures and interact thereafter with specific cell membrane receptors to allow efficient clearance of the activating cell or particle. The degradation fragments of C3b (C3bi, C3dg and C3d) are also important ligands for cellular receptors. Because of its multiple activities, complement activation is involved in one way or another, with most "immune" and inflammatory diseases. The fact that deficiency in almost any component of the complement system can also lead to serious disease, demonstrates that complement is an essential part of the host immune defence system (Kinoshita, 1991).

During *P. falciparum* malaria, there is evidence of complement activation through both the classical and alternative pathways (Stanley *et al.,* 1984). The formation of specific antibodies requires several days, during which defense against infection has to rely on natural immunity, i.e. the alternative and MBL pathways, opsonization and phagocytosis, and other plasma defense proteins. Rosenberg *et al.* (1973) showed that antibodies are produced late during infection with *P. falciparum* whilst complement factors are used up rapidly in the early stage of the infection. Thus C3 levels decreased progressively during the study, whereas mean IgM concentrations increased linearly from 100 to 500 mg/ml during 24 days following onset of malaria. Under *in vitro* conditions, infected erythrocytes opsonized by complement are efficiently phagocytosed by macrophages and neutrophils (Ferrante *et al.,* 1990; Turrini *et al.*, 1992), but seem to be resistant to complement –mediated lysis (Stanley *et al.*, 1984). The activation of complement usually culminates in the formation of membrane attack complexes, which consist of the complement factors C5b, C6, C7, C8 and polymeric C9, forming a lytic pore in the targeted membrane (Muller-Eberhard, 1984). Lysis by complement is an important effector mechanism in the destruction of many pathogens (Kinoshita, 1991).

In as much as the activation of the complement system is important during infection it is necessary that they be regulated to avoid any immunopathological phenomenon, thus calling for complement regulators/inhibitors.

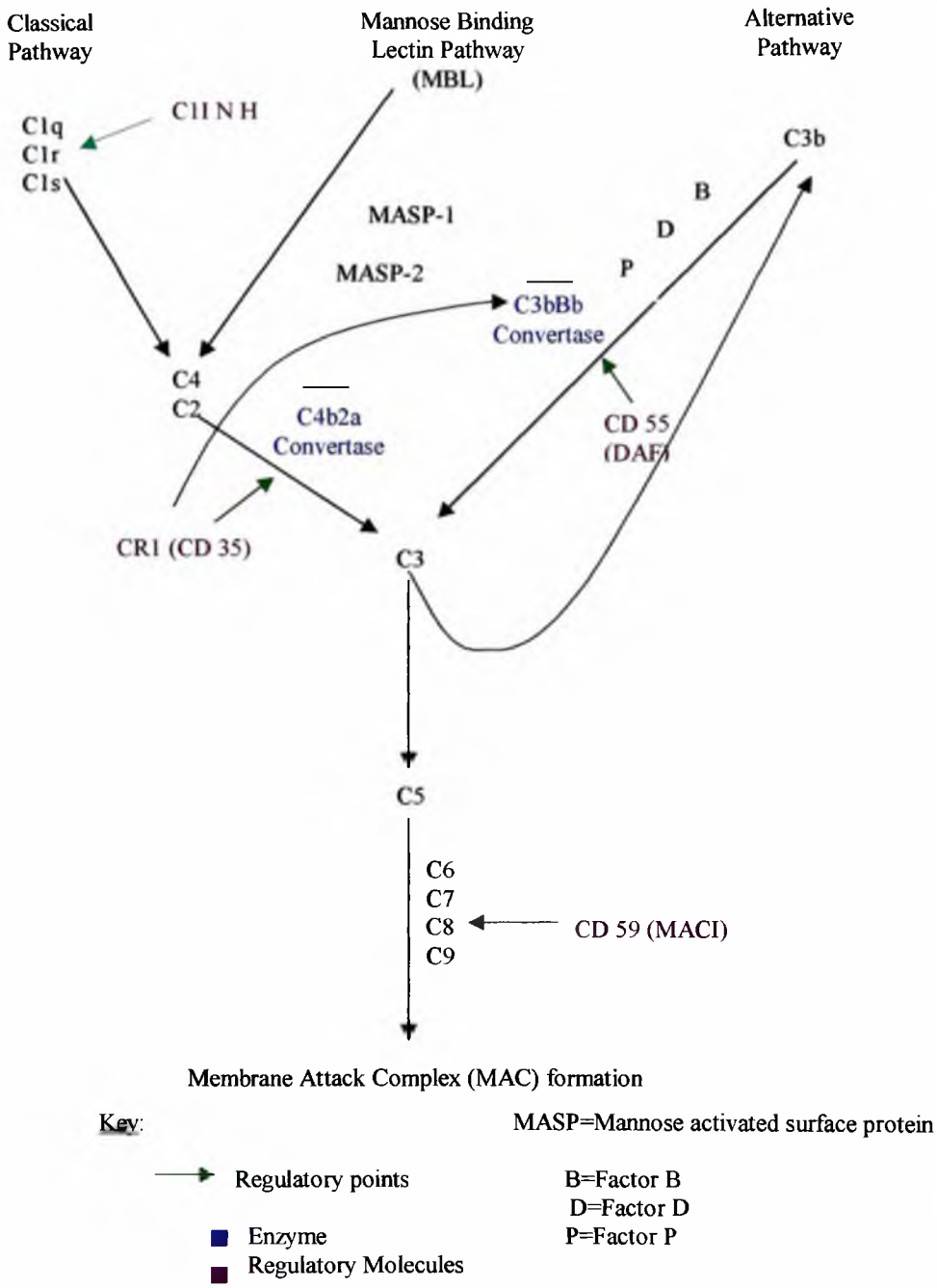


Figure 2.2:Illustration of Complement Activation Pathways and Regulatory Points

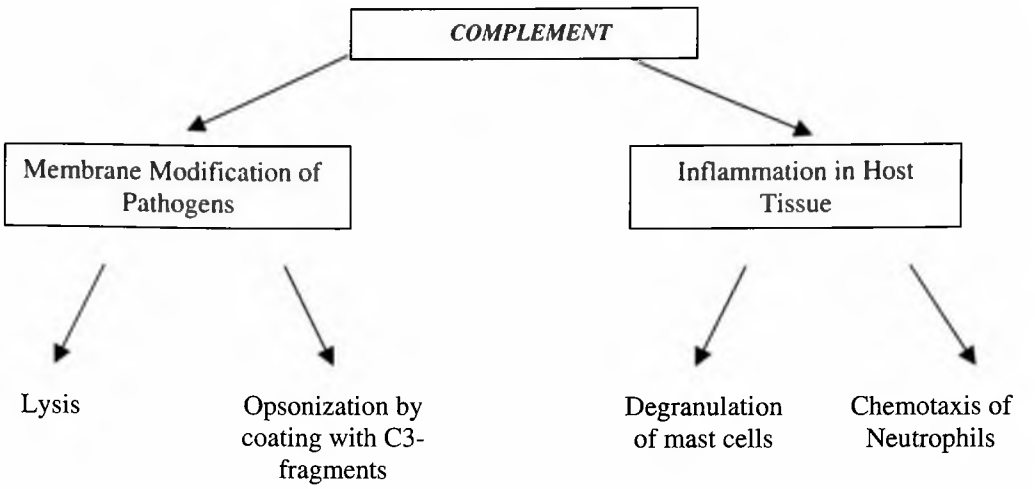


Figure 2.2: The consequences or outcome of Complement activation during infection

2.8.2 Inhibitors of complement

There are two types of complement regulators:

- The Soluble regulators of complement and
- Membrane regulators

2.8.2.1 Soluble regulators of complement

The complement cascade is a rapidly activating (within minutes) and self-amplifying system. To avoid extensive consumption and self-depletion it requires control at several levels. C1-inhibitor (C1INH) prevents C1 activation by forming a covalent complex with C1r and C1s (Ziccardi, 1982). This prevents overactivation of the classical pathway and leaves C1q free to interact with C1q receptors. The latter interaction can launch some effector functions like phagocytosis and stimulation of oxidative metabolism in polymor-phonuclear leukocytes (Ghebrehiwet, 1989).

C4b-binding protein (C4bp) is another inhibitor of the classical pathway. It is composed of one β-chain and seven identical α-chains whose carboxy-terminal ends form a central core in a "spider-like" molecule. C4bp has multiple binding sites for the classical pathway. Factor H is a single-chain glycoprotein that binds to C3b. This binding greatly accelerates the decay of the alternative pathway C3 convertase C3bBb. Factor H has an important role in the discrimination between activators and nonactivators of the alternative pathway (Meri and Pangburn, 1990).

It has been shown that sialic acid and acidic glycosaminoglycans on cell membranes enhance the interaction of factor H with C3b and prevent formation of the C3bBb complex. Lack of these polyanions will promote activation of the alternative pathway on the surface of activators (Pangburn and Müller-Eberhard, 1984; Meri and

Pangburn, 1990; Meri and Pangburn, 1994). Factor I (C3b/C4b inactivator) is a specific serine protease that regulates the C3/C5 convertases of both pathways by inactivating C4b and C3b. It uses C4bp or factor H as a cofactor and splits the α-chains of C4b and C3b causing the loss of biological functions of these molecules (DiScipio, 1992). The fate of the nascent C5b-7 complex is regulated by plasma vitronectin (Podack and Müller-Eberhard, 1979). Up to three vitronectin molecules can bind to the C5b-7 complex keeping the resulting SC5b-7 soluble. SC5b-7 can bind C8 and C9 but the polymerization of C9 does not occur. Another molecule that keeps C5b-7 complex soluble is clusterin, also called SP40, 40 or apo J (Jenne and Tschopp, 1989).

2.8.2.2 Membrane regulators of complement

Activation of the alternative or the classical pathway generates complement activation fragments and complexes, which can bind not only to an appropriate target but also to the surface of host cells. To protect themselves against complement damage, the host cells express several membrane-bound glycoproteins, which regulate complement **(Fig. 2.1).** These regulators fall into two main groups; inhibitors of the alternative and classical pathway C3/C5 convertases, and regulators of the membrane attack complex. The three main C3/C5 convertase inhibitors are decay accelerating factor *(DAF; CD55),* membrane cofactor protein *(MCP; CD46)* and the C3b receptor *(CR1; CD35).* The two known membrane-bound regulators of the terminal pathway are homologous restriction factor *(HRF)* and protectin *(CD59).*

2.8.2.3 Decay Accelerating Factor (DAF, CD55)

Human DAF is a 70-80 kDa glycoprotein that is present on the membranes of peripheral blood cells, vascular endothelial cells and on many types of epithelial cells (Kinoshita *et al.*, 1985; Medof *et al.*, 1987). The amino terminal part of the protein contains four "short consensus repeats" (SCRs) each with about 60 amino acids held together in a single domain by two internal disulfide bridges. The carboxy-terminus of the protein contains 70 amino acids rich in serine, threonine and proline residues (STP -rich region). A similar region is found in Membrane cell protein (MCP) (Lublin *et al.*, 1988) and in many surface receptors like the Low-density lipoprotein (LDL)-receptor and the IL-2 receptor (Nikaido *et al.*, 1984; Yamamoto *et al.*, 1984). As in MCP, many O-linked carbohydrate side chains are attached to the STP regions and probably form a supportive "collar" structure for the DAF polypeptide close to the cell membrane (Lublin *et al.*, 1986; Lublin *et al.*, 1988). A single N-linked complex -type oligosaccharide side chain is present between the first and second SCRs. Unlike MCP, DAF is anchored to the cell membrane via a glycosyl-phosphatidylinositol (GPI) moiety. Soluble forms of DAF have been found in many body fluids including plasma, tears, saliva, urine, synovial and cerebrospinal fluids (Medof *et al.*, 1987). DAF binds to and dissociates both the classical (C4b2a) and the alternative (C3bBb) pathway C3/C5 convertase enzymes (Nicholson-Weller *et al.*, 1983; Pangburn *et al.*, 1983). It seems likely that DAF has a higher affinity for C4b and C3b when they are complexed with their respective catalytic subunits. Otherwise DAF would probably not recycle from a decayed C4b site to an active C4b2a enzyme. DAF does not act, as a cofactor for factor I-mediated cleavage of C3b or C4b. Therefore depletion of DAF on erythrocytes will suggest that there has been

complement activation requiring the use of some molecules of DAF to down-regulate the complement activation.

2.8.2.4 Membrane-attack-complex-inhibitor factor (MACIF, D59)

The human CD59-antigen (protectin) is an 18-25 kDa glycoprotein inhibitor of complement lysis. It was isolated independently in a number of laboratories during the late 1980's (Okada *et al.*, 1989). Subsequently this protein was termed the membrane-attack-complex-inhibitory factor (MACIF). Other groups gave other names such as homologous restriction factor 20 (HRF20) (Okada *et al.*, 1989) and membrane inhibitor of reactive lysis (MIRL) (Holguin *et al.*, 1989). The functional name "protectin" for CD59 was proposed since the other names were complex and inaccurate in describing the size and function of the molecule. Protectin is widely expressed in human cells and tissues. It is present on all circulating blood cells (Davies *et al.*, 1989), endothelial cells (Brooimans *et al.*, 1992), in most epithelial cells (Meri *et al.*, 1991) and spermatozoa (Rooney *et al.*, 1992). CD59 regulate the complement pathway at the terminal stage **(Fig. 2.1).** It binds to C8, in which case the polymerization of C5, C6, C7, C8 and C9 is not possible and no lysis of the membrane. Thus a lack of CD59 or low expression of CD59 will therefore lift the inhibitory function on complement and allow activation of complement.

2.8.2.5 Complement receptor type 1 (CR1, CD35)

CR1 is present on erythrocytes, monocytes, macrophages, eosinophils, neutrophils, follicular dendritic cells and T- and B-lymphocytes. The number of CR1 on erythrocytes is only about 500 per cell, in contrast to leukocytes, where up to 50,000 CR1 per cell can be found (Wolfgang *et al.*, 1999). Nevertheless, more than 85% of

CR1 in blood is present on the RBC because of the vast number of erythrocytes. CR1 expressed on macrophages and polymorphonuclear cells serves as an opsonin receptor. Most probably one of the major defense mechanisms against systemic bacterial infection is C3b- and iC3b-independent phagocytosis. On inactivated phagocytes, CR1 alone cannot mediate phagocytosis but efficiently cooperate with Fc receptors and CR3 to bind and ingest opsonized particles. CR1 also regulates complement activation by the inhibition of C3 convertase activity (**Fig. 2.1**); thus protecting host cells from complement mediated damage. CR1 has also been found to help in the formation of rosettes where infected RBCs bind with uninfected RBCs and both are eliminated by the spleen or destroyed by direct lysis (Rowe *et al.*, 1995, 1997). Thus low expression of CR1 on RBC surfaces will suggest the activation of the complement and/or use of CR1 to inhibit or regulate the increased activity of the C3 convertase and/or to form rosettes leading to elimination of infected and uninfected RBCs resulting in anaemia.

2.9 Pathology and Pathogenesis of Malaria (CM and SA)

The pathology of malaria takes various forms. It is usually manifested as fever associated with coma and/or anaemia. In other instances hypoglycaemia could result. Relatively little attention has been paid to the role that may be played by humoral immune responses, such as antibody and complement activation, in the generation of the pro-inflammatory cytokine response. The antibodies bind to malaria antigens, neutralizing them or facilitating their elimination. Since antibody synthesis takes some time, complement is activated to clear these parasites and could lead to direct lysis and/or opsonization, followed by erythrophagocytosis by monocytes and neutrophils or by the cells of the spleen (Facer *et al.*, 1979).

Antibody-dependent protection is primarily mediated by cytophilic IgG antibodies activating cytotoxicity (Perlmann and Troye-Blomberg, 2000). Malaria infection also involves elevated production of IgE antibodies. However, IgE-containing immune complexes are pathogenic rather than protective by cross-linking IgE receptors (CD23) on monocytes, leading to local overproduction of tumour necrosis factor (TNF), a major pathogenic factor in this disease (Troye-Blomberg *et al.*, 1999; Perlmann and Troye-Blomberg, 2000).

It is also found that initial infection with *P. falciparum* parasites preferentially stimulates synthesis of IgM (Rosenberg *et al.*, 1973). However, IgM antimalarial antibodies have not been shown to be protective or may be due to the smaller quantities produced. A potentially important factor is the high level of Immunoglobulin E (IgE) found in many populations that are exposed to malaria (Desowitz, 1989; Perlmann *et al.*, 1997). Although these high levels of IgE in the plasma are also caused by some helminth infection, high levels of specific IgE against a variety of plasmodial antigens have been demonstrated in exposed populations (Perlmann *et al.*, 1997). Studies in a malaria-endemic area of Africa have indicated that the serum level of malaria-specific IgE is correlated with the ratio of interleukin 4 (IL-4) to IFN-γ producing cells following *in vitro* stimulation with the polyclonal activator leucoagglutinin. The high serum level of IgE is predominant in cerebral malaria patients but can also be observed in severe malaria anaemia without impaired consciousness and seems to be correlated with high circulating TNF levels (Perlmann *et al.*, 1997). Since there is evidence that IgE-induced cross-linking of CD23 gives rise to the production of TNF and other cytokines by monocytes via the NO transduction pathway (Mossalayi *et al.*, 1994; Dugas *et al.*, 1995), it is proposed

that high IgE levels in malaria sera may act to enhance TNF production by a similar mechanism.

2.9.1 Cerebral malaria

In most published studies the term 'cerebral malaria' has been restricted to the syndrome in which altered consciousness associated with a malaria infection, could not be attributed to convulsions, sedative drugs or hypoglycaemia alone or to other infections (Warrel *et al.*, 1982). A child with loss of consciousness after a febrile convulsion should not be considered to have cerebral malaria unless coma persists for more than one hour after the convulsions. Similarly, in a child with comatose, diagnosis of cerebral malaria cannot be sustained if consciousness is promptly restored by administration of glucose (WHO, 2000).

2.9.1.1 Cause of coma

Many hypotheses have been proposed to explain loss of consciousness in severe malaria as seen in CM. None is completely satisfactory but currently two major hypotheses have been proposed (WHO, 2000). These are the mechanical and the humoral hypotheses. The mechanical hypothesis asserts that a specific interaction between a *P. falciparum* erythrocyte membrane protein (PfEMP-1) and ligands on endothelial cells, such as ICAM-1 or E-selectin, reduces microvascular blood flow and induces hypoxia. This selective cytoadherence of parasitized RBCs (PRBCs) and rosetting of non-PRBCs around PRBC can account for CM's histopathological hallmark and its characteristic coma condition. However, this hypothesis is inadequate in explaining the relative absence of neurological deficit even after days of unconsciousness. The humoral hypothesis suggests that a malarial toxin may be

released that stimulates macrophages to release excess of pro-inflammatory cytokines (e.g. TNF-α, IFN-γ) and other cytokines such as IL-1 which modulates the expression of adhesion molecules, as well as nitric oxide (NO) and Fc epsilon R11/CD23 (Mazier and Idrissa-Boubou, 1999; Menendez *et al.*, 2000). Cells other than the infected red blood cells, such as platelets, monocytes and lymphocytes, have the ability to adhere to these enthothelial receptors and to one another via different ligands, leading to a more complex situation and an increase in the degree of vessel occlusion i.e. local ischaemia and merges the 2 hypotheses which are not mutually exclusive.

These cytokines (TNF-α, IFN-γ, IL-1) may induce additional and uncontrolled production of nitric oxide. This ubiquitous messenger would diffuse through the blood-brain barrier and impose similar changes on synaptic function, as do general anaesthetics (White and Ho, 1992) and high concentrations of ethanol, leading to a state of reduced consciousness. The biochemical nature of this interaction would explain the reversibility of coma. Similar cerebral metabolic findings to those in CM have been reported in volunteers breathing low concentration of oxygen (reviewed by White and Ho, 1992). Inflammatory processes are not compatible with the time course or pathology. Increased systemic levels of pro-inflammatory cytokines may obtund but do not lead to profound coma (WHO, 2000). Abnormalities of neurotransmitter synthesis, release or binding may eventually be implicated (Clark and Rockett, 1996). However, it is also worth noting that coma may be neuroprotective. Neurones stressed by an inadequate supply of oxygen and nutrients, and unfavorable metabolic milieu, may preserve themselves by reducing energy demands. Premature reversal of coma might increase the risk of neuronal damage

2.9.2 The mechanisms of malaria anaemia

Anaemia is a multifactorial condition, particularly in malarial-endemic areas; therefore it has always been difficult to quantify the role of malaria infection in anaemia. Individuals might become anaemic as a result of several factors, such as dietary deficiencies, helminths and other infections (Menendez *et al.*, 2000). The contributions of the different aetiological factors to anaemia vary with age. Malaria and nutritional iron deficiency are more frequently responsible for the anaemia of infants and young children than that of older children. In older children (> 3years), hookworm infestation, malnutrition and the anaemia of acute and chronic infections are more important contributors to anaemia (Menendez *et al.*, 2000). Two main mechanisms have been proposed to account for anaemia in malaria. These are:

(i) direct destruction of the RBCs (both infected and uninfected) and

(ii) dyserythropoiesis-inhibition or decreased RBC synthesis

2.9.2.1 Destruction of the RBCs (infected and uninfected) and the consequences

At the end of their normal life span (about 120 days), RBCs are removed by components of the mononuclear phagocyte system, principally in the spleen, where haemoglobin degradation takes place. The essential feature of haemolysis is a shortened RBC life span; haemolytic anaemia results when bone marrow production can no longer compensate for the shortened RBC survival. There are several causes of shortened RBC life span; some of which are sickling positive cases, lack of iron and some bacterial infection. In other instances, intravascular haemolysis (IVH) occurs but is uncommon; it results in haemoglobinuria when the haemoglobin released into plasma exceeds the haemoglobin-binding capacity of plasma-binding proteins (e.g., haptoglobin). Haemoglobin is reabsorbed into renal tubular cells

where iron (Fe) is converted to haemosiderin, part of which is assimilated for reutilization and part of which reaches the urine when the tubular cells are incapable of reabsorbing them. Identification of haemosiderinuria in a fresh urine specimen provides evidence of intravascular haemolysis.

Many published studies relating to malaria anaemia due to *P. falciparum* are concentrated on mechanisms of RBCs destruction at higher rate. The conclusive studies to support these are the formation of spherocytes after removal of parasites by the spleen and interference with the red cell sodium pump (Fogel *et al.*, 1966; Dunn, 1968; Conrad, 1969) hyperactivity of the reticuloendothelial system and hypersplenism (George *et al.*, 1966; Sheagren *et al.*, 1970) and autoimmune red cell destruction (Woodruff *et al.* 1973; McGregor, 1978; Facer *et al.*, 1979). Malaria anaemia takes various forms. The relative contributions to anaemia by the various mechanisms differ according to age, pregnancy state, antimalarials, immune status and genetic constitution of infected individuals and the local endemicity of malaria. In general, haemolysis is of greater importance in non-immune children experiencing acute malaria, whereas dyserythropoiesis is seen in persons experiencing recurrent or frequent *falciparum* malaria, although, in any one individual, several mechanisms are likely to operate (Menendez *et al.*, 2000). The malaria anaemia is most marked in *P. falciparum* infection, which achieves much higher levels of parasitaemia than other plasmodial species that infect humans. In an acute attack of malaria, haemoglobin levels may start to fall after one or two days, often continue to fall for some days after antimalarial treatment has been given, and can take several weeks to recover completely (Abdalla *et al.*, 1980; Philips *et al.*, 1986). The most important impact is the child chronically infected with *P. falciparum*

2.9.2.2 Dyserythropoiesis

Inhibition of red cell production, caused either by depression of erythropoiesis (Kuvin *et al.*, 1962; Srichaikul *et al.*, 1969; Woodruff *et al.*, 1973), inhibition or delayed release of the reticulocytes (Menendez *et al.*, 2000) or ineffective erythropoiesis (Srichaikul *et al.,* 1973; Rencrica *et al.,* 1974; Kurtzhals *et al.*, 1997) may also play a part in causing anaemia in different types of malaria. There are several probable causes of the decrease in red blood cell production (**Fig. 2.3**). One is the suppression of erythropoietin (EPO) synthesis. This has been demonstrated in children in Ghana (Kurtzhals *et al.*, 1998). It was observed that serum EPO was markedly high in children with severe malaria-related anaemia (Menendez *et al.*, 2000). There is also a high probability that inflammatory mediators such as tumour necrosis factor (TNF) suppress EPO synthesis in adults with malaria (Clark and Chaudhri, 1988). The second possible cause of the decrease in RBC production could be the imbalances of some cytokines namely TNF and interleukin 10 (IL-10). Unexpectedly low IL-10 concentrations have been described in Ghanaian and Kenyan children with severe malaria anaemia (Kurtzhals *et al.*, 1998; Othoro *et al.*, 1999; Akanmori *et al.*, 2000). Also Akanmori *et al.* (2000) did find the dysregulation of these cytokines (IL-10 and TNF) in the children with severe malarial anaemia. Similarly, defective interleukin 12 (IL-12) has been shown experimentally to be a mechanism causing fatal rodent malarial anaemia (Mohan and Stevenson, 1998).

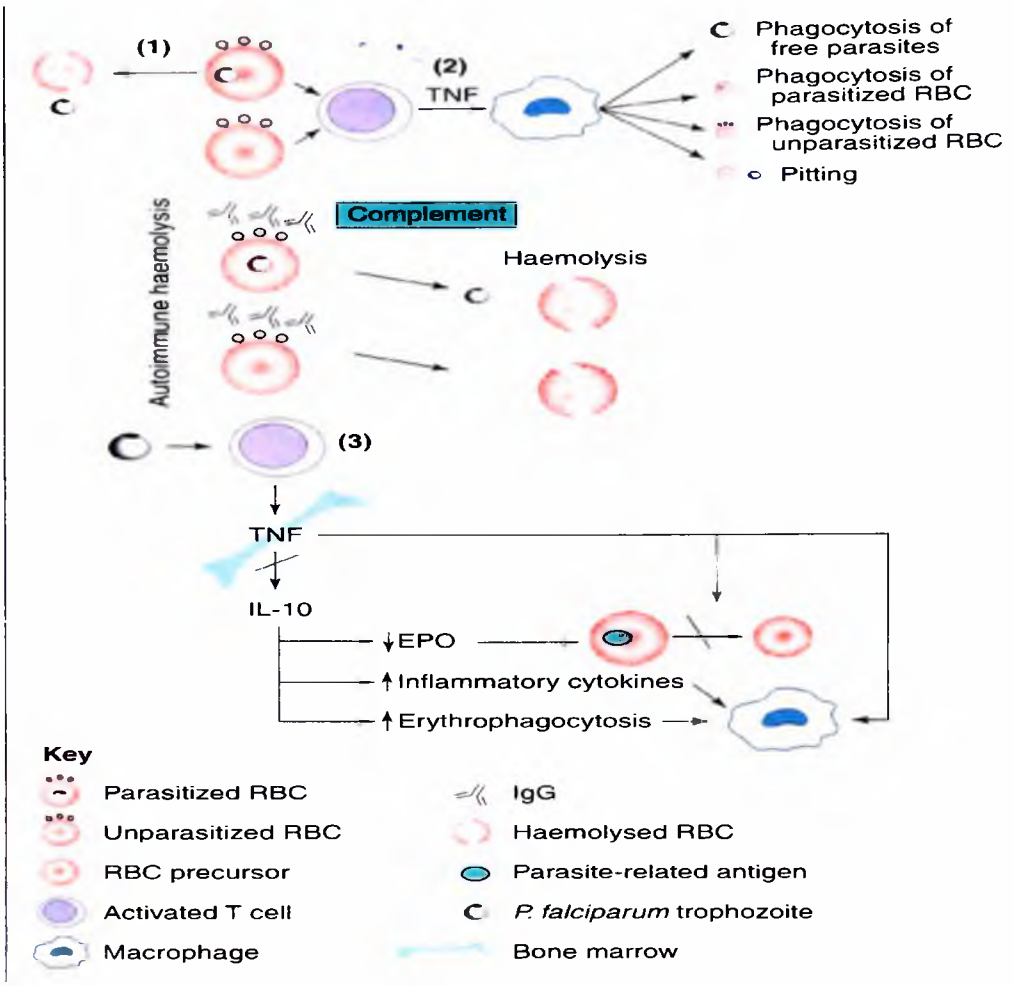


Fig. 2.3: *Main mechanisms of anaemia in malaria.* Intraerythrocytic multiplication of merozoites leads to the rupture of red blood cells (RBC) (1). The binding of the parasite components to the membrane of parasitized and unparasitized RBC leads to their phagocytosis by activated macrophages. In the spleen, parasites are also extracted from RBC without lysis or immediate ingestion by macrophages ('pitting'). Deposition of immunoglobulin and complement components on the surface of parasitized and unparasitized RBC leads to autoimmune haemolysis (2). Parasite antigen-stimulated T helper cell type 1 (Th1) cells produce tumour necrosis factor (TNF) (3); there is a negative feedback mediated through TNF promoting the secretion of interleukin 10 (IL-10) by Th2 cells. IL-10 down regulates phagocytic responses: it inhibits the synthesis of many inflammatory cytokines; it stimulates humoral immunity and acts synergistically with erythropoietin (EPO) to promote erythropoiesis. As a result of this cytokine imbalance, there is bone marrow depression, dyserythropoiesis, erythrophagocytosis and a lack of IL-10 synergism with EPO.

Credit: Menendez et al., (2000). Parasitology Today, vol. 16, no. 11, pg 471.

2.9.2.3 The role of the immune system in malarial anaemia

IgG, IgM and/or complement factors have been found on RBCs (Facer *et al.*, 1979; Facer, 1980), and it has been suggested that these cause increased phagocytic function of monocytes of patients with malaria. Topley *et al.* (1973) found that 13 of 23 malaria patients had a positive DAT with an anti-γ-globulin reagent. Rosenberg *et al.* (1973) showed that 56 patients had a significant rise in serum IgM titers and reduced C3 levels, 52 had IgM and 31 had IgG on their red cells as demonstrated by a fluorescent antibody technique. McGregor (1978) demonstrated that of 1200 people tested in a survey in a Gambian village 47 had a positive DAT. All the subjects were children below 14 years and the majority had malaria parasites in their blood. 13 of these children were studied in greater detail and 11 were shown to have C3d on their RBCs surface. The Coombs' test (DAT) is sensitive in detecting the presence of IgG, C3d and other complement fragments on the infected RBCs surface giving an evidence of immune mediated RBC destruction. However, this (DAT positivity) may not be due to *P. falciparum* infection only as it has been reported that other factors do contribute to this DAT positivity. Some of these factors are drugs like, chloroquine, methyldopa and related drugs, which lead to autoimmune haemolysis (Croft *et al.*, 1968).

2.9.2.4 Dead merozoites and rosetting implicated in the pathogenesis of malaria anaemia

With the infection and multiplication of the parasite, RBCs are ruptured. The merozoites which are released, may bind to the parasitized RBCs (PRBCs) or uninfected RBCs. Complement is activated in which case C3b binds to these dead merozoites on the RBCs where they are opsonised for erythrophagocytosis by the

monocytes after the activation of the corresponding C3b receptors. Rosetting has also been implicated in the removal of the uninfected erythrocytes due to *P. falciparum* infection. Studies in Africa have shown that severe malaria is associated with the ability of RBCs infected with *P. falciparum* to bind uninfected erythrocytes and form rosettes (Rowe *et al.*, 1995). Rowe *et al.* (1997) reported that complement-receptor 1 (CR1) on erythrocytes plays a role in the formation of these rosettes and that erythrocytes with a common African CR1 polymorphism (SI (a-)) have a reduced adhesion to the domain of PfEMP1 that binds normal RBCs.

2.9.2.5 The contribution of the spleen in red cell elimination in the causation of malaria anaemia

There has been considerable evidence that the spleen is involved in the elimination of PRBC (reviewed by Wyler 1983), which shows the importance of the spleen for host defense against malaria infection. According to Allred (1995) the phenomenon of sequestration has evolved as a strategy to enable mature forms of *P. falciparum* to avoid passing through the spleen. With this sequestration has been observed enlargement of the spleen, which is being used by epidemiologist as a measure of the endemicity of malaria in a population. This sequestration causes the parasites to escape the mechanism of being eliminated from the blood stream. Studies by Goka *et al.* (in press) also showed that there is a correlation between C3d binding and increase in spleen size.

In animal models of malaria, the splenic microcirculation is altered, myelomonocytic cells are recruited to the spleen and there is increased intravascular clearance of red cells. Although the cellular activity of the spleen cannot be monitored during the

clinical episodes of falciparum malaria in humans, almost certainly there is active erythrophagocytosis since this is a conspicuous feature within the bone marrow (Abdalla *et al.*, 1980; Wyler, 1983).

Cytokines such as TNF are implicated in activating human monocytes for erythrophagocytosis (Kitawaga *et al.*, 1996). According to Taverne *et al.*, (1994) there was over expressing of human TNF constutively in transgenic mice that had become anaemic and has been attributed to a large extent to erythrophagocytosis. One convincing interpretation that may be attributed to the above observation is that pro-inflammatory cytokines such as TNF act to promote the clearance of iRBC by erythrophagocytosis in the spleen and other organs. One risk is hypersplenism that causes removal of uninfected RBCs thus reducing the survival of uninfected RBCs, which may persist for some time after the acute infection. What this goes to emphasize is that, anaemia is a price the host has to pay in order to curtail excessive parasite growth.

2.9.2.6 Defective bone marrow response

It has been observed that the reticulocyte response in malaria is often weak or totally absent resulting in low reticulocyte counts in malaria. This is strange, as the bone marrow should undergo erythropoiesis by responding to haemolysis. This, however, is accompanied by gross dyserythropoietic changes in the bone marrow, which may persist for several weeks after an acute *P. falciparum* infection. Cytokines such as TNF (Roodman *et al.*, 1987), IL-10 and EPO may have a possible influence on the bone marrow for erythropoiesis to occur. Recent studies done independently by Kurtzhals *et al.* (1998), Othoro *et al.* (1999) and Akanmori *et al.* (2000) have

reported some interesting clinical data, that in African children with severe malaria anaemia, there is a considerable relatively low circulating level of IL-10 in relation to TNF in the pathogenesis of severe malaria.

2.10 The incidence of DAT results and anaemia due to *P. falciparum* malaria

In one group of children with anaemia that were studied, (Facer *et al.*, 1979) there was a correlation between a positive DAT and anaemia, but in another group from a different village no correlation was found. In both groups there were many patients with a negative DAT who were anaemic and similarly there were many children with a positive DAT who were not anaemic. The other observation made was that most of the anaemic children who were DAT positive were younger than those who were not anaemic; showing a strong association between age and both anaemia and a positive DAT (Abdalla and Weatherall, 1982), simply reflect this association. Facer *et al.* (1979) also described four patients who were severely anaemic and who showed positive DAT with active C3 components on their RBCs. They were able to demonstrate monocyte erythrophagocytosis of non-parasitized erythrocytes on the blood films of these patients. It thus seems that acute malaria may sometimes be the result of opsonization and phagocytosis of RBCs, but other mechanisms may be implicated in other cases.

In two separate studies (Abdalla *et al.*, 1980; Abdalla and Weatherall, 1982) it was found that there is little or no correlation between a positive DAT due to IgG alone and IgG plus various complement components and the degree of anaemia, reticulocytosis or any other haematological changes. Moreover, there was no phagocytosis of RBCs from patients with a positive DAT by normal adult peripheral

blood mononuclear cells or by their own mononuclear cells. In the same holoendemic area of West Africa where Facer *et al.* (1979) did their test to find C3 complement fragments on the red cells, Abdalla (1986) found no increase in the number of IgG molecules bound to red cells in patients with malaria compared to that in controls. There was no correlation between severity of anaemia and red blood cell-associated IgG levels. Similarly, in Thailand, patients with falciparum malaria showed no increase in IgG coating of their red blood cells and there was no correlation between the severity of anaemia and these levels (Merry *et al.*, 1986). Looareesuwan *et al.* (1987), also in Thailand, demonstrated increased rates of RBCs destruction following clearance of *P. falciparum* and *P. vivax* parasitaemias.

2.11 Methods for studying surfaces of erythrocytes

Studies of the surface changes of the RBCs have been interesting as this study lends more support in understanding the fate of the RBCs, and a number of methods have been employed to make this possible. Two methods that are widely used are:

(i) DAT (Coombs' Test) and

(ii) Flow cytometry technique

2.11.1 DAT

Between 1944 and 1945, the veterinarian immunologist R.R.A. Coombs worked on the problem of visualising incomplete red cell antibody, and came up with a simple yet effective solution. By adding anti-human-globulin antibody produced by immunisation of rabbits, he was able to specifically agglutinate human red cells coated *in vitro* with incomplete RhD antibody (Coombs *et al.*, 1945). This test has been in use consistently for some time due to its simplicity, reproducibility, low cost

and sensitivity. It is important for the detection of Immunoglobulins (IgG and its subtypes, IgE and IgM) and complement fragments, namely C3b, C3d and C4b attached to the RBC surface. This detection is possible by the use of poly-antihuman sera and mono-specific antihuman anti-sera. In order to study these markers in isolation, monospecific anti-serum such as anti-C3d or anti-IgG can be used. Poly specific antiserum is used to identify two or more markers at the same time (Facer *et al.*, 1979).

There are 4 types of DAT currently in use. These are:

(i) tube technique

(ii) tile technique

(iii) well technique

(iv) gel technique

All these methods make use of the principle underlining the DAT. The differences among these methods are in the preparation of the RBCs, percentage cell suspension used and incubation period.

2.11.1.1 Principle underlining the DAT

Washed RBCs bearing immunoglobulin or complement absorbed on their surface can be agglutinated by the addition of a suitable antiglobulin serum. If an antiglobulin serum reacts with more than one class of immunoglobulin or reacts with immunoglobulin and complement the reagent is said to be polyspecific or to have a 'broad spectrum' of activity. If activity is confined to one immunoglobulin class or component of complement it is a mono-specific antiserum.

In performing the DAT most of the antiglobulin reagents used are

(i) a broad-spectrum antiserum containing anti-IgG and anti-C3d, which is essential.

(ii) monospecific IgG and C3d antisera for further characterization of a positive DAT obtained with the broad-spectrum reagent.

(iii) anti-IgA for use when negative results are found with IgG and C3d antisera

(iv) anti-IgM may be used but is not essential in that C3d, if present, always accompanies IgM (Hall and Malia, 1986).

2.11.2 General description of DAT

Red cells are washed four times in comparatively large volumes of saline (some workers recommend phosphate-buffered saline, pH 7.4, to reduce possible antibody dissociation during washing). Saline at room temperature is usually satisfactory though unexpected negative result may be found due to antibody dissociation that may be prevented by using saline at 4^0C. Washed cells must be tested immediately to prevent further dissociation

2.11.2.1 Tube technique

To one drop of 2-5% washed RBCs are added one or two drops of anti-human globulin (AGS) depending on the volumes used in the standardization procedure or according to the manufacturer's instructions. This is followed by centrifugation at 1,000 rpm for 1 minute and examination done immediately for agglutination. Negative or doubtful results are checked microscopically.

2.11.2.2 Tile technique

To one drop of 10-20% washed cells on a thoroughly cleaned tile is added one drop of AGS. This is then mixed and allowed to stand for 2 minutes. The tile is rocked gently and agglutination observed after 5minutes.

An additional control in both tube and tile methods to ensure that cells have been washed adequately is the addition of one drop of appropriately sensitized cells to all negative tests. After re-centrifugation or further observation on the tile the reaction should now be positive. The strength of reaction does not necessarily correlate with the severity of haemolysis but a good estimate of protein molecules adsorbed on to the cells can be obtained by the 'semi-quantitative DAT' by testing against dilutions of AGS. The choice of dilutions depends on the source of the AGS.

2.11.2.3 Well technique

This is performed in special plates similar to those used for Enzyme Linked Immunosorbent Assays (ELISA) (Facer *et al.*, 1979; Abdallah and Weatherall, 1982). The wells are coated with the AGS to detect the antibody or complement fragment adsorbed unto the RBC surface. One or two drops of 5-10% of washed RBCs is added unto the coated wells and incubated for 10 minutes. After, the plates are shaken gently for any agglutination observed.

2.11.2.4 Gel technique

This is a special kind of technique, which employs the principle of chromatography. The gel is packed in small tubes with the AGS as a mixture. The RBCs are not washed, instead one or two drops of packed RBC is added to the gel in the small

tubes and spun in a micro centrifuge at 2,000rpm for 5minutes. Depending on the position of the RBC in the column, the degree of agglutination can be assessed. The agglutination is strongest if it is still deposited on the gel column. When the packed RBC is observed in the middle of the column, the degree of agglutination could range from +2 and +3. If the RBC is packed at the bottom it means no agglutination is detected. For the detection of C3d, it has been reported by Tissot *et al.*, (1999) that the tube method is more reliable than the gel technique. However, the gel column has been found to be more sensitive than the others (Dittmer *et al.*, in press).

2.11.3 Sensitized cells as controls

In all these techniques control-sensitized cells are necessary to ensure that the test is efficient. IgG-coated cells, IgA, IgM and C3d coated cells are available commercially or can be prepared in the laboratory (Hall and Malia, 1986). For a positive control, monospecific AGS are controlled by including appropriately sensitized cells as numerated earlier with each test or batch of tests. Broad-spectrum AGS need only be tested with IgG-sensitized cells, as there is no evidence that selective deterioration of anti-C3d occurs. In the case of a negative control, washed patient's cells plus saline should give a negative result and also normal washed cells should be negative.

2.12 Flow cytometry

The use of antibodies as sensitive and specific probes for detecting cell-surface antigens has provided the foundation of the understanding of the cellular basis of the immune response. Recent protocols described (Coligan *et al.*, 2000) make use of the most highly technological and expensive equipment available to the immunologist at the present time. The approach to studying this field was described almost 60yrs ago by Coons *et al.* (1941,1942). In these early studies, the feasibility of using chemically labeled antibodies as reagents was demonstrated for detecting antigenic material in tissue sections of mammalian organs. The basic principles established at that time are still valid today:

(i) The antibody molecule must retain specificity after the chemical manipulation

(ii) The label must be stably coupled to the antibody

(iii) The labeled antibody must be easily separated from unbound tracer material

(iv) The label should be detected when present in minute quantities

(v) The labeled antibody should only allow identification of the antigen in a given organ, but should also precisely define which cells within an organ.

Flow cytometry, also called flow microfluorometry, employs instrumentation that scans single cells flowing past excitation sources in a liquid medium. It is a widely used method for analyzing the expression of cell surface and intracellular molecules (on a per cell basis), characterizing and defining different cell types in heterogeneous populations, assessing the purity of isolated subpopulations, and analyzing cell size and volume. This technique is predominantly used to measure fluorescence intensity

produced by fluorescent-labeled antibodies or ligands that bind to specific cell-associated molecules.

Flow cytometry has 2 general applications; quantitative analyses and cell separation, which are dependent upon the analytical capabilities of flow cytometry, in those cells of interest, which must be identified analytically in order to be separated. A graphical representation of flow cytometry analytical measurements made on individual cells is known as a histogram. Data plotted on a histogram include the number of cells and values of one or more measurements made on individual cells. Protocols have been developed that help in the flow cytometric analysis of intracellular antigens in single-cell suspensions (e.g., mononuclear cells from human or murine peripheral blood or bone marrow, lymphoid cell suspensions, cells grown in suspensions cultures, or dissociated tissues) are also included. The basic procedures outlined involve successive steps of fixation, membrane permeablization, staining with directly labeled or unlabeled antibody. Accurate quantitative results on each staining experiment depend on the correct determination of the reactivity of the antibody with the intracellular antigen. This requires optimization of antibody staining by titration of the reagents and the use of appropriate staining controls.

CHAPTER THREE

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 Study design and Patient population

This is a clinical case controlled study conducted in July-August, 2000 at the Emergency Unit of the Department of Child Health (DCH), Korle-Bu teaching Hospital. The DCH is the main referral center for paediatric cases, within the neighborhood, the whole of Accra and Ghana in general. Children fulfilling the criteria for severe malarial anaemia (SA), intravascular haemolysis (IVH), cerebral malaria (CM) and uncomplicated malaria (UM) were compared with healthy age and sex-matched or children with asymptomatic *P. falciparum* infection. In addition, a longitudinal study was done on the IVH cases where they were followed up on days 3, 7, 30 and 90. This was to see how the DAT positivity lasts and develops; and how the parasite density and haemoglobin value have a role to play on the DAT results.

3.1.1 Subjects

The children recruited were aged between 1-10 years and from the following patient categories: SA, CM, IVH, all severe forms of malaria and UM. A control group of healthy, uninfected or asymptomatically infected children (CC), who were matched age and sex wise were also recruited from Dodowa, a nearby town within the Dangbe West district of the Greater Accra Region of Ghana. Malaria transmission in this district, which is similar to what pertains in Accra, is perennial with considerable seasonal variation, peaking during and immediately after the rains (May-October). Residents are estimated to receive 20 infective bites per year and *P. falciparum* constitutes 98% of all infections (Afari *et al.*, 1995).

3.2 Categorization of Patients

The children were categorized as patients with SA, UM, IVH or CM based on clinical observation, haematological and other indices as previously established (Kurtzhals *et al.*, 1998). The patients who were recruited satisfied the following general inclusion and exclusion criteria: Informed consent was obtained from the parents or guardian and children had *P. falciparum* infection before treatment with parasite density of 10,000 parasites/μL. None of the patients were sickling positive based on the metabisulphite test, and no traces of glucose 6-phosphate dehydrogenase deficiency were included, as these two conditions can give rise to anaemia. The other haemoglobinopathies were not taken into consideration since their prevalence is relatively low. Presence of fever (temperature > 37°C) with no other infectious disease like typhoid, or upper respiratory tract infections was also one of the criteria for recruiting patient. All patients were treated with chloroquine, the first line treatment as practiced at the DCH.

3.2.1 Specific Inclusion and exclusion criteria

The SA patients had haemoglobin < 5g/dL and were fully conscious with no cases of severe bleeding, reported or observed convulsions. Those of CM cases were unconscious, with a Blantyre coma scale, score of < 3 and duration of coma which was > 60 minutes. The CM patients had any haemoglobin value and no record of recent severe head trauma and other cause of coma or neurological diseases. Patients with UM had a haemoglobin value > 8g/dL and were fully conscious. With regards to the IVH patients there was evidence of haemoglobin or blood in the urine (bloody urine) detected by the urine dipstick test (Roche Diagnostics Ltd, Great Britain).

3.3 Blood sampling

Peripheral blood was collected into 5 ml K_3EDTA vacutainer tubes (Becton Dickinson vacutainer systems, UK) using sterile, one-use butterfly needles (Becton Dickinson vacutainer systems, UK) by a qualified clinician for haematology, parasitology and immunological assays.

3.3.1 Haematology

Haematological profile was determined on each patient blood sample using an 18-parameter automatic haematological analyzer (Sysmex KX-21, Japan). The haematological parameters considered include the haemoglobin values, total white blood cell (WBC) and total red blood cell (RBC) counts.

3.3.2 Parasitological Examination

To detect the malaria parasite species and parasitaemia, both thick and thin blood films were prepared. After the films were dried, the thin film was fixed with methanol. The films were stained with 2.0% Giemsa (BDH Laboratory Supplies, Poole BH15 ITD, England), for 10 minutes, washed with water, dried and viewed under the microscope (Olympus BH2 Microscope, Japan) at 100X magnification. To determine parasite density (parasites per ul), malaria parasites were counted against 300 WBCs and the number multiplied by each individual's WBCs count from the automatic haematology analyzer.

3.3.3 Preparation of erythrocyte suspension

One half of the K_3EDTA blood was spun at 2,000rpm for 10 minutes, plasma taken and stored at -20^0C while the red blood cells were stored in liquid nitrogen for other

assays. The other half of the K_3EDTA blood was washed with 3 ml incomplete RPMI 1640 (Gibco, Paisley, United Kingdom) once and then resuspended in 3 ml incomplete RPMI and sent to the Laboratory of the Immunology Unit, Noguchi Memorial Institute for Medical Research, NMIMR, University of Ghana for analysis.

3.3.4 Sickling test

A sickling test was done for each patient by mixing a drop of whole blood with a drop of 0.2% freshly prepared sodium metabisulphite on a glass slide. The mixture was then covered with a glass slip and incubated for at least 30 minutes at room temperature. After, the slide was examined under the microscope at a magnification of 40X and the sickling status of the patients determined. Those showing positive sickling were excluded from the study.

3.4 Direct antiglobulin Test.

3.4.1 Preparation of cells for testing

Red cell suspensions from DCH, Korle-Bu were washed four times in comparatively large volumes (4mls) of 0.9% saline, pH 7.2, at 1,200rpm for 2.5 minutes during each wash. The cells were resuspended to 5% for testing by the tube technique and were used immediately to prevent further dissociation of the antibodies from the surface of the RBCs. Saline was kept at room temperature, as it is satisfactory and stored in the fridge at 4°C if it is to be kept for a long time to avoid unexpected negative result due to antibody dissociation.

3.4.2 Antiglobulin reagents

The antibodies used for the study are made up of a broad-spectrum antiserum (DIAGAST Laboratories, Copenhagen, 76105) containing anti-IgG and anti-C3d. Another set of antibodies used were monospecific antibodies anti-IgG (MAESTRIA, 76206) and anti-C3d antisera (DIAGAST Laboratories, Copenhagen, 76103) for further characterization of a positive DAT obtained for IgG and C3d with the broad-spectrum reagent. The broad-spectrum and the monospecific anti-globulin sera (AGS) were controlled by including sensitized red cells (Valicombs, DIAGAST Laboratories, Copenhagen, 51099). This served as a positive control to confirm the positive cases. Internal positive controls were also included. Washed patients cells (which did not show any agglutination) plus saline and normal washed cells were used as negative controls.

3.4.3 Tube technique for DAT

To two drops of 5% RBCs suspension was added two drops of poly-antiglobulin sera and centrifuged at 1,200rpm for 1 minute. Microscopic examination was done immediately for agglutination to check all negative or doubtful positive/weak results. The positive results for DAT were further characterized by using mono specific antiserum. Two drops of the 5% RBCs suspension was added to 2 drops of anti-IgG and anti-C3d in different tubes and spun at low speed for 1 minute. The tube was shaken gently to observe for agglutination and graded accordingly as +4 (strongest agglutination), +3 (several large agglutinates, few cells), +2 (large agglutinates in a sea of smaller clumps and free cells), +1 (many small agglutinates) or 0 (no agglutination).

3.5 Procedure for storage of RBCs for flow cytometry

The blood in the K_3EDTA tube was spun at 1,500rpm for 10 minutes. The supernatant was aspirated and 50µl of the packed RBCs pipetted into an eppendorf tube, and cells stabilized as described by the manufacturer (BioErgonomics, Inc.). To the 50µl of the packed RBCs was added 250µl of phosphate buffer saline (PBS) and an equal volume of Stabilizer Buffer A (BioEgornomics, Inc.). The suspension was placed on a rocking platform at low speed and the cells incubated for 1.5 hours at room temperature. After incubation, the RBC suspension was centrifuged at 1,000rpm for 10 minutes at room temperature. The supernatant was aspirated as much as possible without disturbing the cells pellet. A volume of Stabilizing buffer B (250µl) was added and incubated on rocking platform at low speed for 1.5 hours at room temperature. After incubation, the cell suspension was again centrifuged at 1,000rpm for 10minutes. The supernatant was aspirated and a volume of fresh stabilizing buffer B (250µl), added and resuspended completely. Cells were stored at 4°C and used when ready.

3.5.1 Flow cytometry

The stored RBCs were centrifuged at 1,000rpm for 10 minutes after being allowed to attain room temperature. Exactly 20µl of the packed RBCs was pipetted into a test tube. These were washed with 4 mls of PBS twice. The supernatant was aspirated and the pellet resuspended in 2 ml of PBS. The RBC suspension was counted in the haemocytometer under the microscope and diluted to obtain 2.0 x 10^7 cells/ml. Ten microlitres of ethidium bromide (5mg/ml) solution was added per ml of RBC suspension, and incubated in the dark. While incubating, 15 tubes were labeled and 10µl of antibody added according to Table 6.1 in appendix. After the incubation,

100µl of the RBC suspension, which was stained with the ethidium bromide, was added to each tube and further incubated for 20 minutes in the dark. Tube 15, which also has RBC suspension, however, did not contain any ethidium bromide and antibody and served as negative control for ethidium bromide and antibody. These cells with the respective antibodies were washed twice. The cells were pelleted by centrifuging at 1,200rpm for 5 minutes after each step of washing with 4ml cellwash (optimized PBS, Becton Dickinson, 349524). The supernatant was aspirated after each centrifugation and cells in tubes 1,5,7,12,14 and 15 were resuspended in 1ml cellwash. These were set aside in the refrigerator at 4°C till its acquired on the flow cytometer.

To the rest of the tubes, 8µl of the specified secondary antibody was added according to Table 6.2 in appendix and was incubated for 20 minutes in the dark. These were also washed twice in the same manner as done above, and resuspended in 1ml of cellwash. The other tubes were recovered from the refrigerator, allowed to attain room temperature, vortexed and 10,000 cells acquired on the FACScan using the Cell Quest programme. The RBC surface staining was done on ten (10) patients from each of the following patient categories SA, UM, CM, IVH, DAT positive and DAT negative and healthy, asymptomatic controls (CC) from Dodowa. A total of 70 samples were analyzed.

3.6 Statistical analysis

Data obtained from the DAT results and the flow cytometry (mean FITC values obtained for the categories of children) were entered into Microsoft excel and reorganized into Sigma stat 5.0 program (Sigma stat, Jandel, Denmark). Both

descriptive and Dunn's method of pair wise multiple comparative statistics were prepared using the sigma stat program. Kruskal-Wallis One Way Analysis of Variance (ANOVA) on Ranks was used when normality test failed otherwise 1-way ANOVA was preferred. Correlation between parameters was determined by using the Spearman Rank Order.

Descriptive statistics obtained using this program include the mean, median, standard deviation and standard error. Non-parametric variables (e.g. parasite density) were normalized by logarithmic transformation when necessary. Thus the geometric mean was computed instead of the arithmetic mean. For comparative statistics between the patient categories, the student t-test was used and values were considered significant if $p<0.05$. 95% confidence intervals were included.

CHAPTER FOUR

RESULTS

4.0 RESULTS

4.1 Clinical characteristics of the patient categories (SA, IVH, UM, CM) and controls (CC)

The summary of the clinical data is presented in Table 4.1. A total of 484 patients were recruited for the study, yet only 52, 35, 31 and 25 fulfilled the criteria for SA, CM, IVH and UM respectively as shown in the Table. More males than females reported to the DCH. In the IVH and CM cases the ratio of males to females was 2:1 and 1.5:1 respectively whilst a 1:1 male to female ratio was observed in the SA and UM cases as well as the CC. The SA cases were younger (mean age=2.8yrs.) than the other categories of patients and the CC (mean age 5.7yrs.) ($p<0.001$). The lowest mean Hb was observed in the SA cases, mean Hb=4.0g/dL, followed by IVH, mean Hb=6.3g/dL with the highest mean Hb found to be associated with the CC, mean Hb=10.9g/dL. All the patient categories had high peripheral blood malaria parasite density (geometric mean) with CM and IVH having the highest (173.1 and 115.8 parasites $x10^3/\mu L$ respectively) whilst the CC had the lowest parasite density of 1.4 parasites $x10^3/\mu L$. However, the parasite density of the recruited SA and UM cases (95.0 and 92.2 parasites $x10^3/\mu L$ respectively) was significantly lower than IVH and CM cases, $p<0.05$

4.2 Evaluation of DAT against Flow Cytometry for C3d

RBC suspensions from 30 DAT positive and negative individuals were stained with ethidium bromide and monoclonal antibodies to detect infected RBCs and C3d (expressed on the RBCs surface), respectively. Thus comparing the DAT with flow cytometry data for C3d, it was interesting to note that there was a positive correlation ($r^2=0.53$), which was significant ($p<0.0001$) Fig. 4.1.

4.3 Frequency of DAT positivity in different clinical categories of patients and controls (CC)

DAT positives formed 27.0% (131) of total numbers of patients studied (434). Most of the sensitisation was due to C3d alone (87.8%), with a small proportion being ascribed to IgG alone (2.2%) or both (9.9%), Table 4.2. The DAT negative malaria patients, who formed 73%, were significantly older than the DAT positives, p=0.03 (Table 4.3). Table 4.3 also shows that the mean Hb was significantly higher in the DAT negatives than the DAT positives (p<0.001). Although the parasite density was lower in the DAT positive children (55.2 parasites $x10^3/\mu L$) when compared to the DAT negative (78.4 parasites $x10^3/\mu L$), this was statistically not significant, p=0.109. The spleen sizes were comparable in the patients with DAT positive and negative results, p=0.548. There was a high prevalence of DAT positive cases in the SA (48.1%) and IVH (74.2%) patients recruited whereas the UM and CM had 28.0% and 42.8% prevalence respectively, with 26.3% in the CC (Table 4.1). In addition, of all the DAT positives recorded, 25 (19.1%) were SA cases, 23 (17.5%) were IVH cases, 15 (11.4%) were CM cases and 7 (5.3%) were UM cases with the lowest being the 5 (3.8%) CC (Table 4.1). The rest (42.9%) were uncharacterised. Also in the DAT positives, it was observed that the IVH category of patients had higher parasite density than SA, p<0.05. In the DAT positive cases, parasite density was seen to be higher in the severe forms of malaria namely SA, CM, IVH compared with the UM (Table 4.1). There were 19 deaths recorded for the 484 malaria patients representing a mortality rate of 3.9%. Of these 19 deaths, 15 (78.9%) were DAT negative as compared to 4 (21.1%) DAT positive on admission (Table 4.3).

There was no positive correlation between parasite density and DAT positivity as shown in Fig. 4.2. Patients who had relatively lower parasite density (1-100 parasites $x10^3/\mu L$) seemed to have high agglutination grades (+2, +3, +4). Another interesting observation made was that, about 90% of all the children below the age of 5 years were DAT positive ($p<0.001$), Fig. 4.3 and this age group was also found to have higher agglutination grade +2, +3 and +4. The results also showed that most of the DAT positive cases were either SA or IVH patients who also had higher agglutination grades (+3 and +4) Fig. 4.4.

4.3.1 Frequency of DAT and clinical characteristics in the followed up subjects

Sixteen patients were successfully followed up over a period of 90 days. There was a gradual reduction in the number of DAT positive cases during the period of follow-up (Fig. 4.5). On day 0, 9 of the patients were DAT positive cases and 10 on day 3. However, on days 7, 30 and 90 there were 7, 8 and 2 DAT positive cases, respectively (data not shown). The mean Hb of the DAT positive cases was lower than the DAT negative cases until day 90 when the mean Hb for DAT positive cases became higher, and was almost even with the mean Hb of the DAT negative at day 30. However, there was a steady increase in the mean Hb for both DAT positive and negative cases (from day 3 to day 90). Though there was an increase in the parasite density after day 7 (i.e. days 30 and 90), it was not statistically significant when compared with days 0, 2 and 7, $p>0.05$ (data not shown).

4.4 Flow Cytometry

Flow cytometric histograms and dot plots show the binding of C3d to infected and uninfected erythrocytes (Fig. 4.6). Panels 'a' and 'b' show populations of RBCs

(each represented by a red dot) in four quadrants, lower left (LL), lower right (LR), upper left (UL) and upper right (UR) populations. These quadrants are shown on two axes, the ethidium bromide intensity on the y-axis, showing the infected RBCs, and on the x-axis expression of a marker e.g. C3d on the RBCs surface. The LL quadrant shows population of uninfected RBCs (low mean fluorescence intensity (MFI) for ethidium bromide), which express no marker for C3d (low MFI for C3d) on their surface. The LR on the other hand shows uninfected RBCs with C3d expressed on their surfaces (high MFI for C3d). Infected RBCs that do not express C3d are shown by the population of RBCs in the UL. The population of RBCs in the UR shows infected RBCs with the expression of C3d on their surface. Thus Figs. 4.6a and 4.6b represent dot plots for DAT negative and DAT positive samples respectively whilst Figs. 4.6c and 4.6d represent the histogram plots for the same DAT negative and DAT positive samples respectively.

4.4.1 Results of flow cytometry for IgG, IgM, C3d, C3α/β, CD35, CD47, CD55 and CD59

There were high levels of IgG binding to RBCs of UM and CC compared to SA, IVH and CM cases (Fig. 4.7). Similarly, relatively high levels of CD59 were found in UM and CM as compared to SA, IVH and CC. Also higher levels of C3d were detected on the surface of RBCs in the SA, IVH and CC. The levels of CD47 were significantly higher in the SA than UM and CC ($p<0.05$). However, the levels of CD35, CD55, C3α/β and IgM were very low in the patient categories and CC (Fig. 4.8), and differences between categories were found not to be statistically significant ($p>0.05$). Fig. 4.9 shows that high IgG and C3d levels were detected in the DAT positives, whilst higher CD59 levels were found in the DAT negative samples.

Table 4.1: Clinical characteristics of patients and healthy controls (CC)

Characteristics	Subject Categories					p value
	SA	IVH	UM	CM	CC	
n	52	31	25	35	19	-
M: F	1:1	2:1	1:1	1.5:1	1:1	-
(DAT Positive)	25.0* (48.1%)[a]	23.0* (74.2%)[a]	7.0 (28.0%)[a]	15.0 (42.8%)[a]	5.0 (26.3%)[a]	<0.05
Mean Age (yrs.)	2.8** (2.1-3.4)°	3.8 (2.9-4.7)°	4.7 (3.4-6.1)°	4.1 (3.3-4.8)°	5.7 (4.4-7.1)°	<0.001
Mean Hb (g/dL)	4.0 (3.8-4.2)°	6.3 (5.4-7.1)°	8.9 (8.3-9.6)°	7.1 (6.3-7.9)°	10.9 (10.6-11.4)°	-
GMPD (parasites x$10^3/\mu$L)	95.0*** (63.2-126.8)°	115.8 (25.5-206.1)°	92.2*** (41.7-142.8)°	173.1 (118.6-227.6)°	1.4 (-0.2-2.9)°	<0.001

[a] Values in brackets are percentages
° Values in brackets are 95% confidence intervals
*Significantly higher than UM, CM and CC (however, there was no significant difference between SA and CM).
** Significantly lower than CM, UM and CC.
*** Significantly lower than IVH and CM.
GMPD= Geometric mean parasite density; n = number; M=male; F=female.
The IVH group does not overlap with the other patient categories.

Table 4.2: Proportion of IgG and/or C3d DAT positive subjects

Total number of patients	**484.0**
DAT positives	**131.0 (27.0%)**
C3d + IgG DAT positive	**13.0 (9.9%)**
C3d DAT positive only	**115.0* (87.8%)**
IgG DAT positive only	**3.0 (2.2%)**

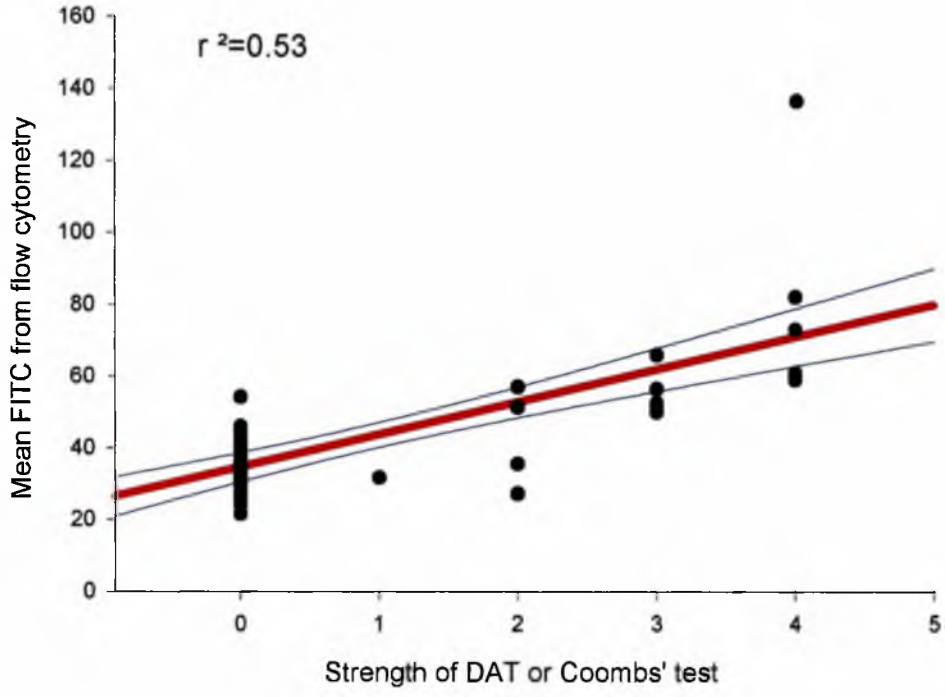
*Significantly higher than C3d + IgG DAT positive and IgG DAT positive only ($p<0.05$).

Table 4.3: Clinical characteristics of patients who were DAT positive and DAT negative

Characteristics	DAT positives	DAT negatives	p value
n (percentage)	131 (27.0%)	353 (73.0%)	-
Mean age (years)	2.9*(2.4-3.3)	3.7*(3.4-4.1)	0.030
Mean Hb (g/dL)	5.7*(5.3-6.1)	7.2*(6.8-7.4)	<0.001
GMPD (parasites x10^3/μL)	55.2*(37.4-73.1)	78.4*(63.7-93.2)	0.109
Haemolysis	23 (70.0%)	8 (30.0)%	<0.05
Mortality	4	15	-
Spleen size (cm)	3.0*(2.6-3.5)	2.9*(2.7-3.1)	0.548

*Values in brackets are 95% confidence intervals

Figure 4.1: DAT results compared with flow cytometry data for C3d



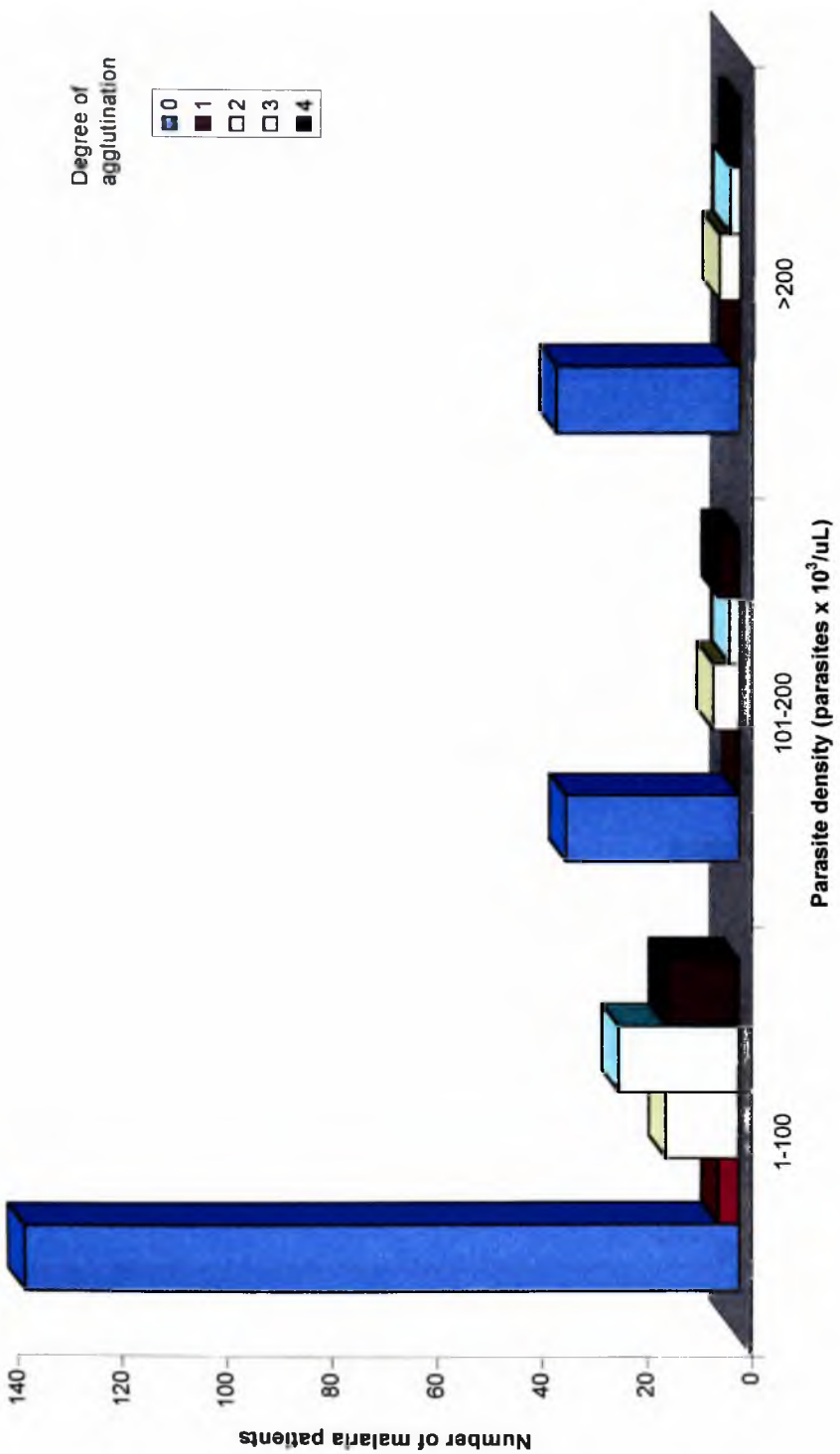


Figure 4.2: Effect of parasite density on the degree of agglutination

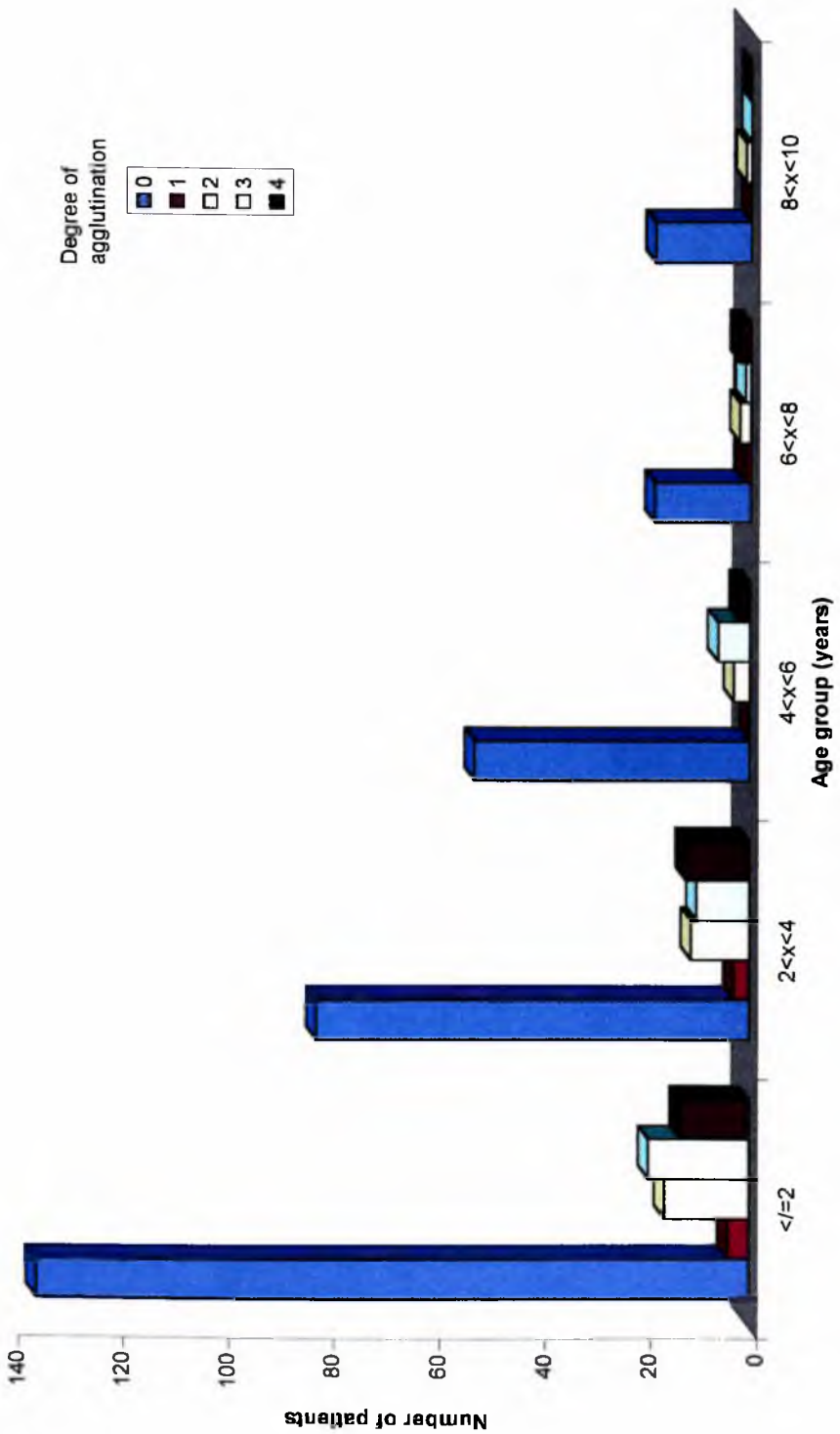


Figure 4.3: Degree of agglutination in the various age groups

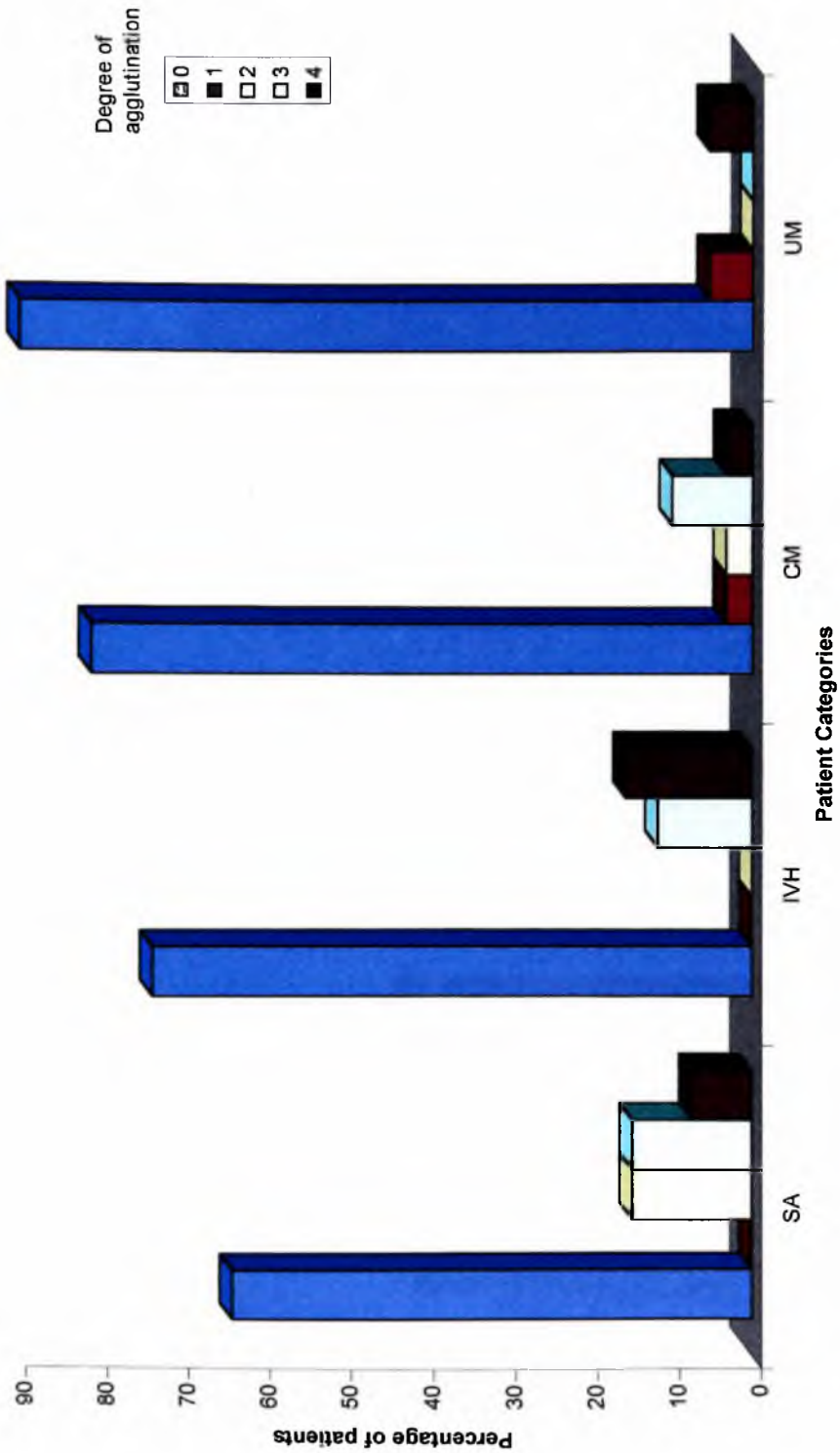


Figure 4.4: Degree of agglutination in the patient categories

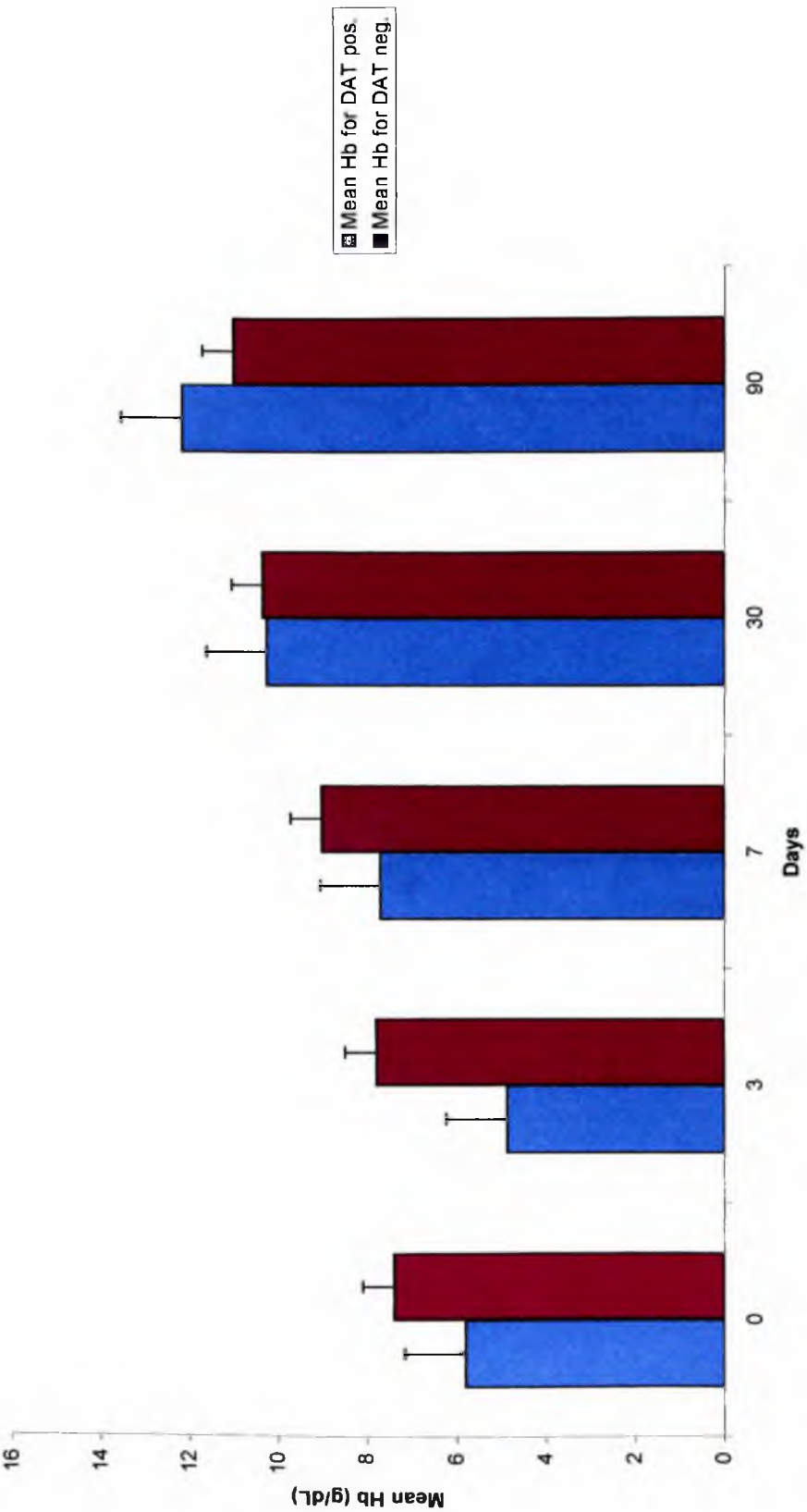


Figure 4.5: Mean Hb in the DAT positives and negatves in the followed up malaria patients

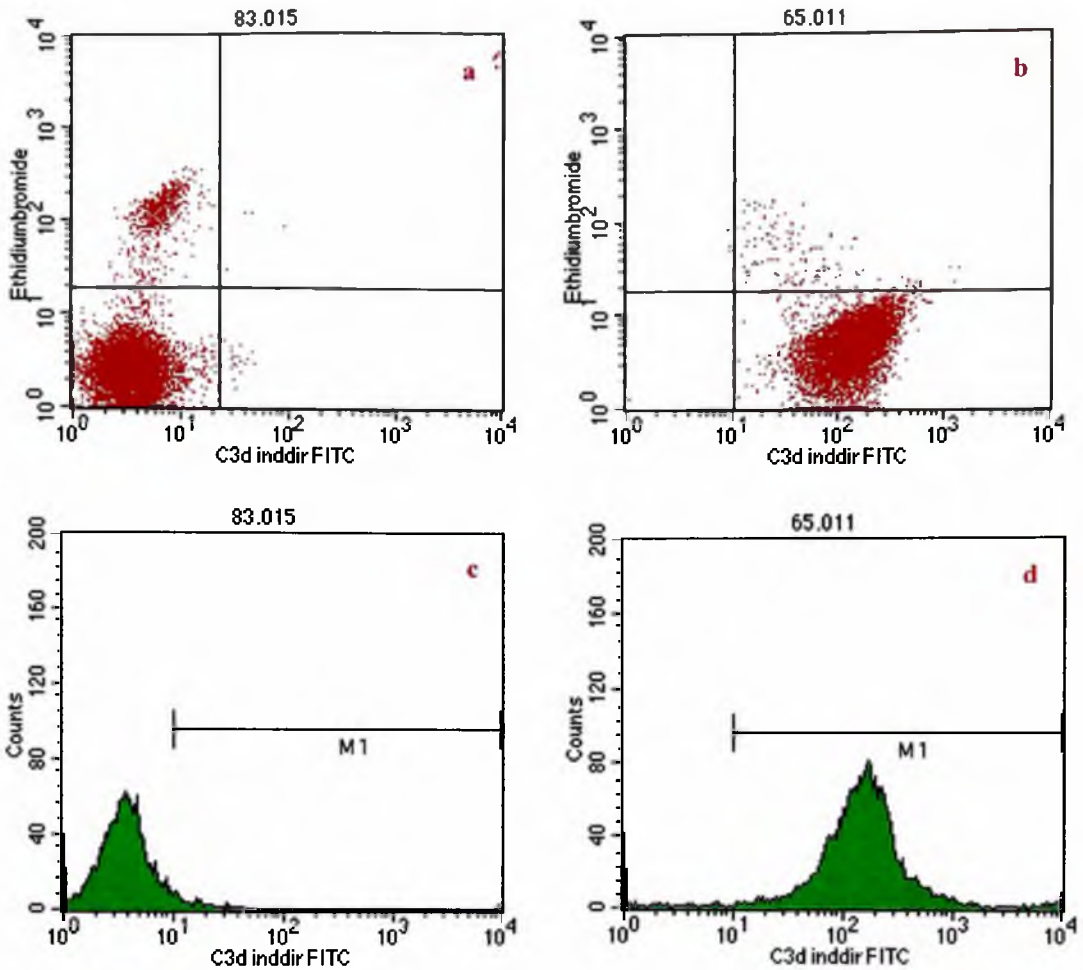


Fig 4.6: Flow cytometric histograms and dot plots showing C3d binding to infected and uninfected erythrocytes.
Infected RBCs are detected according to ethidium bromide flourescence in the upper left and right quadrant in (a) and (b).

a: Population of RBCs (infected and uninfected) which do not express the C3d marker on their surfaces.
b: Population of RBCs (infected and uninfected) which expresses the C3d marker on their surfaces.
c: Histogram plot of the C3d negative RBCs.
d: Histogram plot of the C3d positive RBCs.

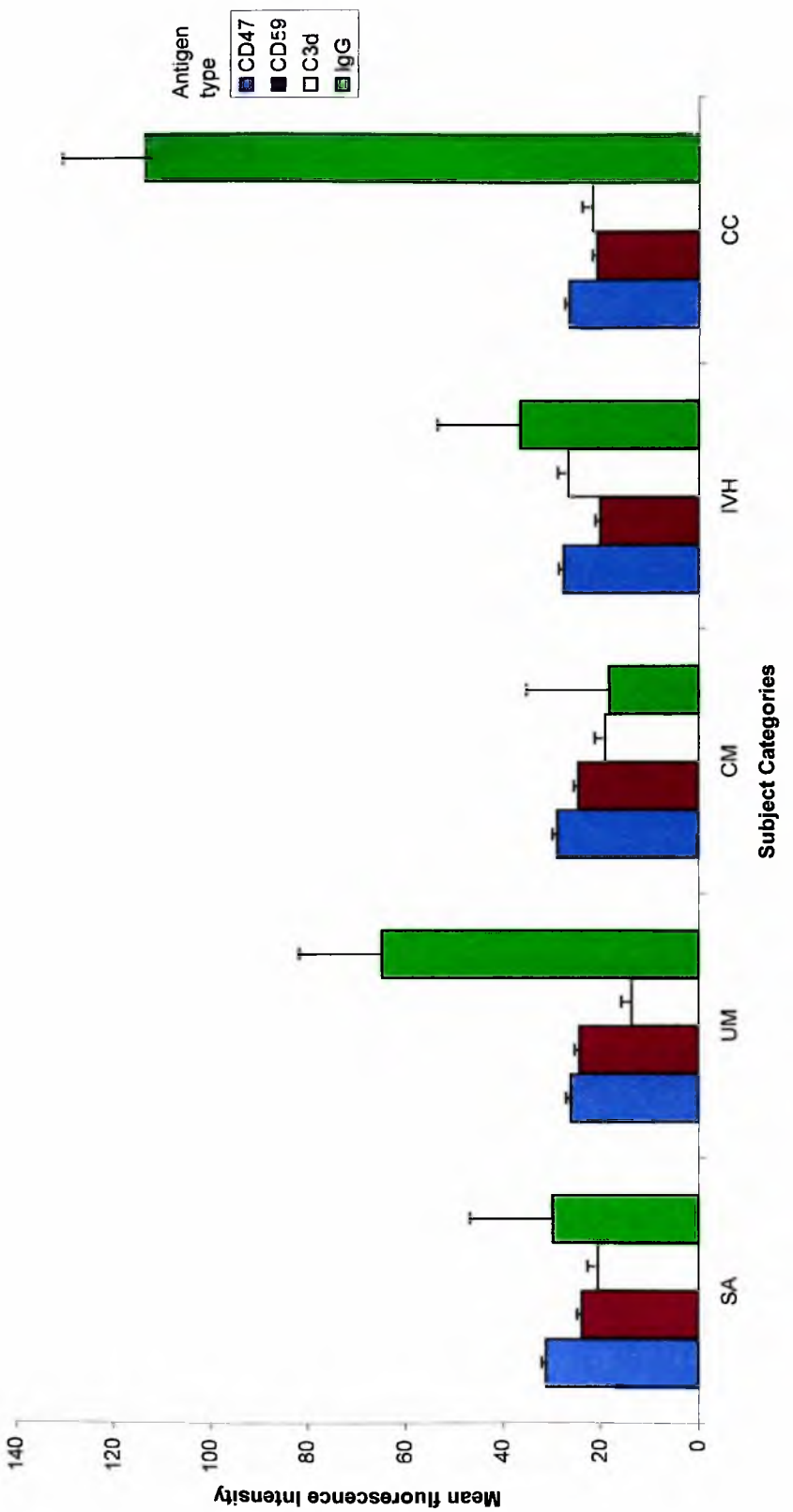


Figure 4.7: Mean fluorescence intensity of CD47, CD59, C3d and IgG expressed on the surface of RBCs in the subject categories

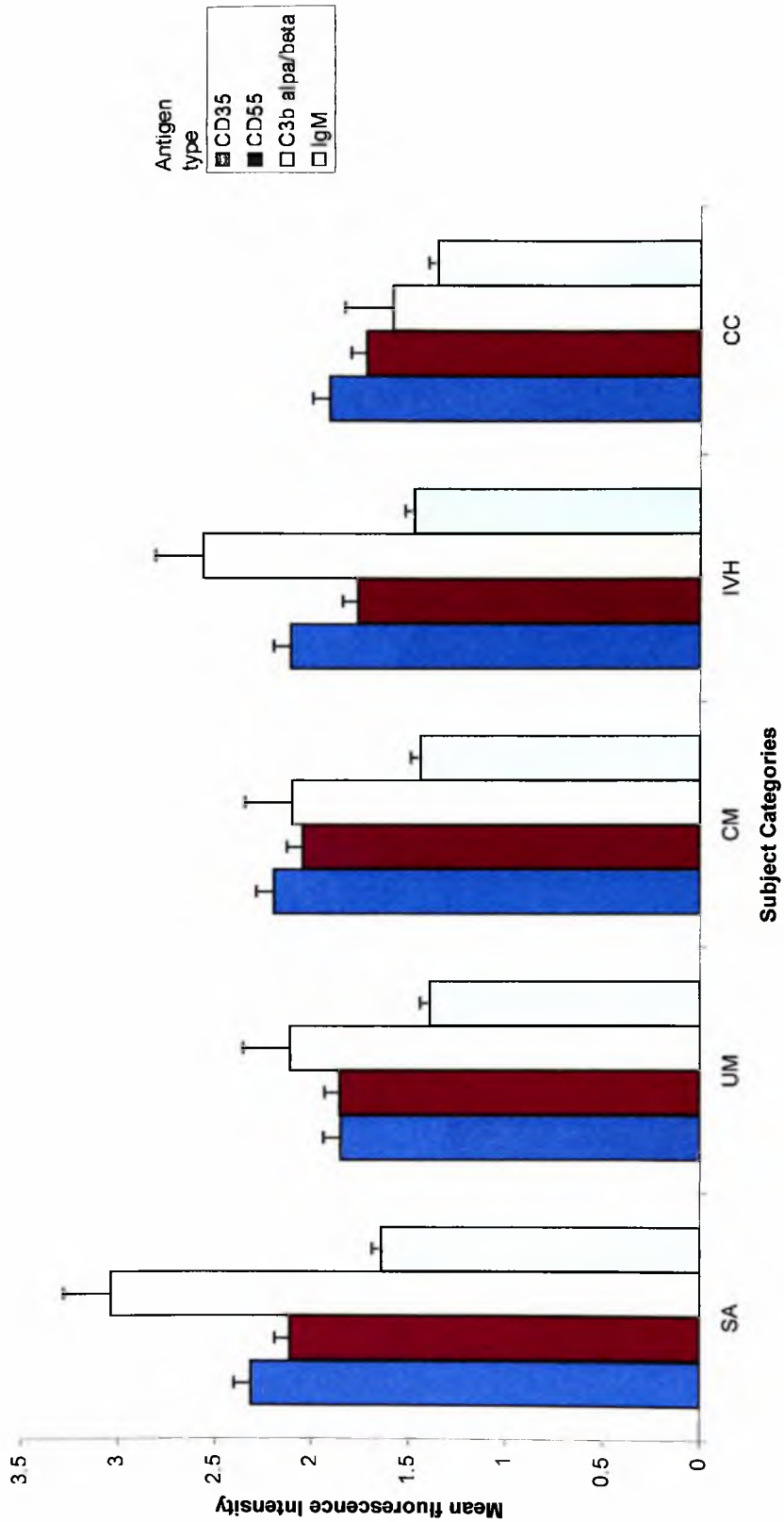
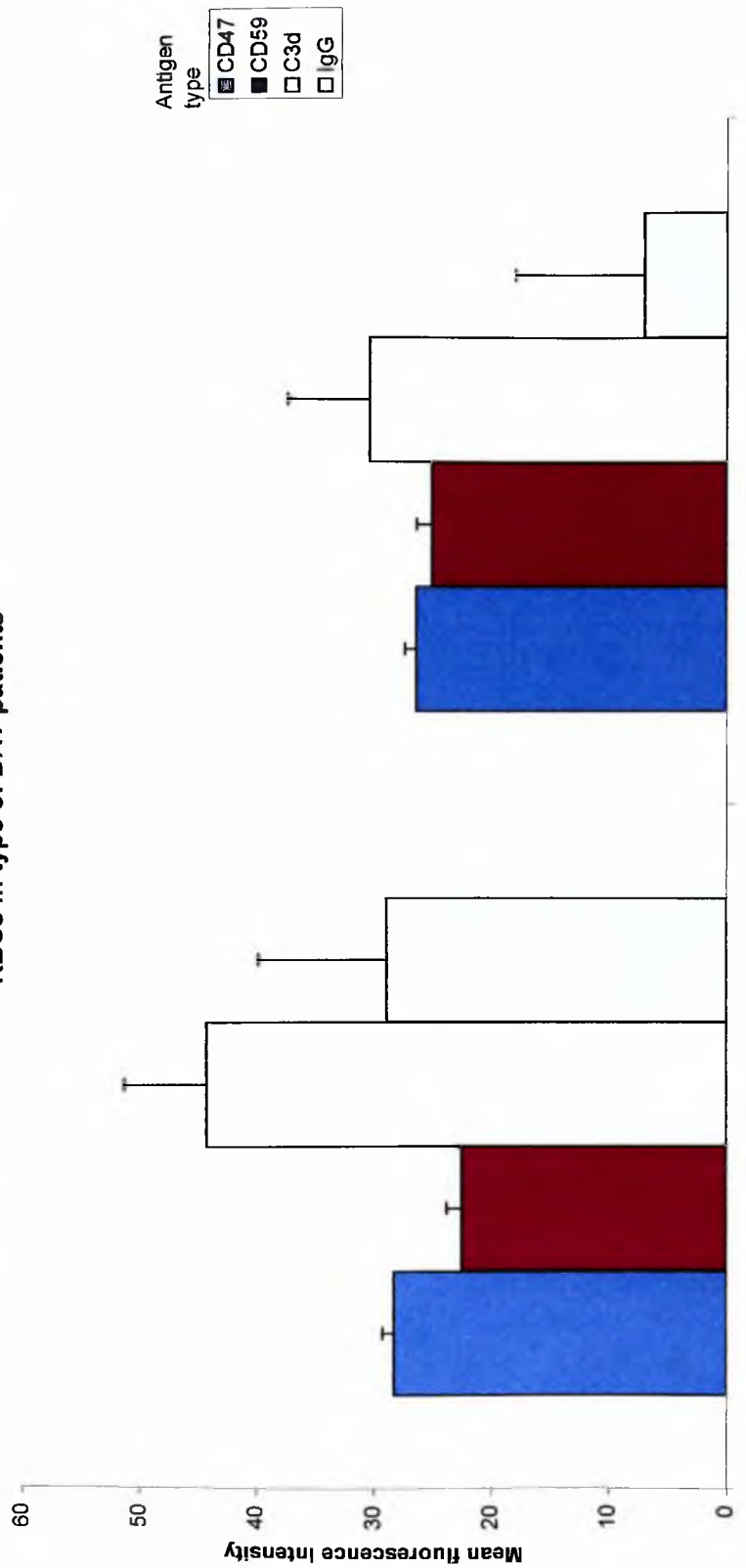


Figure 4.8: Mean fluorescence intensity of CD35, CD55, C3alpha/beta and IgM expressed on the surface of RBCs in the subject categories



Figuer 4.9: Mean fluorescence intensity of CD47, CD59, C3d and IgG expressed on the surface of RBCs in type of DAT patients

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.0 DISCUSSION AND CONCLUSIONS

5.1 Discussion

Results from this study indicate that infection with *P. falciparum* parasites stimulates complement activation. These findings are consistent with those of other investigators (Facer *et al.*, 1979; Wenisch *et al.*, 1997; Wiesner *et al.*, 1997). Of the 484 patients 118 had severe malaria (SA, CM, IVH) compared to 25 who had uncomplicated malaria (UM), Table 4.1. This underlines the fact that severe malaria is an important cause of morbidity in children in Ghana. A higher proportion of the DAT positive cases were found in the SA and IVH patients. The haemolysis in the IVH is critical as the kidney will be burdened with the removal of the haemolysed RBCs and may lead to kidney toxicity. Thus measures must be taken to manage this condition, which may lead to death in children with haemolysis.

The traditional DAT assay (Coombs, 1945) is highly sensitive, easy to perform and relatively inexpensive, and was used to detect the binding of C3d and IgG on the RBCs surfaces in this study. However, some researchers have reservations about this assay due to its subjectivity. This study, however, affirms that the DAT assay is sensitive and can be relied upon as it correlated significantly (53.0%, $p<0.0001$) with the highly specific flow cytometry method. Thus, in situations where flow cytometry cannot be used, the DAT assay may prove to be a viable alternative with dependable results. The DAT assay will therefore help the clinician to predict such conditions as haemolysis of RBCs in children with malaria thereby enabling immediate medical attention to be given to such children in order to save their lives.

It is also interesting to note that most of the DAT positive cases in this study were recorded in the younger age groups, indicating that in younger children, complement activation plays a significant role in protection against malaria. Children of age 5 years and below have been shown to have poor immunity to *P falciparum* infection due to the fact that their immune system is not well developed. These children rely mostly on their innate immunity as opposed to older children and adults whose immune system is more developed (Abdalla and Weatherall, 1982). Thus, one way by which this group of children fight most infections including *P. falciparum* infection is by the use of complement and later antibodies, which require several days to be synthesized. However, it seems that on activating the complement pathway, RBCs are coated with C3d and/or IgG and are lysed rapidly. This situation is reflected by the occurrence of high agglutination grade mostly +2, +3 and +4 in this age group, (Fig. 4.3). This can be deleterious and may eventually lead to death in most cases. Though some deaths were recorded in the course of this study, it cannot be clearly stated whether the degree of agglutination or blood transfusion had any direct influence in that regard. Blood transfusion has been of help in saving the life of such individuals (Lackritz *et al.*, 1992).

It was observed from the results obtained that the control group had lower parasite density and did not develop any clinical signs of malaria. Thus development of clinical symptoms could be attributed to higher parasite density. However, in the development of malaria anaemia, factors other than the degree of parasitaemia alone are responsible, thus implicating immune-mediated mechanisms in the destruction of both infected and uninfected RBCs. In this case, low DAT positivity cases were observed in the high parasitaemia group as compared to the low parasitaemia group,

which rather had high DAT positivity cases. Thus, confirming the low contribution played by the degree of parasitaemia in the development of anaemia through destruction of RBCs as a result of parasite multiplication or schizogony (see Fig. 2.0). Thus, the level of parasitaemia alone cannot explain the degree of anaemia (Woodruff *et al.*, 1973).

The DAT positivity was found to be mostly due to C3d alone, with a small proportion being C3d together with IgG or IgG alone. Similar findings were reported by Facer *et al.* (1979) and Abdalla and Weatherall (1982). This observation may be due to the fact that C3d fragments cover up IgG molecules attached to the parasite antigens, thus forming immune complexes which are associated with severe malaria (Giha *et al.*, 2000). Also the low levels of IgG observed in this work could be attributed to the type of DAT assay (tube technique) used. Abdallah and Weatherall (1982), Facer *et al.* (1979) and Facer (1980) used the microtitre plate whilst Abdallah (1986) and Merry *et al.* (1982) used the ^{125}I-labelled anti-IgG, which allowed detection of IgG present on the red cell surface. With this latter method, Merry *et al.* (1982) were able to find a correlation between the number of IgG molecules on the RBC surface (from 100 to 500 molecules per cell) and the degree of agglutination (rising from 1+ to complete agglutination).

The high degree of agglutination in association with the high DAT positivity cases in children with low haemoglobin levels showed a significant negative correlation. Thus higher degrees of agglutination cause more RBCs to be lysed rapidly or opsonized for erythrophagocytosis by monocytes and neutrophils, leading to an inevitable drop in haemoglobin level. It is therefore not surprising that the DAT

positive cases were found to have lower haemoglobin as compared to the DAT negative cases regardless of patient categories. Though there was no significant difference between the spleen sizes of the DAT negative and DAT positive cases, previous studies have shown that the spleen size in the DAT positive cases was significantly bigger than the DAT negative (Goka *et al.*, in press). There is no apparent reason to explain the difference in the two results.

Results from the experiments conducted on the cases followed up showed a gradual decrease in the number of DAT positive cases while the mean haemoglobin values increased (Fig. 4.5), which may be due to recovery of haematopoiesis restoring RBCs level as parasites are cleared by the administration of chloroquine. In addition, fewer RBCs are coated with the complement fragment, C3d and eliminated by lysis or phagocytosis. This trend suggests the role of complement in the elimination of RBCs. The parasitaemia also dropped on days 2 and 7, then increased on day 30 and 90, which may be due to reinfection but none showed any clinical symptoms (data not shown).

Most tests done to ascertain the role of complement in *P. falciparum* infection have been done in isolation (i.e. the complement fragments were not studied with the regulatory proteins). Some workers have studied complement fragments like C3d and C4b on RBC surfaces (Woodruff *et al.*, 1973; Facer *et al.*, 1979; Facer, 1980; Abdalla and Weatherall, 1982; Merry *et al.*, 1982) while others have studied the levels of complement in serum (Greenwood and Brueton, 1974) without considering the levels of complement regulatory proteins. On the other hand, Waitumbi *et al.* (2000) were able to use flow cytometry to study the complement fragment C3d in

close association with the complement regulatory proteins. In this study the DAT assay was used alongside flow cytometry to determine the relationship between the levels of complement fragments and regulatory proteins. With this approach, it was observed that relatively high levels of C3d and low levels of CD59 were found in the DAT positive cases than in DAT negative cases (Fig. 4.9). This may indicate that some amount of CD59 has been consumed due to complement activation in the DAT positive cases causing direct lysis of the RBCs resulting in low haemoglobin values observed in these cases. CD59 is used up to reduce complement activity (Wiesner *et al.*, 1997). The relatively high level of IgG (from the flow cytometry) in the DAT positive cases suggests that complement activation was occurring via the classical pathway as this pathway is activated solely by the antigen-antibody interaction (Wolfgang *et al.*, 1999). The specificity of the IgG involved was, however, not determined.

Direct lyses of the RBCs and/or opsonization for erythrophagocytosis are some of the consequences of complement activation. Thus depending on the level of CD59 and C3d and/or C3b fragments on surfaces of RBCs, the degree of lysis or erythrophagocytosis can be deduced. The high levels of CD59 observed in all the patient categories (Fig. 4.7) may be explained by the fact that most of the RBCs must have been eliminated by phagocytosis after being opsonized with C3d and to a smaller degree by direct lysis. This is because CD59 inhibits the membrane attack complex at the terminal stage of the complement pathway where direct lysis occurs. The high mean CD59 level in the SA cases was unexpectedly high. This could be explained by the fact that the RBCs with low CD59 might have been eliminated whereas those with higher levels of CD59 resisted lysis (Waitumbi *et al.*, 2000). The

high expression of CD47, an immunological self marker (irrespective of patient category) on the surfaces of most cells including RBCs, also suggests that autoimmunity may not be involved in the mechanism of malaria anaemia (Rosenberg *et al.*, 1973).

Complement receptor type 1, CR1, functions by regulating the activity of the C3b convertase along the classical pathway. CR1 also initiates the opsonization of the RBCs (or any pathogen) for erythrophagocytosis by monocytes and neutrophils. The expression of CR1 on the RBC surface can lead to rosetting, a condition where infected RBCs expressing CR1 form rosettes with uninfected RBCs (Rowe *et al.*, 1997) resulting in the elimination of these rosettes, leading to low haemoglobin. CD 55 (DAF) on other hand inhibits C3b convertase activity through the alternative pathway. In this study, it was observed that relatively low levels of CD35 and high levels CD55 were detected (Fig. 4.8), suggesting that activation of complement on the RBCs of subjects was to a larger extent via the classical pathway than alternative pathway. This also goes to confirm the involvement of complement in the elimination of RBCs in *P. falciparum* infected children.

During infection, IgM is produced earlier than IgG, which is rather protective. Subsequently, the level of IgM decreases while IgG increases. Rosenberg *et al.* (1973) detected high levels of IgM both on the RBCs surface and in the serum of malarial patients. Contrary to the above finding, low levels of IgM were detected in all the patient categories in this study, though mean IgG observed in the UM cases were high. This could probably be due to detection of other IgG, which are non-specific to the *P. falciparum* antigens. On the other hand the high mean level of IgG

of the CC could be protective to *P. falciparum* infection due to the fact that some of the CC had a high parasite density but did not show any clinical symptoms.

5.2 Conclusions

It can be concluded that high C3d is associated with low Hb and younger age; there is also evidence of complement-mediated removal or destruction of RBCs as the cause of SA and IVH. Erythrophagocytosis is involved in the removal of RBCs in children with acute malaria and there is no evidence of autoimmunity in the pathogenesis of anaemia in malaria. The flow cytometry results correlated significantly with DAT results for C3d. Further studies are required to determine whether there is a genetic basis for the activation of complement and to identify children at risk before they develop clinical malaria.

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APPENDIX

2% Giemsa stain solution:

2.0ml of the stain was added to 8.0ml of distilled (pH=7.1-7.2) and vortexed to mix thoroughly. Giemsa stain was obtained as an already prepared commercial product.

0.2% sodium metabissulphite:

0.2g of sodium metabissulphite was weighed and dissolved in 100.0ml of distilled water.

0.9% Saline:

9.0g NaCl was weighed and dissolved in 1.0ml distilled water and stored at 4.0°C.

RBC suspension preparation:

After the last washing and the supernatant aspirated, 50.0ul of packed RBC was pipetted and added to 1.0ml of 0.9% saline and vortexed to obtain the 5% RBC suspension.

Table 6.1:Table showing tube number and primary antibody type

Tube	Antibody
1	Mouse IgG_1 FITC (Pharmigen International, 33814X)
2	CD35 (Pharmigen International, 30961A)
3	IgG (Becton Dickinson, 345140{5140})
4	IgM (Becton Dickinson, 345160{5160})
5	Mouse IgG_{2a} FITC (Pharmigen International, 33034X)
6	CD55 (Pharmigen International, 33571A)
7	CD59FITC (Pharmigen International, 33864X)
8	Mouse IgG_1pure (DAKO, X 0931)
9	C3bα+β pure (Cymbus Biotechnology Ltd., CBL 189 for α; CBL 190 for β)
10	Rabbit IgG pure (Zymed Laboratories, INC., 81245172)
11	C3d pure (DAKO, A 0063)
12	C3d FITC (DAKO, F 0323)
13	
14	CD47FITC (Pharmigen International, 36734X)
15	No ehidium bromide, no antibody

Table 6.2: Table showing Tube number and secondary antibody type

Tube	Antibody
1	
2	Goat anti-mouse FITC (DAKO A/S, F0479)
3	Goat anti-mouse FITC (DAKO A/S, F0479)
4	Goat anti-mouse FITC (DAKO A/S, F0479)
5	
6	Goat anti-mouse FITC (DAKO A/S, F0479)
7	
8	Goat anti-mouse FITC (DAKO A/S, F0479)
9	Goat anti-mouse FITC (DAKO A/S, F0479)
10	Swine anti-rabbit FITC (DAKO A/S, F0054)
11	Swine anti-rabbit FITC (DAKO A/S, F0054)
12	
13	
14	
15	