EFFECTS OF *PLASMODIUM FALCIPARUM* INFECTION ON MONOCYTE AND NEUTROPHIL FUNCTION: A BASIS FOR INCREASED DESTRUCTION OF UNPARASITIZED ERYTHROCYTES

A Thesis submitted to the Board of Graduate studies, University of Ghana, Legon, Ghana.

In partial fulfilment of the requirements for the award of the Master of Philosophy degree in Biochemistry

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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 under the supervision of Prof. B. D. Akanmori (Immunology Unit, NMIMR), 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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THIS WORK IS DEDICATED TO MY PARENTS FOR SUPPORTING ME THIS FAR.
ACKNOWLEDGEMENT

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>SECTION</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title page</td>
<td>i</td>
</tr>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>iv</td>
</tr>
<tr>
<td>Table of contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Tables</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>x</td>
</tr>
<tr>
<td>Abstract</td>
<td>xii</td>
</tr>
</tbody>
</table>

## CHAPTER ONE

### INTRODUCTION

1

## CHAPTER TWO

### LITERATURE REVIEW

2.1 The Disease 7

2.2 The parasite 7

2.3 Malaria in Ghana 10

2.4 Clinical manifestations of malaria 11

2.5 Immunity to Malaria 12

2.5.1 Natural Resistance 12

2.5.2 Acquired Immunity 13

2.6 Phagocytosis 19
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Subjects 38
3.2 Inclusion Criteria 38
3.3 Blood Collection 39
3.4 Haematological Analysis 39
3.5 Parasitology 40
3.6 Sickling Test 40
3.7 Phagocytic Function Assay 40
3.8 Surface Marker Staining 42
3.9 Cytokine Assay 43
3.10 Statistical Analysis 44
CHAPTER FOUR
RESULTS

4.1 Summary 46
4.2 Characteristics of Patients 47
4.3 Phagocytic Activity 48
4.4 Monocyte Surface Marker Expression 54
    4.4.1 HLA-DR 54
    4.4.2 CD16 56
4.5 Plasma levels of Cytokines on admission 58
    4.5.1 IL-10 58
    4.5.2 TNF-alpha 59
    4.5.3 IL-8 60
    4.5.4 Neopterin 61
    4.5.5 MIP-1alpha 62
    4.5.6 MIP-1beta 63
    4.5.7 Correlation between cytokine levels 64
    4.5.8 Correlation between cytokines levels and clinical characteristics 65
4.6 Longitudinal Study of Cytokine levels 67

CHAPTER FIVE
DISCUSSION AND CONCLUSIONS

5.1 Discussion 70
5.2 Conclusions 79

REFERENCES 80
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Clinical and laboratory characteristics of study subjects</td>
<td>48</td>
</tr>
<tr>
<td>4.2</td>
<td>Showing p-values of test for differences in expression of HLA-DR between categories</td>
<td>56</td>
</tr>
<tr>
<td>4.3</td>
<td>Showing p-values of test for differences in expression of CD16 between Categories</td>
<td>58</td>
</tr>
<tr>
<td>4.4</td>
<td>Showing Spearman’s Rank Order correlation between pairs of Cytokines</td>
<td>65</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 3.1</td>
<td>Showing distinct cell populations and fluorescence of neutrophils</td>
<td>45</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Showing gated monocytes and their expression of surface markers</td>
<td>46</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Showing increase in phagocytosis with time</td>
<td>50</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Box plot showing the distribution of PhIs for the subjects by Category (2000)</td>
<td>51</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Box plot showing phagocytic indices of patients by category (2001)</td>
<td>52</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Association between Phi and Hb</td>
<td>53</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Longitudinal study of Phagocytic Index</td>
<td>54</td>
</tr>
<tr>
<td>Figure 4.6a</td>
<td>Percentage CD14+ cells expressing HLA-DR</td>
<td>55</td>
</tr>
<tr>
<td>Figure 4.6b</td>
<td>MFI of HLA-DR of CD14+ expressing CD16</td>
<td>56</td>
</tr>
<tr>
<td>Figure 4.7a</td>
<td>Percentage of CD14+ cells expressing CD16</td>
<td>57</td>
</tr>
<tr>
<td>Figure 4.7b</td>
<td>MFI of CD16 for CD14+ cells</td>
<td>58</td>
</tr>
<tr>
<td>Figure 4.8</td>
<td>Plasma levels of IL-10</td>
<td>59</td>
</tr>
<tr>
<td>Figure 4.9</td>
<td>Plasma levels of TNF-α</td>
<td>60</td>
</tr>
<tr>
<td>Figure 4.10</td>
<td>Plasma levels of IL-8</td>
<td>61</td>
</tr>
<tr>
<td>Figure 4.11</td>
<td>Plasma levels of neopterin</td>
<td>62</td>
</tr>
<tr>
<td>Figure 4.12</td>
<td>Plasma levels of MIP-1α</td>
<td>63</td>
</tr>
<tr>
<td>Figure 4.13</td>
<td>Plasma levels of MIP-1β</td>
<td>64</td>
</tr>
<tr>
<td>Figure 4.14a</td>
<td>Graph of Hb versus neopterin (SA)</td>
<td>66</td>
</tr>
<tr>
<td>Figure 4.14b</td>
<td>Graph of Hb versus neopterin (CM)</td>
<td>67</td>
</tr>
<tr>
<td>Figure 4.14c</td>
<td>Graph of Hb versus neopterin (UM)</td>
<td>67</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Complement fragment</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>Cerebral Malaria</td>
<td></td>
</tr>
<tr>
<td>DAT</td>
<td>Direct Antiglobulin Test</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked Immunosorbent Assay</td>
<td></td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
<td></td>
</tr>
<tr>
<td>FL-1</td>
<td>Fluorescence channel 1</td>
<td></td>
</tr>
<tr>
<td>FSC-H</td>
<td>Forward Scatter Height</td>
<td></td>
</tr>
<tr>
<td>G-6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
<td></td>
</tr>
<tr>
<td>HbS</td>
<td>Haemoglobin type S</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
<td></td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
<td></td>
</tr>
<tr>
<td>IVH</td>
<td>Intravascular haemolysis</td>
<td></td>
</tr>
<tr>
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<td>Lipopolysaccharide</td>
<td></td>
</tr>
<tr>
<td>LT</td>
<td>Leucotriene</td>
<td></td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemotactic Protein</td>
<td></td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
<td></td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
<td></td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
<td></td>
</tr>
<tr>
<td>NAP</td>
<td>Neutrophil Activating Protein</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Term</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
<td></td>
</tr>
<tr>
<td>PABA</td>
<td>para-aminobenzoic acid</td>
<td></td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet Activating Factor</td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
<td></td>
</tr>
<tr>
<td>PMNs</td>
<td>Polymorphonuclear cells</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
<td></td>
</tr>
<tr>
<td>Phl</td>
<td>Phagocytic Index</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
<td></td>
</tr>
<tr>
<td>RBCs</td>
<td>Red Blood Cells</td>
<td></td>
</tr>
<tr>
<td>SA</td>
<td>Severe Anaemia</td>
<td></td>
</tr>
<tr>
<td>SSC-H</td>
<td>Side Scatter Height</td>
<td></td>
</tr>
<tr>
<td>TDR</td>
<td>Tropical Disease Research</td>
<td></td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
<td></td>
</tr>
<tr>
<td>UM</td>
<td>Uncomplicated Malaria</td>
<td></td>
</tr>
</tbody>
</table>
Abstract

It has become certain that part of the haemolytic component of the anaemia of *Plasmodium falciparum* malaria results from increased removal of unparasitized red blood cells. There have been several reports of associations between positive Coombs’ DAT and anaemia, and also of observations of unparasitized erythrocytes in monocytes of patients with *P. falciparum* infections. Perturbations in cytokine balance have also been blamed for reduced red cell survival as well as for defective erythropoiesis.

This study sought to ascertain to what extent increase in monocyte and neutrophil function and the general inflammatory response to *P. falciparum* infections contribute to the anaemia of malaria. Neutrophil phagocytic activity, monocytes surface expression of HLA-DR and FcγRIII, and plasma levels of IL-10, TNF-α, IL-8, neopterin, MIP-1α and MIP-1β were compared among groups of children with severe *P. falciparum* malaria anaemia (SA), others with other forms of malaria (cerebral, CM or uncomplicated, UM) and healthy or asymptomatic controls (AC). Phagocytic activity was measured as ability of neutrophils to take up fluorescent latex particles. All groups of children with symptomatic malaria showed higher neutrophil phagocytic activity than the control group (p<0.005). Further, among the children with symptomatic malaria, children in the SA group showed a higher phagocytic activity than the others (SA vs. CM, p=0.029; SA vs. UM, p=0.002).

There was a marked reduction in monocyte expression of HLA-DR in all groups of patients compared to controls (p<0.001), but differences among patient groups were not significant. Plasma levels of all cytokines measured were not significantly different in the SA group compared to other groups of patients (p>0.05), but were markedly elevated in all patient groups compared to controls (p<0.05).
CHAPTER ONE

1.1 INTRODUCTION

Malaria continues to be a global health concern. According to WHO (1992), there are 300 million to 500 million clinical cases of malaria annually. Of these, 1.5 million to 2.7 million result in deaths. Approximately 1 million of those malaria-associated deaths occur in children younger than 5 years.

A protozoan of the genus *Plasmodium* causes the disease. Four species of the genus infect humans, namely *P. vivax, P. ovale, P. malariae* and *P. falciparum*. Practically all the mortality due to malaria worldwide is associated with *P. falciparum*. However, the importance of the other species must not be underestimated, particularly *P. vivax*. Around 90% of all *P. falciparum* related morbidity and mortality falls on children in sub-Saharan Africa. The disease is estimated to take the life of 1 out of every 20 children before the age of 5 years in Tropical Africa (WHO, 1992).

In Ghana, malaria continues to be a major cause of morbidity and mortality affecting both the young and the old. It accounts for 8% of certified deaths and ranks as the commonest cause of death in the 0-4 year age group. It is the most commonly reported disease in the outpatient units of the country’s health institutions, accounting for about 42% of all attendances (Ahmed, 1989). Findings by Koram et al. (1997) suggested that children less than 10 years account for almost 50% of the total number of malaria patients, with the intensity of infection being highest among children less than 5 years old.
Malaria control is limited to methods including use of insecticide-impregnated bed-nets and insecticide spraying to eliminate the mosquito vector, prompt treatment of patients and use of chemoprophylaxis to protect those at risk, for example pregnant women and travelers.

Malaria parasites are developing resistance against the available antimalarial drugs at an alarming rate, making it increasingly important to understand the protective immune response to malaria so that new treatment strategies, vaccines and interventions can be developed.

Infections with *Plasmodium falciparum* can cause a wide spectrum of illness ranging from apparently symptomless infections, through acute febrile episodes to life-threatening conditions such as cerebral malaria and severe anaemia. Malarial anaemia can have a diverse clinical presentation. Abdalla *et al.* (1980) identified two common clinical scenarios: a chronic smouldering anaemia and a more acute presentation with a precipitous drop in haematocrit accompanied by bilirubinaemia.

Anaemia is a major factor in child deaths due to *P. falciparum* infections in endemic areas (Weatheral *et al.*, 1983). However, despite a great deal of work, the pathogenesis of this anaemia remains uncertain. Three mechanisms have been proposed to explain the anaemia.

i. Destruction of parasitized red cells

ii. Defective erythropoiesis

iii. Increased destruction of uninfected red blood cells
There is considerable evidence from both animal and human studies that direct damage to the red cells by invasion and growth of parasites is the major mechanism for premature red cell destruction in all acute forms of malaria (Seed and Kreier, 1980). Defective red-cell production results from either depression of erythropoiesis, inhibition of reticulocyte release or ineffective erythropoiesis (Weatherall and Abdalla, 1982). Kurtzhals et al. (1997) suggested that *P. falciparum* infection caused a rapidly reversible suppression of the bone marrow response to erythropoietin.

Following suggestions made by Zuckerman (1966), there has been considerable interest in the possibility that at least part of the haemolytic component of the anaemia of *P. falciparum* malaria results from immune destruction of red cells. Reports of findings of a positive Coomb’s direct anti-immunoglobulin test (DAT) in patients with malaria has been the main stimulus. Facer et al. (1979) found patients who were severely anaemic and who showed a positive DAT with active C3 components on their red cells. They were able to demonstrate monocyte erythrophagocytosis of non-parasitized cells on the blood films of these patients. They suggested therefore, that anaemia in acute malaria might sometimes be the result of opsonization and phagocytosis of sensitized cells. Also Kreier and Green (1980) found increased destruction of uninfected erythrocytes to result from increased phagocytosis of erythrocytes due to the erythrocyte membrane being damaged by either parasite or host proteolytic enzymes activated through antigen-antibody and complement reactions. This theory is supported by the observation of non-parasitized erythrocytes in bone marrow monocytes and macrophages during and following infection (Phillips et al., 1986).
Recently Goka et al. (2001) showed a strong association between C3d binding to the erythrocyte membrane and the severity of anaemia in children with falciparum malaria in Ghana. They found evidence of a role for complement activation and erythrophagocytosis in the pathogenesis of anaemia in this type of malaria. There was therefore the need to identify the cells involved in the elimination of these erythrocytes and the mechanism involved.

The present study thus sought to test the hypothesis that increased destruction of uninfected red blood cells that contributes to the anaemia in P falciparum infection results from increased monocyte and neutrophil function.

Monocytes and neutrophils are both types of leukocytes that are derived from the bone marrow and together are called professional phagocytes because their major function is the phagocytosis of invading pathogens and damaged autologous cells. Monocytes are mononuclear phagocytes that circulate for a few hours and develop into tissue macrophages. Neutrophils on the other hand are polymorphonuclear (PMN) with an array of granules, and are short-lived.

Human mononuclear phagocytes bear a group of cell surface receptors that bind the Fc domain of IgG. These Fc gamma receptors are utilized in the phagocytosis of opsonized cells and immune complexes and are involved in the pathogenesis of several haematologic and immunologic disorders. There are three such Fc receptors namely, I (CD64), II (CD32), and III (CD16). Of these, CD16 has been shown to play the most critical role in regulating opsonic phagocytosis. Work by Liao and Simon (1994) and
Liao et al. (1994) have shown that mechanisms regulating opsonic phagocytosis target the Fc gamma receptor III (FcγRIII). Also CD16 positive monocytes show enhanced phagocytic ability compared to CD16 negative ones.

In addition to phagocytosis, monocytes also play an important function of presenting antigens to T-cells. This is by processing small antigenic fragments of invading organisms and placing them on the host cell surface in combination with major histocompatibility complex (MHC) molecules for subsequent recognition and binding by T-cells. Monocytes thus express MHC class II molecules constitutively, but the level of class II expression is increased when the monocytes are activated.

Following an infection, there is an inflammatory response involving mobilization and activation of phagocytes, and also secretion of proinflammatory cytokines by activated T-cells and phagocytes. Plasma levels of such cytokines can serve as useful markers of phagocyte stimulation. Neopterin is one of such markers. It is a pyrazino-pyrimidino derivative, and is produced and released in excess by activated macrophage-like cells. Also, activated macrophages express a form of nitric oxide synthase, which generates nitric oxide. Tumour necrosis factor (TNF) is another cytokine released by activated monocytes and has been found to stimulate erythrophagocytosis, whilst IL-10 has been shown to be a TNF antagonist. Mobilization of monocytes and neutrophils to sites of inflammation is elicited by a special group of chemotactic cytokines called chemokines. Notably, the chemokines macrophage inflammatory protein-1α (MIP-1α) and MIP-1β are specific for monocytes, whilst IL-8 is selective for neutrophils. Plasma levels of some of these chemokines would give an indication of the extent of inflammation and phagocyte function.
The overall aim of this study was to compare monocyte and neutrophil function in children with malaria anaemia and a reference group of children without the condition so as to ascertain whether the anaemia of *P. falciparum* is associated with an increased monocyte and neutrophil activity or not. The reference group consisted of children with malaria without severe anaemia (cerebral or uncomplicated malaria), and also others with asymptomatic infections or no parasitaemia.

The specific objectives therefore, were to:

1. adapt a flow cytometric assay for determination of neutrophils phagocytic function.
2. compare phagocytic capacity of neutrophils of children with anaemia of *P. falciparum* malaria to that of children without the condition,
3. compare expression of the activation marker HLA-DR (MHC II molecule) and the phagocytic cells-specific marker FcγRIII (CD16) on surfaces of peripheral blood monocytes in these children,
4. compare levels of plasma markers of inflammation and macrophage activation namely, neopterin, TNF-α, IL-10, and the chemokines MIP-1α, MIP-1β and IL-8 in these children, and
5. study the nature of changes in phagocytic activity and plasma levels of cytokines TNF-α, IL-10, neopterin, MIP-1β and IL-8 during seven days after start of malarial treatment.
CHAPTER TWO
LITERATURE REVIEW

2.1 The Disease

Malaria is a febrile illness caused by a protozoan of the class sporozoan, genus *Plasmodium*. The disease has been in existence for centuries. It was initially referred to simply as “fever” and in Africa it was strongly linked to superstition. The discovery of the use of “Peruvian bark” for the treatment of certain fevers but not all fevers marked an important milestone as it led to a better understanding of the disease. The disease was later given the name ‘malaria’ by the Italians, relating the association of the disease with marshy areas.

2.2 The parasite

Over 120 species of the genus *Plasmodium* cause malaria in mammals, birds and reptiles. Of these 22 are found in the primates and four occur naturally in humans, namely: *P. falciparum, P. vivax, P. ovale, P. malariae*.

*Vivax* malaria covers the widest geographic area, including temperate, tropical and subtropical zones. It is however absent in large areas of tropical Africa. *Malariae* malaria is widely distributed, but it is not as common as *vivax* malaria (TDR, 1987).

*Falciparum* malaria is the most lethal and most widespread, occurring throughout the tropics and sub-tropics. A report by Walliker (1982) showed that *falciparum* malaria accounted for up to 80% of all malaria cases worldwide.
LIFE CYCLE

The discovery by Ronald Ross of some of the developmental stages of the parasite in a mosquito (female anopheles) that had previously fed on an Indian patient with *Plasmodium* in his blood, triggered intensive research that led to a better understanding of the mode of transmission and life cycle of the malaria parasite.

The life cycle of the malaria parasite is very complex involving several developmental stages in the vertebrate host and the vector (female anopheles mosquito).

Garnham (1966) summed the life cycle of the parasite to include one cycle of asexual division in the vertebrate liver, another cycle of pigment-producing asexual division in the red blood cells of the vertebrate host, and a sporogenic development in the body of the mosquito. Human malaria is transmitted solely by female mosquitoes of the genus anopheles, of which there are about 60 important vectors in nature.

The cycle begins with the bite by an infected mosquito inoculating the parasites in the form of sporozoites into the bloodstream of the human host. After circulating in the bloodstream for not more than 1 hour, the sporozoites enter liver cells (hepatocytes).

In the hepatocytes, *P. falciparum* parasites grow, multiply and develop directly into schizonts referred to as pre-erythrocytic (PE) schizonts. PE schizonts of *P. falciparum* take 5-7 days to develop. When matured the schizont and liver cell rupture and the parasites known as merozoites enter the bloodstream. To survive, the merozoites must invade red cells within a few minutes of being released from the schizonts.

Entry of the parasites into the red cells starts a cycle in the blood (erythrocytic cycle), which for *P. falciparum* takes 36-48 hours to complete. The merozoite then develops
into a trophozoite within a vacuole formed by the internal membrane of the host red cell. The trophozoite feeds on haemoglobin by ingesting small amounts of red cell cytoplasm, which leads to formation of malaria pigment (hemozoin) as an end product of haemoglobin breakdown. Hemozoin accumulates as a dark-brown granule in the trophozoite.

When the trophozoite is fully developed it divides to form a schizont containing 8-32 merozoites and malaria pigment. Mature schizonts rupture from the red cells 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’. Those merozoites that are not destroyed by the host’s immune system invade new red cells and the cycle is repeated destroying red cells further.

Occasionally, some of the trophozoites develop into male and female gametocytes. These sexual stages of the parasite cannot divide in humans but are found in the peripheral circulation to be taken up by the mosquito in its blood meal. In the midgut of the mosquito, the gametocytes differentiate into gametes, fertilization takes place to form a zygote, which also transforms into a motile ookinete, which penetrates the gut epithelium and matures to form the oocyst. Inside the oocyst, large numbers of sporozoites are formed. The mature sporozoites leave the oocytes and some find their way into the salivary gland of the mosquito, ready to be transmitted when the insect next takes a blood meal.
2.3 Malaria in Ghana

The history of malaria studies in Ghana can be traced to about a century ago when Connal (1912) showed that in Accra 50% of children below the age of 3 years were infected with malaria parasites. Several years on, studies by Ahmed (1989) showed that the hyperendemicity of malaria in Ghana followed an uneven distribution along distinct climatic and ecological zones. The middle forest ecological zone (Ashanti, Brong Ahafo, Eastern and greater parts of Western regions) had the highest occurrence of clinical disease, followed by the coastal zone (Central, Greater Accra, and parts of Western regions), and the Northern savanna area had the lowest. Malaria occurrence in Ghana is also affected by seasonal variations, being higher during the rainy season when mosquito breeding is favoured than in the dry season when there are fewer breeding grounds for mosquitoes.

According to Ahmed (1989), the disease is predominantly found in children below 3 years of age and the commonest malaria parasite involved is *P. falciparum*, and to a lesser extent *P. vivax* whilst *P. ovale* rarely occurs. This was consistent with reports by Coulboune and Wright (1955) of *P. falciparum* infection rates of between 90-98% from various parts of the country. Also Morrow (1981) found *P. falciparum* malaria as the cause of many of the serious complications of malaria in Ghana. Recently, Koram et al. (1997) also reported from a study conducted in southern Ghana that *P. falciparum* accounted for over 90% of asymptomatic, and more than 95% of clinical cases respectively.
2.4 Clinical Manifestations of Malaria.

The clinical features of *P. falciparum* infection cover a spectrum from asymptomatic infection to fulminate disease leading to death. Important determinants of the clinical pattern are species of parasite, age, immune status and degree of malaria endemicity (vector population, inoculation rates etc.).

The vast majority of cases present as a relatively mild, non-specific febrile illness, which resolves rapidly if treated appropriately. The fever rarely follows classical descriptions of cyclical fevers with rigors and chills and is essentially indistinguishable from many other common childhood infections. Additional symptoms that are common include a cough, abdominal pain, vomiting and mild diarrhoea. Older children, who are more vocal, may complain of headache and general body pains.

Severe, life-threatening malaria has a complex pathogenesis, but for management purposes can be defined by simply applied bedside criteria based on the level of consciousness and the degree of respiratory distress. Important features of severe malaria include metabolic acidosis, hypoglycemia, severe anaemia, multiple convulsions and coma (WHO, 2000).

The two most severe complications of clinical malaria responsible for most of the malaria-associated mortality in Ghana are severe anaemia and cerebral malaria. Anaemia is one of the major clinical manifestations of malaria apart from fever and splenomegaly. Cerebral malaria is the most profound manifestation of severe malaria in humans caused by *P. falciparum* infection and is characterized by neurological symptoms including deep coma (WHO, 2000).
Although all ages are affected, clinical attacks are more frequent in children. Similarly, severe disease is more common in children.

2.5 Immunity to Malaria

2.5.1 Natural Resistance

A disease that kills mainly children would be expected to select strongly for any genetic trait that reduces susceptibility. A number of genetic factors play a role in natural immunity with most of the factors being those that affect the main site of development (the red blood cells) directly. 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. Parasite growth has been shown to be significantly inhibited in HbS carrier red blood cells and not in homozygous individuals (Friedman, 1978). Akanmori et al. (1991) also showed that the titres of IgG and complement C3 were lower in the serum of homozygous individuals (SS) as compared to heterozygous (AS) individuals. This suggests that the carriers (AS) are likely to mount a stronger complement mediated immune response against malaria parasites. Also, certain human leukocyte antigens (HLA), which are important in the initiation of immune responses, have been found to be associated with protection from severe malaria. HLA class I antigen, HLA-Bw53 and an HLA class II haplotype, DRB*1302-DQB*0501 have been linked to reduced susceptibility to severe malaria (Hill et al., 1991).
The comparative resistance of newborn mice and humans up to 4-6 months was traced to a lack of p-amino benzoic acid (PABA) in milk (Review by Playfair, 1982). PABA is an essential factor in the synthesis by the parasite of tetrahydrofolate and thence thymidine. Of course, in most cases, milk will contain protective antibodies too.

Also the natural resistance of a particular host to a particular parasite can sometimes be traced to immune cells in the spleen. For example, *P. falciparum* will infect splenectomized but not intact gibbons and marmosets (Playfair, 1982).

2.5.2 Acquired Immunity

2.5.2.1 Non-specific Immunity

The innate immune system is built up of certain types of cells and humoral factors that recognize, inactivate and kill infectious agents immediately after entering the host organism. By definition, these processes are rapid, non-specific (in the sense of being active against a broad spectrum of microbial pathogens), and operate before an antigen-specific (adaptive) immune response has developed. Accordingly, natural resistance is mainly due to constitutively available mechanisms of defense that can be activated within seconds or minutes. These include the phagocytosis of bacteria or protozoa by neutrophils or macrophages; the release of antimicrobial peptides, of hydrolytic enzymes or of reactive oxygen intermediates ("oxidative burst") by phagocytes; activation of the complement system via the alternative or the mannose-binding protein pathway; or the cytotoxic activity of natural killer (NK) cells against infected target cells (Fearon and Locksley, 1996).
Like many other parasite infections, malaria can be affected by stimulation of the immune system with quite unrelated agents, especially of microbial origin. These include endotoxin, *Corynebacterium parvum* and several known inducers of interferon. Studies have shown that only a small fraction of immunoglobulin represents malaria specific antibodies, with the majority being ‘non-malaria specific’ and some autoantibodies reacting with cellular as well as extracellular self-components (Freeman *et al.*, 1980, Marsh and Greenwood, 1986). Playfair *et al.* (1990) suggested that the induction of immune responses by endotoxin-like malaria exoantigens may be important in the anti-malaria immunity. During the rupture of schizonts, a large amount of soluble parasite antigens are released into circulation. These antigens share some chemical and biological characteristics with bacterial endotoxins, like lipopolysaccharide. These exoantigens trigger macrophages and monocytes to release endogenous pyrogens such as interleukin-1 and tumour necrosis factor (Bate *et al.*, 1988, Kwiatkowski *et al.*, 1989, Jakobsen *et al.*, 1991). Antibodies against these exoantigens are believed to lead to reduction in severity of clinical symptoms. In general it appears that parasites inhabiting the adult red cell are more susceptible to this kind of non-specific immunity than those that parasitize the reticulocyte (Playfair, 1982).

2.5.2.2 Specific Immunity

The period of greatest risk in human malaria is from six months to five years, after which the disease becomes progressively less severe, despite continued exposure. There is considerable evidence that infants in the first 6 months of life are protected from malaria attacks by antibodies believed to be from their immune mothers (McGregor, 1984, Akanmori *et al.*, 1995). As the maternal antibodies wane, the children become
increasingly susceptible to *P. falciparum* infections. By the third year in life, the children begin to show increasing signs of tolerating higher parasitaemia in the absence of clinical illness. During this period there is increased levels of malaria specific immunoglobulin in serum (McGregor *et al*., 1965), suggestive of the development of acquired protective immunity.

From this age through adolescence, natural immunity begins with decrease in severity of clinical episodes but with high levels of parasitaemia. Studies by Riley *et al*., (1990) in the Gambia showed that parasite rates did not seem to decline until the age of 10 to 12 years, whilst the incidence of clinical disease peaked at the age of 6 years. This suggests that clinical or anti-disease immunity may be different from anti-parasite immunity because the incidence of severe disease and death from malaria decline in young children at a time when they rather harbour considerable parasite loads.

The immune responses induced as a result of the acquisition of anti-malarial immunity are said to be species, stage and strain specific (Howard, 1986). Malaria infection causes an increase in immunoglobulin synthesis, which is evidenced by the elevated levels of immunoglobulin in residents of malaria endemic areas (McGregor, 1974).

2.5.3 Mechanisms of Malaria Immunity

Immunity to malaria is acquired as a result of the activity of both humoral and cellular factors with some contribution from the physiological condition of the host. The humoral factors are represented by antibodies that appear in the blood whereas the
cellular factors are the macrophages and other cells produced by the lymphoid-macrophage system of the spleen, liver and bone marrow.

Humoral Factors
Antibodies are the effector molecules of the humoral response to malaria infection. They bind to malaria antigens, neutralizing them or facilitating their elimination. The gamma globulin fraction of serum contains five types of immunoglobulin: IgG, IgM, IgE, IgD and IgA. The distinction between each major class of immunoglobulin lies in the type of heavy chain. Studies done in residents in highly endemic malaria areas as well as in experimentally infected volunteers have shown antibody responses induced by malaria infection to involve only the immunoglobulin isotypes IgM, IgG and IgA (Targett, 1970, Collins et al., 1971). No specific malarial antibody has been detected in IgD and IgE immunoglobins. Collins et al. (1971) in their studies also noted that patent parasitaemia usually triggered the simultaneous production of IgM and IgG antibodies and that, where this was not so, IgG responses usually preceded IgM. They also found IgA response to be often more delayed. Measuring the persistency of these antibodies, these workers also found IgM and IgA responses to be transient, while those involving IgG were more persistent. Targett (1970) in his study also showed IgM levels to rise abruptly in association with parasitaemia and often fell dramatically when chemotherapy terminated infection, even when malarial antigens continued to circulate.

Antibodies acquired by man as a result of infection with a single species of malarial parasite have been found to cross react with heterologous parasites (Diggs and Sadun,
1965), but antiserum usually yield their highest titres when reacted against homologous parasites indicating species specificity of antibodies (Collins et al., 1967).

The protective value of antibodies has been demonstrated in humans. McGregor et al., (1963) showed IgG from West Africa immune serum to have protective value against infections caused by *P. falciparum* parasites from East Africa. Cohen and colleagues (1969) deduced that immune serum acted mainly by blocking the invasion of red cells by merozoites. Other antibody-mediated mechanisms for which a protective effect has been claimed include antibody dependent cytotoxicity, opsonization and IgM autoantibodies.

**Cellular Immunity**

Chemotaxis and activation of macrophages are features of cell-mediated immune response in malaria. Parasitized red cells have been clearly shown to be removed more rapidly from the circulation than normal red cells, and both the spleen and the liver can be responsible (Playfair, 1982). Playfair in that review noted that macrophages from infected animals were more phagocytic for parasitized cells *in vitro* and in millipore chambers implanted in the peritoneal cavity, and also immune serum enhanced phagocytosis by both malarious and normal macrophages. Playfair also noted that a proposal that NK cells were important in killing intra-erythrocytic malaria parasites was not supported by experiments in which NK activity was deliberately reduced.
T cells

Evidence from studies of correlations between T cell responses and protection, cell transfer experiments and T or B cell depleted animals suggest that T cells play an important role in protection against malaria (Reviewed by Playfair, 1982).

Both T lymphocytes carrying alpha/beta and gamma/delta antigen-receptors play a role in malaria immunity. However, although gamma/delta T cells may expand 40-fold or more in the peripheral immune system in acutely infected humans and also inhibit parasite growth \textit{in vitro} and \textit{in vivo}, their relative importance for protection or pathogenicity is presently unclear. Ho \textit{et al.} (1990a) found that gamma/delta T cells were elevated during the acute infection of \textit{P. falciparum} infection and remained elevated for at least four weeks during convalescence. They suggested that T cells might participate in the immune response against \textit{P. falciparum} by functioning as non-MHC restricted cytotoxic cells against intraerythrocytic parasites. Alternatively, lymphokines such as TNF alpha, TNF delta and IFN gamma may be produced on antigen stimulation, which may have antiparasitic activity. Of the two major T cell subsets (CD4+, CD8+) carrying alpha/beta T cell receptors, the role of CD8+ T cells in blood stage infections appears to be limited. Instead CD4+ T cells are of major importance. These cells comprise at least two functionally different subsets (Th1, Th2), distinguished on the basis of lymphokine secretion.

Th1 cells upon activation produce IL-2, TNF, lymphotoxin, and interferon gamma, which initiate cell-mediated immunity recognized for the elimination of intracellular pathogens. Th2 cells, which produce the lymphokines IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, mediate humoral immune responses (Mosmann and Coffman, 1989). In some
rodent malarials, Th1 cells producing IFN-gamma and IL-2 are important for controlling infection in its early phases, whilst Th2 cells, producing IL-4 and IL-10, together with antibodies, are important for parasite clearance in later phases of infection (Troye-Blomberg et al., 1994). The balance between these two subsets is critical for the outcome of an infection.

2.6 Phagocytosis

The vertebrate host defence system is best viewed as a set of highly integrated layers of response to any threats. Evolutionarily early “primitive” systems, such as lysis via the complement cascade, are very rapid but not very discriminatory on their own and are relatively easily evaded by potential pathogens. Phagocytosis and killing of invading organisms by polymorphonuclear and mononuclear leukocytes is a marked improvement in both the range of effector mechanisms and the detection of noxious agents.

Phagocytosis is the process by which cells internalize other cells, cell fragments, protein aggregates, and foreign bodies (Jones et al, 1999). While targets for engulfment by the process of phagocytosis may be any size, the term phagocytosis is generally reserved for those particles that are more than 1 μ in diameter, because at this size the mechanisms for internalisation clearly differ from those used for endocytosis of soluble material (Brown and Steinberg, 1996). According to Jones et al. (1999), phagocytosis was first described by Elie Metchnikoff who observed that there were cells in starfish larvae capable of ingesting and destroying foreign substances and invaders. In metazoans, the most prominent roles for phagocytosis include removal of tissue debris after injury, removal of apoptotic cells during development, and destruction of invading pathogens.
As an essential element of host defence, phagocytosis is a part of the mechanism by which most potential pathogens are ultimately destroyed. The host defence aspects of phagocytosis require recognition of potential pathogens and are a function primarily of specialized leukocytes, the myeloid cells known as polymorphonuclear neutrophils (PMNs) and monocytes or macrophages, collectively referred to as “professional phagocytes”

PMNs and monocytes migrate through blood and tissues, surveying for disruptions of homeostasis, which require repair. All phagocytes are bone marrow derived. PMNs mature from committed precursors for 6 days in the marrow and then are released into the vasculature to circulate for only 6 to 12 hours (Bainton, 1992), following which they enter various tissues, where they ultimately apoptose and are efficiently removed by resident macrophages. During infection, PMNs are released from the marrow in greater numbers and sometimes prior to full maturation.

Myeloid granulocytes are divided based on the staining properties of their granules into neutrophils, eosinophils, and basophils. PMNs are the most abundant; constituting about 95% of the total granulates. The granules usually contain lysosomal enzymes and/or bacteriostatic or bactericidal molecules and act as a repository for membrane molecules, which can be rapidly mobilized to the cell surface for host defence.

Mononuclear phagocytes are released from the bone marrow as monocytes, which migrate into different tissues, where they further differentiate into mature macrophages. The phenotype of these cells is highly dependent on the tissue in which they are found.
Much of the role of these cells is to remove dead and damaged cells and particulate matter from the tissues. As Kupffer cells in liver and spleen sinusoids, they remove senescent erythrocytes from the bloodstream. Kupffer cells also remove other debris, such as fibrin degradation products and immune complexes, from the circulation. As alveolar macrophages, they ingest and remove inhaled particles that are small enough to reach the terminal airways. As microglia in the central nervous system, they ingest degenerated myelin. They also are found in the submucosa of the gastrointestinal and urogenital tracts, in the skin, and in the synovial membranes lining the joint cavities. At these sites as well as in the peritoneal and pleural cavities, they remove the small numbers of bacteria that frequently leak into these tissues from the bowel or the lung.

Monocytes circulate with a half-life of 16 to 20 hours prior to migration into tissue to become tissue macrophages (Van Furth, 1992). Fully differentiated resident macrophages have a lifetime of 4 to 15 days in their home tissues. During inflammation, monocyte accumulation at the perturbed site is greatly increased. This is due primarily to increased influx from circulating monocytes and only minimally to proliferation of resident macrophages. These inflammatory macrophages have a different phenotype from the resident tissue cells, including expression of myeloperoxidase, increased phagocytic capacity, and enhanced ability to generate toxic oxygen and nitrogen metabolites (Jones et al., 1999). The host response to plasmodia includes the production of enlarged populations of peripheral blood monocytes and tissue macrophages in the spleen and the liver (Taliaferro and Mulligan, 1937 and Arese et al., 1991). This is believed to be a result of activation and mobilization of blood monocytes and suggests that the macrophage is a major immunologic effector cell in malaria. Mononuclear
phagocytes are able to recognize and ingest parasitized red blood cells, and have the potential to dispose of up to 40-80% of the total red cell mass in a few days (Arese et al., 1991). In addition to increased phagocytosis, macrophages and neutrophils have shown significant ability to produce toxic oxygen radicals, which have been suggested to participate in parasite killing (Dockrell and Playfair, 1983), as well as much of the clinical symptoms associated with malaria infection (Clark et al., 1981).

2.7 Major Histocompatibility Complex

Another role of monocytes/macrophages is that of antigen presentation. This function is most important as an initial trigger of an antigen-specific T cell response during an immune challenge. Antigens are presented to T cells in combination with certain proteins known as major histocompatibility complex molecules.

The major histocompatibility complex (MHC) is a large genetic complex with multiple loci. Molecules within this complex were originally recognized through their ability to provoke vigorous rejection of grafts exchanged between different members of a species. The MHC loci encode two major classes of membrane molecules: class I and class II MHC molecules. The class I molecules are glycoproteins found on the membrane of nearly all nucleated cells, always in association with a small protein called β2-microglobulin. Class II MHC molecules are glycoproteins expressed by the various specialized cells that function as antigen-presenting cells. There are three class II loci in humans, DP, DQ and DR. Each class II locus has two genes, an α gene and a β gene which respectively encode the α and β chains of the class II MHC molecules.
In order for a foreign protein antigen to be recognized by a T cell, it must be degraded into small peptides that form physical complexes with a class I or class II MHC molecule. The MHC molecule bearing the peptide is then exported to the cell surface. The human MHC is also known as the human leukocyte antigen (HLA) complex, thus the class II proteins are HLA-DP, -DQ and -DR. In bacterial or parasitic infections such as malaria, upregulation of MHC class II for antigen presentation is important to ensure adequate helper-T-cell development and to determine the outcomes of disease and secondary infections. Class II is upregulated in human macrophages by factors including IFN-gamma, tumour necrosis factor and IL-4 (Glimcher and Kara, 1992), all of which are important players in malaria infections.

2.8 Cytokines

Cytokines are regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of cytokines include numerous effects on cells of the immune system and modulation of inflammatory responses. Cytokines are simple polypeptides or glycoproteins with molecular weight not more than 30kDa.

Cytokines share many of their properties with two other groups of protein mediators, namely growth factors and hormones. However, cytokines are different from growth factors in that their production is tightly regulated whereas that of growth factors tends to be constitutive. Also unlike cytokines actions, the major actions of growth factors are targeted at nonhaematopoietic cells. Although the range of actions displayed by individual cytokines can be broad and diverse, at least some action(s) of each cytokine is (are) targeted at haematopoietic cells.
It is also not easy to distinguish clearly between cytokines and classical polypeptide hormones. One of the major distinguishing features is that classical hormones are produced by specified cells e.g. insulin is produced by cells of the pancreas, growth hormone by the anterior pituitary and parathormone by the parathyroid. In contrast, cytokines tend to be produced by less specialized cells, and more often than not, several unrelated cell types can produce the same cytokine. For example IL-1 is produced by monocytes/macrophage, mesangial cells, NK cells, B cells, T cells, neutrophils, endothelial cells, smooth muscle cells, fibroblasts, astrocytes and microglial cells. However, there are exceptions, for example IL-2, IL-3, IL-4, IL-5, lymphotoxin and IFN-γ are produced only by lymphoid cells, especially T cells. Perhaps the most characteristic features of cytokines, those that distinguish them from hormones, are the redundancy and ambiguity of cytokines actions, i.e. the fact that structurally dissimilar cytokines (e.g. TNF-α/β and IL-1α/β) show remarkable similarities in their actions (Vilcek and Le, 1994) and that individual cytokines tend to exert a multitude of actions on different cells and tissues.

Chemokines

Chemokines are chemotactic cytokines. They are a large family of structurally and functionally related proteins. Although the properties of these molecules have only recently begun to be elucidated, the bulk of the evidence to date suggests that the chemokines function as regulators of inflammatory and immunoregulatory processes particularly through their specific leukocyte chemoattractant effects.
Structurally these proteins form a super family of proteins that are related by a four-cysteine motif. The super family is subdivided into two branches based upon whether the first two cysteines in the motif are either adjacent (termed the C-C branch) or spaced by an intervening residue (the C-X-C chemokines). Members of the C-X-C family include platelet factor 4 (PF4) and interleukin 8 (IL-8). C-C chemokines include monocyte chemotactic proteins-1, 2 and 3, macrophage inflammatory proteins-1α (MIP-1α) and MIP-1β and RANTES.

The effects of chemokines on monocytes and neutrophils in vitro show a simple, though not absolute, pattern. Generally speaking, C-X-C chemokines affect neutrophils but not monocytes, while C-C chemokines affect monocytes but not neutrophils. The biological activities most actively investigated in vitro with these two cell types include chemoattraction and activation. IL-8, ENA-78 and NAP-2 have all been reported to preferentially attract neutrophils in vitro and to induce activation, as measured by shape change, transient increases in cytoplasmic calcium concentration, degranulation, respiratory burst, increased adhesive properties, and enhanced ability to kill pathogens (Oppenheim et al., 1991). The situation with the C-X-C chemokines is also relatively simple in vivo. Injection studies using rats, mice, and rabbits commonly show an influx of neutrophils in response to the C-X-C chemokines, which are known to be active in vitro.

The monocyte activities of the C-C chemokines, when tested in vitro, are also fairly straightforward: RANTES, MCP-1, MCP-2, MCP-3, human MIP-1α, human MIP-1β
and I-309 have all been reported to induce the migration of monocytes, but not neutrophils in microchemotaxis assays. MCP-1 has also been reported to elicit degranulation and respiratory burst in monocytes and regulate adhesion molecule expression and cytokine production in these cells (Schall, 1994).

IL-8 has been characterized as a neutrophil activating protein (NAP-1) with biological effects similar to known chemotactic substances such as the plasma-derived anaphylatoxin C5a, the cell-derived platelet activating factor (PAF), and bacterial- or synthetic formylmethionyl peptides, e.g. FMLP (Van Damme, 1994). Most researchers originally isolated the molecule from peripheral blood monocytes stimulated with lipopolysaccharide or mitogen. Fibroblasts, endothelial cells, smooth muscle cells, and a variety of more specialized cell types can produce IL-8 in response to the primary cytokines IL-1 and TNF-α. IL-8 can also be produced by neutrophils under specific circumstances. *In vitro*, IL-8 stimulates neutrophils to directed migration, as measured under agarose or in the Boyden chamber and also induces neutrophil degranulation (Van Damme, 1994). Compared with other chemotaxins (C5a, PAF, FMLP, and LTBu), activation of neutrophils by IL-8 seems to be more selective in that eosinophils and monocytes are not responsive (Van Damme, 1994). To date no reports of the levels or roles of monocytes- or neutrophils-specific chemokines in malaria have been published. Such information is important in understanding the contribution of phagocytosis to malaria pathogenesis.
Numerous publications have described the outpouring of pro-inflammatory cytokines and other inflammatory mediators that occurs during the acute phase of malaria, both in experimental murine models and in humans with clinical symptoms of \textit{P. falciparum} or \textit{P. vivax} (Review by Kwiatkowski and Perlmann, 1999). According to that review, the principal clinical manifestation of the pro-inflammatory cytokines in malaria is a systemic response, namely fever. High levels of TNF was named to be mainly responsible for this fever with likely contributions from other pyrogenic cytokines including IL-1β, IL-1α, IL-6 and LT-α which also show elevated levels in patients with malaria fever. In addition to its role in causing fever, TNF has been reported to suppress parasite growth \textit{in vivo}. TNF and IL-1 stimulated macrophages and neutrophils for increased phagocytic ingestion of \textit{P. falciparum} infected erythrocytes in the presence of IFN-gamma, and the two cytokines provided an important stimulus for high output nitric oxide production by combining with other factors such as IFN-gamma to activate expression of inducible nitric oxide synthase in macrophages (Kwiatkowski and Perlmann, 1999). Several reports cited in that review suggested that nitric oxide was potentially inhibitory to all stages of parasite development within the mammalian host.

The overall effects of cytokines in malaria infection is likely to be determined by the acquired immune response, and particularly by the relative dominance of the Th1 subset (which tends to boost TNF production) and the Th2 subset (which acts through IL-10 to suppress TNF production).
2.9 Pathogenesis of Severe Malaria

Traditionally, severe malaria in African children has been considered to fall into two main categories—cerebral malaria and severe anaemia.

2.9.1 Cerebral Malaria

The essential pathological feature of severe falciparum malaria is sequestration of erythrocytes containing mature forms of the parasite in the deep vascular beds of vital organs. Sequestration is not uniformly distributed among the vital organs; in cerebral malaria it is usually greatest in the brain (McPherson et al., 1985) and least in the skin. Sequestration is also not uniformly distributed at the microvascular level.

WHO (2000) suggested mechanisms underlying cerebral malaria to include:

(i) that the cerebral capillaries and venules contained a high proportion of parasitized cells, predominantly large “late” trophozoites and schizonts. These parasitized erythrocytes stuck together and had difficulty passing through the capillary bed, consequently reducing flow gradually to a stop,

(ii) that there was an increase in cerebral capillary permeability with outward leakage of plasma resulting in cerebral oedema and, because of the extravasation of plasma into the cerebral interstition, local haemoconcentration and reduced microcirculatory blood flow,

(iii) and that decreased erythrocyte deformability and cytoadherence cause microcirculatory obstruction, with resulting local hypoxia and substrate depletion (i.e., ischaemia).
2.9.2 Severe Anaemia.

Despite a great deal of work, the pathogenesis of the anaemia associated with *P. falciparum* malaria remains uncertain. Apart from the difficulties inherent in studying the anaemia of parasitic infections, or of any other infection for that matter, Weatherall and Abdalla (1982) noted that the problems in studying the anaemia of *P. falciparum* malaria are compounded by several factors, including the rapidity with which high parasitaemias are achieved in non-immune individuals and the remarkable variability of the clinical course with respect to racial background, age and immune status. Furthermore, many patients in areas where the condition is endemic have multiple pathology, including iron or folate deficiency and associated bacterial or parasitic infections.

The high morbidity and mortality associated with *P. falciparum* infection had previously been attributed to the particular parasite’s ability to invade red cells of all ages, unlike *P. vivax* and *P. ovale* which are limited in their multiplication potential because they can only invade reticulocytes. However data compiled by Pasvol and Wilson (1982) showed that reticulocytes and metabolically younger red cells are more susceptible to invasion by *P. falciparum* than are older cell populations. This may be of some clinical importance since the morbidity and mortality of malaria is directly related to the level of parasitaemia. Experiments in animal models support the notion that the average red cell age, and thus the parasitaemia that develops, can determine the outcome of the infection (Miller and Carter, 1976). For example, it was shown that young rats infected with *P. berghei* had higher parasitaemias and mortality than older rats, which had a lower percentage of reticulocytes (Weatherall and Abdalla, 1982). With respect to *P
falciparum infections, this might provide an explanation for the observed low incidence of *P. falciparum* malaria in the first few months of life; erythropoiesis is markedly depressed for the first two to three months after birth and hence there are relatively few young red cells in the circulation. It might also account for the high incidence of *P. falciparum* malaria in 6-24 month old children.

Malarial anaemia can have different clinical presentations. Abdalla *et al.* in 1980, whilst studying haematological changes in a group of young Gambian children with *P. falciparum* malaria identified three scenarios. In children with acute infection anaemia was most marked during the period after treatment, and many of these patients developed a positive direct Coombs test during the period of the illness. A second group of children appeared to have a more chronic form of *P. falciparum* malaria infection, were profoundly anaemic at presentation, showed gross dyserythropoietic changes in their bone marrows, and had a full reticulocyte response and rise in haemoglobin after treatment. They also found a third group of children whose haematological abnormalities were intermediate to those of the acute and chronic groups.

Although the pathogenesis of this anaemia is uncertain, there seems little doubt that the condition has a multifactorial basis boiling down to two processes: destruction of erythrocytes and the failure of the bone marrow to produce an adequate supply of new erythrocytes (Abdalla *et al.*, 1980; Phillips *et al.*, 1986).
Destruction of Red Cells

Data compiled by Seed and Kreier (1980) gave considerable evidence from both animal and human studies that direct damage to the red cells by invasion and growth of parasites is the major mechanism for premature red cell destruction in all acute forms of malaria. It seems likely that the destruction of red cells occurs both intravascularly and by sequestration of parasitized cells in the spleen and other parts of the microcirculation. Clearly, erythrocytes are destroyed when schizonts rupture, but in addition, Weatherall and Abdalla (1982) named mechanical disruption of the cells, impaired membrane function, and increased rigidity and reduced deformability as possibly the main mechanisms of premature cell destruction. They noted that the peculiar vasculature of the spleen coupled with the fact that erythrocytes become so closely related to macrophages, makes the spleen a highly effective filter of damaged or rigid erythrocytes. These processes become of great significance if the patient has a very high parasitaemia or a very chronic infection, but they cannot easily account for the degree of anaemia seen in acute infections where fewer than 1% of erythrocytes are infected, and it therefore seems likely that uninfected red blood cells are also destroyed (Looareesuwan et al., 1987).

Red-cell survival studies reported in human *P. falciparum* malaria by Rosenberg et al. (1973) provided some evidence that both endogenous red-cell survival, and that of transfused normal erythrocytes, is shortened. They examined the survival of normal cells transfused into patients who were under treatment for *P. falciparum* malaria, thus attempting to rule out the possibility that loss of the normal cells might occur as a result
of the effect of direct parasitization. The normal cells showed a reduced survival time in some cases.

Following suggestions made by Zuckerman (1966), there has been considerable interest in the possibility that at least part of the haemolytic component of the anaemia of \textit{P. falciparum} malaria results from immune destruction of red cells. Reports of findings of a positive Coomb’s DAT in patients with malaria has been the main stimulus. Facer et al. (1979) found patients who were severely anaemic and who showed a positive DAT with active C3 components on their red cells. They were able to demonstrate monocyte erythrophagocytosis of non-parasitized cells on the blood films of these patients. They suggested therefore, that anaemia in acute malaria may sometimes be the result of opsonization and phagocytosis of sensitized cells. Also Kreier and Green in 1980 found increased destruction of uninfected erythrocytes to result from increased phagocytosis of erythrocytes due to the erythrocyte membrane being damaged by either parasite or host proteolytic enzymes activated through Ag-Ab and complement reactions. This theory is supported by the observation of non-parasitized erythrocytes in bone marrow monocytes and macrophages during and following infection. (Phillips \textit{et al.}, 1986).

The poor sensitivity and subjectivity of the Coomb’s test is blamed for disputes on the role of immune mediated haemolysis in \textit{P. falciparum} malaria anaemia. However, new data suggests that binding of complement factor C3b to RBC is an important contributor to this type of anaemia, whereas IgG binding to RBC is rare in children with malaria. Recently Goka \textit{et al.} (2001) reported strong association between C3d binding to erythrocyte membrane and severity of anaemia in Ghanaian children with \textit{P falciparum}.
malaria. In the same area Abdalla (1986) found no increase in the number of IgG molecules bound to red blood cells in patients with malaria compared to the controls. There was no correlation between severity of anaemia and red blood cell-associated IgG levels. However Ho et al. (1990b) found a strong correlation between anaemia and the clearance of erythrocytes coated with relatively low numbers of IgG molecules.

Work by Voller (1974) suggested that the development of autoantibodies to the erythrocytes cause the haemolysis or opsonization of uninfected red cells. Such anti-erythrocyte antibodies have been found in *P. falciparum* infections (Rosenberg et al., 1973). However, relying on observations by Salmon et al. (1997) of anaemia in a mouse model where destruction of uninfected erythrocytes was similar in SCID and nude mice to normal mice, Jakeman et al. (1999) dismissed the involvement of antibodies in the destruction of uninfected erythrocytes. They indicated that since the SCID and nude mice cannot make antibody, neither anti-erythrocyte antibodies nor immune complexes involving anti-parasite antibody could have been involved in this case. Jakeman and co-workers believed that a likely explanation for the destruction is the direct binding of parasite components, possibly whole but non-viable merozoites, to the surface of uninfected erythrocytes followed by antibody-independent phagocytosis.

Defective Red Cell Production.

Defective red-cell production caused either by depression of erythropoiesis (Woodruff et al. 1979), inhibition of reticulocyte release, or defective erythropoiesis (Rencricca et al. 1974) may also play a part in causing the anaemia of the different types of malaria. In any haemolytic process a falling haemoglobin level causes increased production of erythropoietin, which results in erythroid hyperplasia of the marrow and reticulocytosis.
Many workers have noticed that there is a relatively poor reticulocyte response to anaemia in patients with *P. falciparum* malaria. In a detailed haematological analysis of a group of Gambian children with the anaemia of *P. falciparum* malaria, Abdalla *et al.* (1980) observed greater abnormalities in the marrows of groups of children who presented with severe anaemia and a low reticulocyte count associated with a low parasitaemia than in those of children with acute infections and high parasitaemias who showed normal or reduced erythroid precursor populations, which had no gross morphological abnormalities. The marrow appearances of the former were quite remarkable in that they showed marked dyserythropoietic changes including multinuclear erythroblasts, karyorrhexis, incomplete and unequal amitotic nuclear divisions, and cytoplasmic bridging.

Recently, findings by Kurtzhals *et al.* (1997) suggested that *P. falciparum* infection caused a rapidly reversible suppression of the bone marrow response to erythropoietin. Further, they found that the inhibition of bone marrow response was a general finding irrespective of initial haemoglobin levels, suggesting that the severity of anaemia depends on the degree of peripheral erythrocyte destruction in patients with suppressed bone marrow response to erythropoietin.

### 2.9.3 Role of Cytokines in Pathogenesis of Severe Malaria

Inflammatory processes play a central role in the clinical manifestations of malaria. Pro-inflammatory cytokines have been established as causal mediators of malaria fever and
have been strongly implicated in the pathogenesis of cerebral malaria, malaria anaemia, and other severe complications of infection.

Observations on the similarity between severe malaria and endotoxaemia by Clark et al. (1981) led to the concept that the products of activated macrophages may mediate severe malaria. Activated monocyte/macrophage cells secrete predominantly TNF-α, IL-6 and IL-10.

Several studies now have confirmed a positive association between plasma concentrations of TNF and other pro-inflammatory cytokines, and mortality in severe falciparum malaria (Grau et al., 1989, Kern et al., 1989, Kwiatkowski et al., 1990, Krishna et al., 1994), although some studies have failed to confirm such a relationship, (Shaffer et al., 1991). In most of these studies, plasma concentrations of TNF have correlated with several indicators of severity, namely hypoglycaemia, hyperparasitaemia, and anaemia. Plasma concentrations of IL-1 (Kwiatkowski et al., 1990), IL-6 (Kern et al., 1989), and IL-8 (Friedland et al., 1993) have also been shown to be correlated with disease severity. Clark and Chaundhri (1988) suggested that increased TNF-α levels might be implicated in the development of ineffective erythropoiesis or dyserythropoiesis in murine malaria. Recent studies by Kurtzhals et al. (1998), Othoro et al. (1999) and Akanmori et al. (2000) have shown that African children with severe malarial anaemia have relatively low circulating levels of IL-10 in relation to TNF. Since IL-10 is a natural TNF antagonist, this finding would be consistent with a role for TNF in the pathogenesis of severe malaria anaemia.
In view of all these reports implicating the immune response in the pathogenesis of severe malaria anaemia there was the need to identify the cells involved and the mechanisms by which they exert their effects. This study sought to investigate the role of phagocytic cells and inflammatory mediators in the development of anaemia in malaria.

2.10 Measurement of Phagocytic function by flow cytometry.

In many clinical situations evaluation of phagocytic ability has been of considerable interest. Classical techniques are based on the quantification of particle uptake, including viable microorganisms, yeasts, and microspheres, by direct microscopic observation or radioactivity incorporated via radiolabelled substances. These methods have the disadvantages of being tedious and subjective, making them unsuitable for routine practice in clinical laboratory.

Recently, flow cytometry has been used extensively in clinical laboratories because it provides a means of rapidly, objectively and simultaneously evaluating different parameters in a large number of cells. However, this method is difficult to use in functional studies, e.g. the assessment of phagocytic function, because the non-specific adherence of certain cells, including PMNs to a variety of substances interferes with the measurement of the numbers of particles actually internalised by the cell. Several researchers have overcome this problem by using substances such as methylene blue, trypan blue, sodium azide or iodoacetate to quench superficial fluorescence, thus reducing non-specific adherence. Santos et al. (1995) developed a protocol that overcame the problem by comparing the results in the experimental sample (incubated at
37°C) against those obtained in a control sample incubated at 4°C. They suggested that fluorescence detected in the control sample corresponded entirely to adherence, since cellular functions, including phagocytosis are inhibited by low temperatures. When the results were checked with scanning electron microscopy (SEM), it was found that latex microspheres remained on the surfaces of cells incubated at 4°C, whereas internalised beads could be distinguished from adhered beads in cells incubated at 37°C. Another advantage of the method of Santos et al. (1995) was that whole blood was used, thus eliminating the need to separate PMN before the assay. Further, the use of whole blood provides a better simulation of the conditions in vivo.

The method of Santos et al. (1995) was used as the basis for the neutrophils phagocytic function assay in the present study.
CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Subjects

Children presenting with malaria at the emergency room of the department of Child Health, Korle-Bu Teaching Hospital, Ghana, during the peak malaria seasons (July to August) of two consecutive years, 2000 and 2001, were recruited into the study. Also healthy children from a nearby community, Dodowa, without detectable Plasmodium in thick blood films or with symptomless P. falciparum infection, were enrolled as controls. All patients and control donors were enrolled in the study only following informed, signed, parental consent. The study was approved by the ethics and protocol review committee at the University of Ghana Medical School and by the Ghanaian Ministry of Health.

In the year 2000, only samples taken on day of admission (Day 0) were tested for phagocytic function and surface marker expression, where as in the year 2001 study samples taken after three days (Day 3) and seven days (Day 7) after start of treatment were included in the phagocytic function assay. Plasma samples from patients recruited in both years were all tested for cytokines by ELISA assays.

3.2 Inclusion Criteria

Children aged between 1 to 10 years were enrolled. General inclusion criteria were asexual P. falciparum parasitemia of more than 10 000 /μL and axillary temperature of more than 37.5°C. Children with any disease other than malaria or with a positive sickling test (metabisulphite method) were excluded from the study.
Patients were categorised into cerebral malaria (CM), severe anaemia (SA) and uncomplicated malaria (UM). The criteria were:

- severe anaemia, haemoglobin < 50 g/L, no other cause of anaemia, and full consciousness (score of 5 on the Blantyre coma scale);
- uncomplicated malaria, full consciousness, haemoglobin more than 80 g/L, and no other complications of malaria, and
- cerebral malaria, unrousable coma with score of 3 or less for more than 60 minutes and no sign of meningitis or encephalitis on assessment of cerebrospinal fluid. Patients with convulsions were excluded from the SA and UM categories because febrile convulsions occur more frequently in malaria than in other febrile illness, which makes a degree of cerebral involvement difficult to exclude (Kurtzhals et al. 1998).

3.3 Blood collection

Venous blood was collected into sterile vacutainer tubes containing heparin and EDTA for routine diagnostic purposes. Plasma was obtained by centrifugation within 30 minutes of blood collection, and stored at the hospital at -20°C before transfer to Noguchi Memorial Institute for Medical Research where the samples were stored at -80°C until use. Some of the heparin samples were used for phagocytosis within 8 hours of collection while portions of the EDTA samples were used for monocyte surface marker expression analysis.

3.4 Haematological analysis

An 18-parameter automated haematology analyser (Sysmex KX-21, Japan) was used for measurement of haemoglobin and counting of white blood cells.
3.5 Parasitology

Thick and thin blood films stained with Giemsa were made for microscopic detection and identification of *Plasmodium*. Briefly blood smears were made, dried and the thin film 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. Parasites were counted against 300 WBCs and the value multiplied by each individual’s WBCs count from the automatic haematology analyzer to obtain parasites per µL of blood.

3.6 Sickling Test

The sickling test was done 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. The slide was examined under the microscope at a magnification of 40X and the sickling status of the patients determined.

3.7 Phagocytic function Assay

Various methods have been described to quantitate phagocytic capability of leukocytes. Basically, fluorescent particles or bacteria are incubated with the cells, and the fluorescence of each cell is measured by flow cytometry. The amount of fluorescence is then a measure of ingested fluorescent particles and hence phagocytic activity.

In this study, a modified flow cytometry technique, first described by Santos *et al.* (1995) was applied. Heparin was used as anticoagulant rather than EDTA because
the latter showed a higher tendency to clump formation and significantly inhibits phagocytosis as it captures Ca\(^{2+}\) ions. The target particles were yellow-green fluorescent latex spheres (2.0\(\mu\)m in diameter; Polysciences, Warrington, PA) diluted in PBS (40\(\mu\)l/ml). The concentration was approximately 5 \(\times\) 10\(^6\) particles/ml. One milliliter of this suspension was mixed with 4ml of glucose medium (0.59% sodium chloride, 0.25% sodium acetate, 0.03% potassium chloride, 0.04% calcium chloride, 0.02% magnesium chloride and 0.13% glucose (w/v)).

Hundred microlitre of whole blood was preincubated at 37\(^\circ\)C for 5 minutes and mixed with 100\(\mu\)l of the target suspension and immediately incubated with gentle shaking at 37\(^\circ\)C. Different samples were prepared and collected after 5, 10, 20, 30, 45 and 60 minutes of incubation. At the same time, another set of samples was prepared under the same conditions to be incubated on ice as a control. After incubation, whole blood samples were lysed for 10 minutes by addition of 2ml of NH\(_4\)Cl, washed and then analysed by flow cytometry.

Analyses were performed using a Becton Dickinson Flow cytometer (B-D, Japan). Neutrophils and monocytes were selected by electronic gating according to forward scatter (size) and side scatter (granularity). The bi-dimensional cytogram obtained from these parameters allowed a clear distinction between populations of lymphocytes (R1), monocytes (R2), and neutrophils (R3), as can be seen from figure 3.1a. The number of cells that incorporated particles was determined with a fluorescence histogram on a logarithmic scale (figure 3.1b).

In order to overcome the problem of superficial adherence, results of experimental samples were compared against those obtained in a control sample incubated on ice.
The fluorescence detected in the control sample corresponds entirely to adherence, which is not affected by low temperatures, while cellular functions, including phagocytosis, are inhibited.

To make up for value fluctuations from one experiment to the next, findings were standardized by expressing them as the phagocytic index (Phi), calculated from the formula:

\[
\text{Phi} = \frac{\%\text{Ph}T_x}{\%\text{Ph}T_0}
\]

where \(\text{Ph}T_x\) represents phagocytic cells after \(x\) min of incubation at \(37^\circ\text{C}\) and \(\text{Ph}T_0\) represents positive cells in the corresponding control culture, incubated on ice. This gives a relative value which reflects the real increase in phagocytic activity with time.

### 3.8 Surface Marker Staining

The following monoclonal antibodies were used: peridinin Chlorophyll Protein (per CP) - conjugated anti-CD14 (Becton-Dickinson, 340585), fluorescein isothiocyanate (FITC)- conjugated anti-CD16 (Immunotech, L06562), phycoerythrin-conjugated anti-HLA-DR (Becton-Dickinson, 347367), mouse anti-IgG1 (Becton-Dickinson) and mouse anti-IgG (Becton-Dickinson).

Whole blood was stained in two tubes in the following combinations: isotype controls; F/PE-conjugated IgG/perCP-conjugated IgG and CD14/CD16/HLA-DR.
All antibodies were diluted 1 in 5, and 10ul of each added to 100ul of blood in the given combinations. These were incubated for 30 minutes in the dark. Red cells were lysed by addition of 4ml of FACS lysing buffer (Becton-Dickinson) and let stand for 10 minutes. The cells were pelleted by centrifugation at 2000g for 10 minutes, and then washed and resuspended in 300ul of FACS flow solution (Becton-Dickinson) containing 0.5% formaldehyde for flow cytometric analysis. Monocytes were first selected by electronic gating according to forward scatter and side scatter, and then by their expression of the LPS receptor CD14. The proportion of monocytes which are CD16 positive and HLA-DR positive as well as the mean fluorescence intensity of each marker were then obtained from the respective histograms (Figure 3.2).

3.9 Cytokine Assay

Plasma levels of six cytokines and chemokines namely IL-10, TNF-α, MIP-1β, MIP-1α, IL-8 and neopterin were determined for eighty (80) Day 0 samples from all categories of patients and fifteen Controls. In addition Day 3 and Day 7 samples from ten (10) patients from each category were tested.

All cytokines were measured using commercial ELISA kits; TNF-α, R&D systems, USA (cat. No. DTA50), detection limit, less than 4pg/ml; IL-10, R&D systems, USA (cat. No. D1000), detection limit, less than 4pg/ml; IL-8, R&D systems, USA (cat. No. D8050), detection limit, less than 10pg/ml; MIP-1α, R&D systems, USA (cat. No. DMA00), detection limit, less than 10pg/ml; MIP-1β, R&D systems, USA (cat. No. DMB00), detection limit, less than 11pg/ml; Neopterin, IBL- Hamburg, Germany, (cat. No. RE 593 21), detection limit, less than 0.7nmol/L.
The Quantikine TNF-α Immunoassay kit used in this work is capable of detection of the total amount of TNF-α in samples, i.e., the total amount of free TNF-α plus the amount of TNF-α bound to soluble receptors. The IL-10 kit used has also been shown not to cross react with viral IL-10. ELISA was preferred for the measurement of the chemokines IL-8, MIP-1α and MIP-1β because bioassays for these chemokines based on their chemotactic abilities are generally tedious and do not differentiate between chemokines of the same family.

All the assays employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for each cytokine had been precoated onto a microplate. Samples and standards were pipetted into the wells and incubated for specified period of time so that any cytokine present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells and incubated for a specified period of time. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of cytokine bound in the initial step. The color development was stopped after 15 minutes and the intensity of the color measured by spectrophotometry.

3.10 Statistical Analysis

Results in different groups were compared by one-way ANOVA on ranks, Mann-Whitney U-tests, or Student’s t test. P values < 0.05 were considered to be significant. Longitudinal data were analysed by two-way repeated measurement ANOVA. Associations between different parameters were analysed by Spearman’s rank correlation.
Figure 3.1 Flow cytometry data showing measurement of phagocytosis. A) Dot plot distinguishing cell populations by their forward scatter (FSC-H) and side scatter (SSC-H) properties. R1=lymphocytes, R2=monocytes and R3=neutrophils. B) Histogram showing fluorescence (FL1-Height) of neutrophils in R3. M1 represents phagocytosis-positive fluorescence, and the percentage of positive neutrophils is given in the histogram statistics as % gated in M1.

Histogram Statistics

File: 21/7-1110 0min.001
Gate: G3
X Parameter: FL1-H FL1-Height (Linear)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Events</th>
<th>% Gated</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>20650</td>
<td>100.00</td>
</tr>
<tr>
<td>M1</td>
<td>1114</td>
<td>5.39</td>
</tr>
</tbody>
</table>

Acquisition Date: 21-Jul-01
Figure 3.2 Flow cytometric data showing analysis of monocyte surface marker expression. A) Dot plot showing selection of monocytes by electronic gating (R1). B) and C) show separation of gated monocytes according to expression of surface markers. Positive cells are separated from negative cells by quadrant markers. The distribution and mean fluorescence of cells is shown in the quadrant statistics. UL=upper left, UR=upper right, LL=lower left and LR=lower right.
4.1 Summary

The study involved a total of ninety-three (93) symptomatic malaria patients and twenty-five (25) parasite-free or asymptomatic community controls recruited during the peak malaria seasons (July – August) of the years 2000 and 2001. Of the patients involved, thirty-five (35) were classified as having cerebral malaria (CM), thirty-three (33) as severe malaria anaemia and twenty-five (25) as uncomplicated malaria (UM) cases.

For samples obtained in the year 2000, tests were made for phagocytic activity and the expression of monocytes surface markers HLA-DR and the FcyRIII (CD16). In the year 2001 study, phagocytic activity was measured longitudinally, three days (Day 3) and seven days (Day 7) after start of anti-malarial treatment. Plasma samples stored from all the subjects involved in the two-year study were tested for levels of IL-10, TNF-α, MIP-1α, MIP-1β, IL-8 and neopterin. In addition Day 3 and Day 7 samples from 10 patients of each category were tested for the levels of these cytokines.

The following graphs and tables show the results of the study. Where box plots are used, the box represents the interquartile range; the line through the box is the median; whiskers show 95% confidence interval range and individual symbols are values lying outside the 95% range.
4.2 Characteristics of Patients

The clinical and laboratory characteristics of the children involved in the study on the day of admission are summarized in table 1 below.

Table 4.1 Clinical and Laboratory Characteristics of study subjects. Presented as \textsuperscript{1}mean (inter-quartile range), \textsuperscript{2}Geometric mean (inter-quartile range) and \textsuperscript{3}Median (inter-quartile range).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (years) \textsuperscript{1}</th>
<th>Haemoglobin (g/dl) \textsuperscript{1}</th>
<th>Parasitaemia \textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe Anaemia</td>
<td>33</td>
<td>4.0 (2.8-5.0)</td>
<td>4.4 (4.2-4.6)</td>
<td>72 (30-177)</td>
</tr>
<tr>
<td>Cerebral Malaria</td>
<td>35</td>
<td>4.5 (3.5-5.5)</td>
<td>7.0 (6.0-8.0)</td>
<td>99 (38-258)</td>
</tr>
<tr>
<td>Uncomplicated</td>
<td>25</td>
<td>5.5 (3.8-7.2)</td>
<td>9.5 (8.8-10.2)</td>
<td>65 (33-110)</td>
</tr>
<tr>
<td>Controls</td>
<td>25</td>
<td>6.5 (4.3-8.5)</td>
<td>11.0 (10.4-11.7)</td>
<td>1 (0-2) \textsuperscript{3}</td>
</tr>
</tbody>
</table>

Parasitaemias were generally high in all the groups on admission but not significantly different (p = 0.359). Whereas haemoglobin concentrations formed part of the definition of uncomplicated and severe anaemia, the haemoglobin concentrations of patients with cerebral malaria fell between those of the other two groups (SA and UM). Severe anaemia patients were generally younger compared to the other groups, but the differences were not statistically significant.
4.3 Phagocytic Activity

Preliminary studies showed that phagocytic activity generally increased in a time dependent manner from 0 - 30 minutes of incubation then stayed level. The first 30 minutes of incubation was thus chosen for the assay. The progression of phagocytosis with time is seen as the increase in fluorescence of neutrophils as shown in figure 4.1, and subsequently by the calculated phagocytic index (Phi).

The phagocytic index was then calculated from the percentages of fluorescent-positive neutrophils at 5, 10, 20 and 30 minutes incubation, by dividing by the percentage of fluorescent positive neutrophils at 0 minute of incubation.

Phagocytic abilities of different patients were compared by their PhIs at 30 minutes.

The phagocytic abilities of the patients tested in the year 2000 study are shown in figure 4.2 according to their various categories.
Figure 4.1 Flow cytometric analysis of increase in phagocytosis with time for a patient. Histograms show the fluorescent intensity (FL-1) and the number of cells (counts). A) 0 minutes B) 10 minutes C) 30 minutes. The marker M1 shows positive cells. The percentage of positive cells is given in the histogram statistics as % gated in M1. Clearly there is an increase in positive peaks with time.

A. 13/7-0027 0Min.002

B. 13/7-0027 10Min.003

C. 13/7-0027 30Min.004

Histogram Statistics
File: 13/7-0027 0Min.002
Acquisition Date: 13-Jul-01
Marker Events % Gated
All 20167 100.00
M1 745 3.69

Histogram Statistics
File: 13/7-0027 10Min.003
Acquisition Date: 13-Jul-01
Marker Events % Gated
All 17344 100.00
M1 3770 21.74

Histogram Statistics
File: 13/7-0027 30Min.004
Acquisition Date: 13-Jul-01
Marker Events % Gated
All 17281 100.00
M1 10756 62.24
All categories of children with symptomatic malaria showed higher phagocytic activity than the control group (SA, Phl=13, p=0.0015; CM, Phl=7, p=0.0029; UM, Phl=9, p=0.0002 vs. AC, Phl=4).

Figure 4.2 Box plot showing the distribution of PhIs for the subjects by category (2000).

Among the patients, the SA category exhibited the highest phagocytic function whilst the CM group showed the least. These differences were however, found to be statistically insignificant (SA vs. CM, P=0.45; SA vs. UM, P=0.74).
As a follow-up to the observed increased phagocytic ability in malaria in the year 2000 study, the assay was repeated for the year 2001 samples but this time including a longitudinal investigation.

The results of the year 2001 were consistent with the findings of the 2000 study. An important development however, was that the difference in Phi between the SA group and the other malaria categories was statistically significant (SA vs. CM, \( p=0.029 \); SA vs. UM, \( p=0.002 \)). The PhIs for the three categories are represented in the box plot in figure 4.3.

![Box plot showing phagocytic indices of patients by category (2001).](http://ugspace.ug.edu.gh)
When the phagocytic indices of all the patients for the two years were pooled, the SA stood out clearly above the other patient categories, giving geometric means (95% confidence interval) of 13.4 (10.4-17.3) for SA, 8.4 (6.1-11.5) for CM and 6.9 (5.2-9.1) for UM.

Phagocytic ability did not correlate with age or parasitaemia. There was however, some extent of negative correlation between phagocytic index and haemoglobin concentration as can be seen from figure 4.4.

Figure 4.4 Association between Phi and Hb
Phagocytic indices of sixteen patients were followed from Day 0, through Day 3 to Day 7. The results obtained are illustrated figure 4.5.

![Figure 4.5 Longitudinal Study of Phagocytic Index](image)

The mean Phagocytic index for the group decreased on Day 3, though the decrease was not statistically significant. The value remained about the same on Day 7.
4.4 Monocyte Surface Marker Expression

4.4.1 HLA-DR

There was a marked reduction in the proportion of monocytes expressing the activation marker HLA-DR (Figure 4.6a) as well as the mean fluorescence intensity (MFI) of the marker (Figure 4.6b) in all categories of patients compared to controls. The reduction in percentage of HLA-DR+ monocytes was more pronounced in the severe malaria (SA and CM) patients than in those with uncomplicated malaria. The p-values from the Mann-Whitney U-test are shown in table 4.2.
Figure 4.6b MFI of HLA-DR of CD14+ cells

![Bar chart showing MFI of HLA-DR of CD14+ cells for different categories.]

Table 4.2 Showing p-values of test for differences in expression of HLA-DR between categories

<table>
<thead>
<tr>
<th>Category Comparison</th>
<th>%HLA-DR+CD14+</th>
<th>MFI HLA-DR CD14+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM vs. AC</td>
<td>&lt;0.0001 *</td>
<td>&lt;0.0001 *</td>
</tr>
<tr>
<td>SA vs. AC</td>
<td>&lt;0.0001 *</td>
<td>&lt;0.0001 *</td>
</tr>
<tr>
<td>UM vs. AC</td>
<td>0.0102 *</td>
<td>0.0001 *</td>
</tr>
<tr>
<td>CM vs. SA</td>
<td>0.9155</td>
<td>0.7670</td>
</tr>
<tr>
<td>CM vs. UM</td>
<td>0.0804</td>
<td>0.9723</td>
</tr>
<tr>
<td>SA vs. UM</td>
<td>0.0576</td>
<td>0.7576</td>
</tr>
<tr>
<td>CM+SA vs. UM</td>
<td>0.0375 *</td>
<td></td>
</tr>
</tbody>
</table>

* Significant (p<0.05)
4.4.2 CD16

There was an expansion in the proportion of CD14+ monocytes expressing CD16 in all categories of patients compared to controls (Figure 4.7a). However this was not accompanied by an increase in mean fluorescence intensity (MFI) of the marker. Indeed MFIs of CD16 were significantly lower in CM and SA patients compared to controls (Figure 4.7b). The p-values from the Mann-Whitney U-test are shown in table 4.3.

Figure 4.7a Percentage of CD14+ expressing CD16
Figure 4.7b MFI of CD16 for CD14+ cells

Table 4.3 Showing p-values of test for differences in expression of CD16 between categories

<table>
<thead>
<tr>
<th>Patient category</th>
<th>%CD16+CD14+</th>
<th>MFI CD16</th>
<th>CD14+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM vs. AC</td>
<td>0.0420 *</td>
<td>0.0257 *</td>
<td></td>
</tr>
<tr>
<td>SA vs. AC</td>
<td>0.0040 *</td>
<td>0.0062 *</td>
<td></td>
</tr>
<tr>
<td>UM vs. AC</td>
<td>0.1743</td>
<td>0.2911</td>
<td></td>
</tr>
<tr>
<td>CM vs. SA</td>
<td>0.5964</td>
<td>0.3298</td>
<td></td>
</tr>
<tr>
<td>CM vs. UM</td>
<td>0.7223</td>
<td>0.6277</td>
<td></td>
</tr>
<tr>
<td>SA vs. UM</td>
<td>0.3620</td>
<td>0.1371</td>
<td></td>
</tr>
<tr>
<td>CM+SA vs. UM</td>
<td>0.0030 *</td>
<td>0.0029 *</td>
<td></td>
</tr>
</tbody>
</table>

* Significant (p<0.05)
4.5 Plasma Levels of Cytokines on admission

4.5.1 Interleukin-10 (IL-10)

Circulating levels of IL-10 as measured in the plasma were enormously elevated in all categories of malaria patients compared to controls (p<0.0001). Among the patient groups, the severe anaemia group showed the lowest levels of IL-10. This was especially significant compared to the cerebral malaria group (CM vs. SA, p=0.007). The box plot in figure 4.8 illustrates the general distribution of IL-10 levels.

Figure 4.8 Plasma levels of IL-10
4.5.2 Tumour Necrosis Factor alpha (TNF-α)

Plasma levels of the cytokine TNF-α were found to be significantly increased in all groups of symptomatic malaria patients compared to the control group (p<0.05). Between the patient categories, both severe groups had significantly higher levels of the cytokine compared to the uncomplicated malaria group (CM vs. UM, p=0.016; SA vs. UM, p=0.05). The levels in the groups are shown in figure 4.9.

Figure 4.9 Plasma levels of TNF-alpha
4.5.3 Interleukin 8 (IL-8)

The plasma levels of IL-8 of the study subjects are shown in figure 4.10. The median value for the severe malaria anaemia category was slightly higher than those for cerebral and uncomplicated malaria groups. This difference was however not statistically significant. Levels of the cytokine in the control group were clearly lower than those in all the patient categories (P<0.05).
4.5.4 Neopterin

Following in the trends for IL-8, TNF-α and IL-10, circulating levels of neopterin were significantly elevated in all groups of subjects with symptomatic malaria compared to the community controls (p<0.001). Again similar to the IL-8 situation, the median value for the severe anaemia category was slightly higher than those for cerebral and uncomplicated malaria groups. However, this difference was not statistically significant.

Figure 4.11 Plasma levels of Neopterin
4.5.5 Macrophage Inflammatory Protein 1 alpha (MIP-1α)

Plasma levels of the chemokine MIP-1α for all the categories of subjects are shown in figure 4.12. The median values of MIP-1α for the categories were in the order AC < CM < SA < UM. The differences however, were not statistically significant (AC vs. CM, p=0.183; AC vs. SA, p=0.119; AC vs. p=0.100).

Figure 4.12 Plasma levels of MIP-1alpha
4.5.6 Macrophage Inflammatory Protein 1 beta (MIP-1β)

There was a significant elevation of MIP-1β levels in all the patient categories using the control group as a reference (SA vs. UM, p=0.005; CM vs. AC, p=0.01; UM vs. AC, p=0.002). Again the median value for the severe anaemia group was slightly higher than those for both cerebral and uncomplicated malaria categories, but the differences were not significant. The data is shown in figure 4.13.

Figure 4.13 Plasma levels of MIP-1beta
4.5.7 Correlation between cytokine levels.

There was strong positive correlation between the levels of all the cytokines measured except neopterin, which failed to show any significant relationship with any of the other cytokines. The various correlation coefficients and p-values for pairs of the cytokines are shown in table 4.4.

Table 4.4 Spearman Rank Order Correlation between pairs of cytokines. Cells are in the form: correlation coefficient, p-value. * Significant i.e. coefficient >0.25 and p<0.05.

<table>
<thead>
<tr>
<th></th>
<th>MIP-1β</th>
<th>TNF-α</th>
<th>IL-8</th>
<th>Neopterin</th>
<th>MIP-1α</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>0.463,</td>
<td>0.595,</td>
<td>0.403,</td>
<td>0.225,</td>
<td>0.260,</td>
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<tr>
<td></td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>0.0559</td>
<td>0.0876</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>0.603,</td>
<td>0.359,</td>
<td>-0.0990,</td>
<td>0.591,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.001*</td>
<td>0.001*</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.504,</td>
<td>-0.148,</td>
<td>0.532,</td>
<td>0.001*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.001*</td>
<td>0.254</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>0.0282,</td>
<td>0.399,</td>
<td>0.004*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.806</td>
<td></td>
<td>0.236</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neopterin</td>
<td></td>
<td></td>
<td></td>
<td>0.174,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.236</td>
<td></td>
</tr>
</tbody>
</table>
4.5.8 Correlation between Cytokine levels and clinical characteristics

There was no significant relationship between the levels of cytokines IL-10, TNF-\(\alpha\), IL-8 and MIP-1\(\alpha\) and any of the clinical characteristics parasitaemia i.e., haemoglobin and age. MIP-1\(\beta\) levels also did not show significant association with haemoglobin and age, but correlated quite significantly with parasitaemia (coefficient = 0.257, \(p=0.0275\)).

Neopterin levels showed a negative association with haemoglobin levels in all categories of patients, but differing in the strength of correlation from one category to another.

Whilst the negative correlation was significant in uncomplicated malaria (coefficient = -0.489, \(p=0.0382\)) and cerebral malaria (coefficient = -0.489, \(p=0.0388\)), it was below significance level in the severe malaria category (coefficient = -0.167, \(p=0.410\)). The trends are shown in figure 4.14.

**Figure 4.14a** Hb versus neopterin (SA)
Figure 4.14b Hb versus neopterin (CM)

Figure 4.14c Hb versus neopterin (UM)
4.6 Longitudinal Study of Cytokine Levels

Kinetic changes in plasma levels of all the cytokines measured were similar in all categories of patients studied. From Day 0 to Day 3, there was a significant reduction in plasma levels of all the cytokines for all patient categories to concentrations as low as that for the controls in some cases.

For IL-10 levels, mean concentration (25th-75th percentiles) for all the patients followed longitudinally reduced from 795 pg/ml (144-1108) on Day 0 to 19 pg/ml (6-23) on Day 3 (p<0.001). The level on Day 3 was not different from the mean level in the controls, which was 18 pg/ml (2-30). IL-10 levels remained within normal levels on Day 7 giving a mean of 28 pg/ml (13-32).

Mean levels of MIP-1β dropped from 610 pg/ml (119-693) on Day 0 to 49 pg/ml (12-61) on Day 3 (p<0.001). This level on Day 3 fell below the mean level of 122 pg/ml (69-134) of the controls (p<0.001). The level rose to 54 pg/ml (16-90) on Day 7.

For TNF-α, mean concentration changed from 104 pg/ml (26-98) on Day 0 to 27 pg/ml (3-52) on Day 3 (p=0.003), which was not different from the mean value of 12 pg/ml (6-17) for the controls (p=0.461).

With IL-8, levels fell significantly (p<0.001) from 111 pg/ml (18-121) on Day 0 to 14 pg/ml (10-20) on Day 3, but this was still above the mean concentration of 9 pg/ml (1-11) of the controls. It was not any lower on Day 7 at 16 pg/ml (9-20).
The levels of neopterin failed to normalize after three days of treatment but reduced from 81 nmol/L (55-102) on Day 0 to 46 nmol/L (36-54), which was still significantly higher than the mean of 23 nmol/L (8-33) of the controls (p<0.001). However, on Day 7 neopterin levels fell further to 34 nmol/L (24-39), which was within control levels (p=0.113).
CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.0 DISCUSSION

This study sought to investigate the effects of P. falciparum infection on monocyte and neutrophils function as a possible basis for increased destruction of unparasitized erythrocytes. Neutrophil phagocytic activity, expression of monocytes surface markers, HLA-DR, and FcγRIII, and plasma levels of mediators of inflammation were compared in children with severe malaria anaemia (SA) to others with other forms of malaria (CM and UM) or without malaria symptoms at all (AC) in two consecutive malaria seasons.

The results of the neutrophil phagocytic function assays from samples of both years indicate clearly that symptomatic malaria is generally associated with an increase in phagocytic activity of neutrophils. As predicted this enhanced phagocytic ability was most pronounced in the severe anaemia category where mean phagocytic index was more than three times that observed in the group of controls. For the year 2000 (figure 4.2), phagocytic indices for the severe anaemia category showed a wide distribution with individual patients recording phagocytic indices higher than 25 whilst others were not different from the controls. The possible reason for this is that the durations of infection in the individual patients in the group could be widely varied, making it a combination of acute and chronic cases as identified by Abdalla et al. (1980).

The results for year 2001 confirmed the observations in year 2000, and further provided evidence that indeed neutrophils of patients with severe anaemia had a greater ability to phagocytose beads than patients with uncomplicated or cerebral malaria (figure 4.3).
Further, there was considerable negative correlation between haemoglobin concentration and phagocytic index (figure 4.4) such that low haemoglobin seemed to be associated with high phagocytic index.

These results add to mounting evidence implicating immune-mediated removal of red blood cells as the major mechanism of anaemia among children in the holoendemic areas. There has been evidence that red blood cells were sensitized with C3 complement in malaria infections (Facer et al., 1979; Facer, 1980), and recently Goka et al. (2001) found a dose-dependent association between C3d binding to the erythrocyte membrane and the severity of anaemia in Ghanaian children with *P. falciparum* malaria. Also, a strong correlation was found between anaemia and the clearance of erythrocytes coated with relatively low numbers of IgG molecules (Ho et al., 1990b). Looareesuwan et al. (1987), in Thailand, demonstrated increased rates of erythrocyte destruction associated with *P. falciparum* and *P. vivax* infections. Red blood cell clearance rates were increased in proportion to disease severity and were highest in patients with severe malaria (Looareesuwan et al., 1991). This enhanced clearance was more marked with compatible donor erythrocytes than with autologous cells and was not mediated by antibody or complement. In the present study the target particles used were non-opsonized latex beads, so the phagocytic activities observed were largely non-specific.

These observations suggest an exoerythrocytic factor such as non-specific activation of the reticuloendothelial system, such that the threshold for recognizing cells to be removed from circulation is lower than normal, both for mechanical modifications and Fc receptor-mediated labeling. The findings in this study then imply that such non-
specific activation is more marked in severely anaemic patients than others with uncomplicated or cerebral malaria.

The increase in non-specific phagocytic activity in the malaria patients in this study is consistent with previously reported observations of increase in colloidal particle clearance shown by the reticuloendothelial system in rodent malaria (Cox et al., 1964 and Lucia and Nussenweig, 1969) and in human malaria (Sheagren et al., 1970). The observations in this study however, contrast the results of Ward et al., (1984) to the extent that they found that the percentage of phagocytic cells was the same in normal individuals and in those with uncomplicated malaria, and there was no significant difference in the number of Candida albicans phagocytosed per cell by monocytes from the two groups.

A longitudinal study of the changes in phagocytic index from the day of admission (Day 0) through Day 3 to Day 7 shows a decline in neutrophil phagocytic activity as patients receive anti-malarial treatment and parasitaemia clears. This is sufficient confirmation that the increased phagocytic activity exhibited by the neutrophils in the malaria patients compared to the controls is associated with the presence of the parasites.

The increased phagocytic activity observed is supported by the pattern of monocytes expression of CD16 shown in figure 4.6a. CD16 is one of a group of cell surface receptors that bind IgG, and has been shown to play the most critical role in regulating opsonic phagocytosis (Liao et al., 1994). The majority of peripheral blood monocytes strongly positive for the lipopolysaccharides (LPS)-receptor CD14 are negative for Fc gamma receptor type III (CD16). However, a subset of monocytes coexpressing CD14
and CD16 accounts for about 8% of all monocytes. This population exhibits features of tissue macrophages, and has been found to be largely expanded (>20%) during acute and chronic inflammatory diseases (Scherberich and Nockher, 1999). This seems to be the situation in malaria as observed in this study. In the controls studied, mean percentage of CD14+CD16+ cells was about 7%, but this doubled in the uncomplicated malaria group, tripled in the cerebral malaria category and was almost four-fold in those with severe malaria anaemia (an increase of about 20%). This expansion in CD14+CD16+ cell population which was most marked in the severe anaemia category is important because Scherberich and Nockher (1999) in their study found that CD14+CD16+ monocytes showed a higher phagocytosis rate than CD14+CD16 negative cells.

The observed reduction in mean fluorescence intensity of CD16 of CD14+ cells in malaria patients compared to controls shows that the expansion in population of CD16 expressing cells in malaria is actually accompanied by an increase in antibody-dependent phagocytosis. The mean fluorescence intensity is a measure of the average number of molecules of CD16 on each CD16+CD14+ cell. It is expected that with an increase in phagocytic activity part of which would be antibody-dependent, there is likely to be involvement of some CD16 molecules, which play a critical role in opsonic phagocytosis, and hence the depletion observed. Further, there has been evidence that the Fc gamma receptor III is even involved in non-opsonic phagocytosis. Liao et al. (1994) found that macrophages that had phagocytosed at least one non-opsonized bead showed reduced levels of surface Fc gamma receptor III but not Fc gamma R-I or Fc gamma R-II when compared with cells that had never been exposed to beads. Moreover, they found cells that were not in direct contact with beads, but that shared
medium with cells that had phagocytosed beads to also have reduced levels of Fc gamma R-III, suggesting a cytokine-mediated mechanism of down regulation.

Indeed it is interesting to note that the pattern of MFIs for the different categories is directly opposite to that seen for the percentages. The severe anaemia group had the lowest average MFI of about 60. That for the cerebral malaria group was a little higher at about 62, whilst those for uncomplicated malaria and controls were high at about 80 and 100 respectively.

HLA-DR is a marker of activation and is upregulated by IFN-gamma, which is one of the key cytokines in the pro-inflammatory response in malaria (Glimcher and Kara, 1992). So it was expected that HLA-DR would be more expressed in the patients compared to the matched controls. However, as can be seen from both figures 4.5a and 4.5b, the proportion of CD14+ cells expressing HLA-DR as well as the average number of HLA-DR molecules per cell were significantly lower in all categories of patients compared to the control group. Further, the severe cases (severe malaria anaemia and cerebral malaria) were found to show a significantly lower HLA-DR expression when pooled against the uncomplicated malaria cases. The levels of significance of these differences are shown in table 4.2.

There are two possible explanations for these observations. First, it could be a manifestation of the effects of hemozoin loading. Hemozoin is the detoxification product of the heme portion of degraded haemoglobin and piles up as electron dense material in the food vacuole of intraerythrocytic malaria parasites so that it is internalized by both circulating and resident phagocytes. MHC class II is upregulated in
human macrophages by TNF, IL-4 and granulocyte-macrophage colony-stimulating factor but the major physiological inducer is IFN-γ. Schwarzer et al. (1998) found expression of MHC class II after IFN-γ stimulation was blocked in hemozoin-loaded monocytes at the protein expression and gene transcription levels but was preserved in control monocytes loaded with opsonized latex beads or anti-D (Rh0)-immunoglobulin G (IgG)-opsonized human erythrocytes. Inhibition of protein kinase C (PKC) was suspected; as PKC mediated phosphorylations have been found to be involved in IFN-γ-mediated enhancement of MHC class II. The phenomenon had previously been reported in other bacterial and parasitic diseases; after ingestion of Mycobacterium, macrophages showed reduced expression of class II antigens (Gercken et al., 1994); Leishmania donovani, an obligate intracellular protozoan, suppressed macrophage expression of class II in response to IFN-γ stimulation (Reiner, 1994).

The finding in this study that this MHC class II down regulation was more pronounced in severe cases of malaria than in the uncomplicated malaria cases could be because the percentages of heavily hemozoin-laden leukocytes and macrophages seem to roughly correlate with the severity of the disease (Metzger et al., 1995).

Secondly, the reduced expression of HLA-DR in malaria patients could be a hint of systemic inflammation. Such observations have been reported in sepsis, where an initial hyper inflammatory phase characterized by excessive release of pro-inflammatory mediators is followed by a hypo-inflammatory one, associated with immunodeficiency (Volk et al., 1996). Such post-aggressive immunosuppression has been found to be characterized by a loss of HLA-class II antigen expression (Volk et al., 1991). Though the average reduction in percentage of HLA-DR+CD14+ seen in the malaria patients in the study (10-15%) falls short of the 30% considered to confirm monocyte deactivation.
(Volk et al., 1989), the results hint of an emerging “immunoparalysis”, and suggest that the inflammatory response in malaria, especially the severe cases, is more than appropriate. Indeed certain individual patients had percentage of HLA-DR+CD14+ cells as low as 61%, and this is alarming because such loss in MHC class II expression leads to deficiency in antigen presentation and T-cell reactivity, giving way to opportunistic infections. The immunosuppression would also favour parasite survival and multiplication in the host. This phenomenon of immunosuppression in malaria was first reported by Greenwood et al. (1972), and has been implicated in associations between malaria and Salmonella infections found in Gambia (Mabey et al., 1987) and recently in Cameroon (Ammah et al., 1999).

The idea of a hyper-inflammatory response in malaria infections is supported by the finding of highly elevated levels of most of the cytokines measured in this study. Moreover there was strong positive correlation between the levels of all these cytokines except neopterin (table 4.4), pointing to a generalized inflammation. The difference with neopterin is understandable since this cytokine is associated specifically with macrophage activation.

Median level of TNF-α was higher in the CM group than the UM and SA patient groups (Figure 4.9), whilst median level of IL-10 was lowest in SA (figure 4.8). This is consistent with recent reports by Kurtzhals et al. (1998) and Akanmori et al. (2000). However, a wider distribution of TNF levels in the SA group was observed as represented by the height of the box in figure 4.8, extending higher than the CM group. TNF presumably constitutes part of the protective immune response to the infection by inducing fever that is detrimental to parasite development, and/or by stimulating effector
cells (Kwiatkowski, 1989), but this pro-inflammatory response needs to be carefully controlled to prevent the damaging consequences of excessive TNF production. IL-10 is one of the cytokines expected to mediate this sort of anti-inflammatory regulation in malaria, so the low levels of this cytokine in the SA group in the midst of moderately high levels of TNF suggest that the excessive pro-inflammatory immune response may be insufficiently compensated by anti-inflammatory cytokines, therefore TNF may be exerting its damaging effects of causing dyserythropoiesis and increasing erythrophagocytosis (Clark and Chaudhri, 1988) in this SA group.

IL-8 is a neutrophils-specific chemoattractant, so the increased plasma levels observed in all malaria patient categories was expected because of the increased recruitment of neutrophils. It was also expected that IL-8 levels would be higher in the SA group than the UM and CM categories, but the differences observed in this study were not statistically significant.

Similarly, MIP-1α and MIP-1β are monocytes-specific chemoattractants and the same trend was expected. However, the median plasma levels of these chemokines in the SA group were not different from those of the CM and UM patient categories. High serum levels of MIP-1α and IL-8 have been reported in patients suffering from \textit{P falciparum} malaria (Burgmann et al., 1995). There have also been reports of increased levels of MIP-1β in HIV infected individuals (Tartakorsky \textit{et al.}, 1998 and Benveniste \textit{et al.}, 2000).

Activation of the human cellular immune system is associated with greatly increased formation of the pteridines neopterin and 7,8-dihydroneopterin and it has been
postulated that pteridines play a role in the pathogenesis of the anaemia of inflammation. Pagel et al. (1999) suggested that the release of pteridines by activated macrophages might inhibit the synthesis of erythropoietin. So in addition to being a marker of macrophage activity, neopterin may play a direct role in causing anaemia. It was therefore not surprising that the UM and CM categories of malaria patients in our study had median neopterin levels that were 3 times that for the control group whilst the SA group had almost a 4 fold amount.

It was significant to find that plasma concentrations of all the cytokines IL-10, TNF-α, MIP-1β and neopterin reduced to levels not different from those of the controls with the disappearance of the disease during seven days from start of treatment. Even for IL-8, which did not exactly normalize, levels were drastically reduced. These observations confirm that the elevated cytokine concentrations were associated with the diseased state, implicating them in the pathogenesis. Indeed it was found that two out of three patients who had persistent anaemia on Day 7 still maintained high levels of neopterin. This is in agreement with findings of Biemba et al., (1998) which showed that prolonged macrophage activation was partly to blame for persistent anaemia in children with complicated malaria.

The lack of significant direct associations between clinical characteristics and many of the cytokines was surprising. But it suggests that the causes of the distinct forms of pathology seen in malaria are multifactorial and cannot be explained by one single factor. This is why I believe that the finding that there is significant negative correlation between haemoglobin and neopterin concentrations in UM and CM patients but not in
SA was due to the multifactorial basis of the SA condition. It seems that macrophage activity plays a major role in the decline in haemoglobin concentrations seen in malaria in general, but the enormous depletion associated with the SA condition is as a result of a combination of other factors. Some of these factors have already been cited elsewhere in this text to include dyserythropoiesis, reversible bone marrow suppression and intravascular haemolysis.

4.2 CONCLUSIONS

The findings in this study confirm that there is an enormous stimulation of monocytes and neutrophils in *Plasmodium falciparum* infections giving rise to an increase in phagocytic activity and elevation of circulating levels of pro-inflammatory cytokines, which may not be sufficiently compensated for by anti-inflammatory cytokines. The results suggest that this activation is likely to have non-specific effects, and is more pronounced in severely anemic patients than in others. This confirms our hypothesis that activation of monocytes and neutrophils contributes to the anaemia of *P. falciparum* malaria. We believe that this exoerythrocytic activation of phagocytes is capable of increasing the destruction of unparasitized red blood cells by lowering the threshold for recognizing cells to be removed.

The study also found indications of post-aggressive immunosuppression in malaria showing as a down regulation in MHC class II expression on monocytes. This also needs further investigation because of the threat of opportunistic infections complicating malaria and leading to risk of death among infected children.
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