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AN IMMUNOHISTOCHEMICAL STUDY OF CYTOKINE AND ADHESION MOLECULE EXPRESSION IN HUMAN BRAIN IN FATAL CEREBRAL MALARIA.

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY (M.Phil.) DEGREE IN BIOMEDICAL SCIENCE (PATHOLOGY).

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DECLARATION

THE EXPERIMENTAL WORK DESCRIBED IN THIS THESIS WAS DONE BY ME, AT THE PATHOLOGY DEPARTMENT OF UNIVERSITY OF GHANA MEDICAL SCHOOL, KORLE-BU AND THE HISTOLOGY AND ELECTRON MICROSCOPY UNIT OF NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH, LEGON, UNDER THE SUPERVISION OF PROF. E. K. WIREDU (ASSOCIATE PROFESSOR OF PATHOLOGY & DEAN OF SCHOOL OF ALLIED HEALTH SCIENCES) AND PROF. A. A. ADJEI (ASSOCIATE PROFESSOR OF IMMUNOLOGY & HEAD OF DEPARTMENT OF MEDICAL LABORATORY SCIENCES).

ALL THE WORK RECORDED IN THIS THESIS IS ORIGINAL, UNLESS OTHERWISE ACKNOWLEDGED IN THE TEXT OR BY THE REFERENCES CITED. THIS WORK HAS ALSO NOT IN IT'S PRESENT FORM OR OTHERWISE BEEN SUBMITTED TO THIS OR ANY OTHER UNIVERSITY FOR THE AWARD OF A HIGHER DEGREE.

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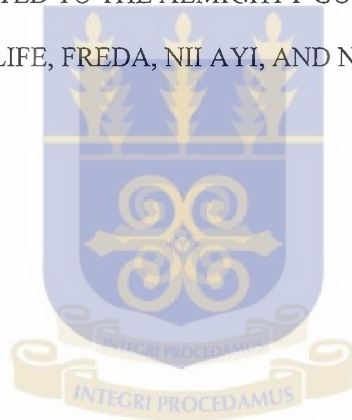
DEDICATION

THIS THESIS IS DEDICATED TO THE ALMIGHTY GOD, AND THE THREE LOVES OF MY LIFE, FREDA, NII AYI, AND NII ARMAH.



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LIST OF ABBREVIATIONS

APAAP	-	Alkaline Phosphatase Anti-Alkaline Phosphatase
BBB		Blood Brain Barrier
BDU		Bleeding Duodenal Ulcer
BS	-	Brain Stem
C	-	Cerebrum
CB	-	Cerebellum
CD	-	Cluster of Differentiation
CIDR	-	Cystine-Rich Interdomain Region
CNS	-	Central Nervous System
CM	-	Cerebral Malaria
CSA		Chondroitin Sulphate-A/Chondroitin-4-Sulphate
CSF	-	Cerebrospinal Fluid
CT	-	Computerized Tomography
DARC	-	Duffy Antigen Receptor for Chemokines
DBL		Duffy Binding-Like
EC	-	Endothelial Cell
FMCM	-	Fatal Murine Cerebral Malaria
GFAP	-	Glial Fibrillary Acid Protein
GHS	-	Ghana Health Service
GPI	-	Glycosphosphatidylinositol
HLA		Human Leukocyte Antigen
HRP		Histidine-Rich Protein
IFN	-	Interferon
Ig	-	Immunoglobulin

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IHC	**-**	**Immunohistochemical/ Immunohistochemistry**
IL		**Interleukin**
iNOS/NOS-2	**-**	**Inducible Nitric Oxide Synthase/Nitric Oxide Synthase-2**
ISH	**-**	***In-situ* Hybridisation**
ITMs	**-**	**Insecticide Treated Materials**
IUGR	**-**	**Intrauterine Growth Retardation**
MHC	**-**	**Major Histocompatibility Complex**
MOH		**Ministry of Health**
mRNA	**-**	**Messenger Ribonucleic Acid**
MSP		**Merozoite Surface Protein**
NCM	**-**	**Non-Cerebral Malaria**
NCNSI		**Non-Central Nervous System Infection**
NI		**Non-Infection**
NM	**-**	**Non-Malaria**
NO		**Nitric Oxide**
NPEs	**-**	**Non-Parasitized Erythrocytes**
P.	**-**	***Plasmodium***
PBM		**Purulent Bacterial Meningitis**
PEs	**-**	**Parasitized Erythrocytes**
PfEMP	**-**	***Plasmodium falciparum* Erythrocyte Membrane Protein**
PTD		**Preterm Delivery**
RBM	**-**	**Roll Back Malaria**
ROS	**-**	**Reactive Oxygen Species**
RT-PCR	**-**	**Reverse Transcriptase-Polymerase Chain Reaction**
sCAMs		**Soluble Cell Adhesion Molecules**
sE		**Soluble Endothelial**

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sICAM	**-**	**Soluble Intercellular Adhesion Molecule**
SMA	**-**	**Severe Malarial Anaemia**
sVCAM	**-**	**Soluble Vascular Cell Adhesion Molecule**
TGF	**-**	**Transforming Growth Factor**
TNF	**-**	**Tumour Necrosis Factor**
TP&S	**-**	**Typhoid Perforation & Septicaemia**
TSP	**-**	**Thrombospondin**
UM	**-**	**Uncomplicated Malaria**
WHO	**-**	**World Health Organisation**

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ABSTRACT

Introduction: Although the role of systemic proinflammatory cytokines, IL-1β and TNF-α, and their up-regulation of adhesion molecules, ICAM-1, VCAM-1 and E-Selectin, in the pathogenesis of cerebral malaria (CM) is well established, the role of local cytokine release remain unclear.

Methods: Immunohistochemistry (IHC) was used to compare the expression of ICAM-1, VCAM-1, E-Selectin, IL-1β, TNF-α and TGF- β at light microscopic level in cerebral, cerebellar and brainstem postmortem cryostat sections from 10 CM, 5 severe malarial anaemia (SMA), 1 purulent bacterial meningitis (PBM), 2 non-central nervous system infections (NCNSI) and 3 non-infections (NI) deaths in Ghanaian children.

Results: Fatal malaria and *Salmonella* sepsis showed significantly higher vascular expression of all 3 adhesion molecules, though their expression in the sepsis case was not as intense as that in the fatal malaria sections. There was highly significant co-localization of receptor expression with sequestration in the malaria cases, though there was negligible difference in their expression between the CM and SMA sections. TGF-β showed intravascular and perivascular distribution in all cases, but expression was most intense in the PBM case and CM group. TNF-α and IL-1β showed prominent brain parenchymal staining, in addition to intravascular and perivascular staining, in only the PBM case and CM group. The increased expression of the adhesion molecules was associated with increased local proinflammatory cytokine release in the CM sections, but not in the SMA group. The maximal expression of all 6 antigens studied was in the cerebellar sections of the malaria cases.

Conclusions: Endothelial activation is a feature of fatal malaria and *Salmonella* sepsis, with adhesion molecule expression being highly co-localized with sequestration in fatal malaria. IL-1β and TNF-α are expressed in only cases with neurodegenerative lesions, whilst TGF-β is present in all cases. Both cytokines and adhesion molecules were maximally expressed in the cerebellar sections of the malaria cases.

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Malaria is the name denoting the disease or condition in man caused by parasites belonging to the genus *Plasmodium (P).* The term was originally restricted to plasmodial infections in man, but it now includes all infections caused by organisms belonging to the family *Plasmodiidae*, which are therefore commonly referred to as the malaria parasites. Malaria cause more death and disease than any other known human pathogen [World Health Organization (WHO), 1984].

The four species naturally infective to man are *P falciparum, P. malariae, P vivax, and P. ovale.* Of these, *P falciparum* is the most widespread in Africa south of the Sahara and is also the principal cause of severe malarial disease, since the other species of malaria rarely cause death or persistent sequelae. Malaria is transmitted to man by *Anopheles* mosquito, and its transmission is directly associated with the feeding habits of the female *Anopheles* mosquito, which sucks blood from man and other animals. The male *Anopheles* mosquito feeds solely on plant juices and does not play any direct role in the transmission of malaria.

Malaria is geographically confined to areas where the average summer temperature is above 16°C, thus most of the world, except Canada and the northern parts of Europe and Asia, has sometime in the past been affected (Wernsdorfer, 1980). Today, all Europe, the United States, most of the former Soviet Union and Australia are free of malaria. *P. falciparum* infections are thus restricted to the subtropical and tropical areas of the World (Wernsdorfer, 1980). Malaria is one of the most common and important parasitic diseases of man worldwide. It continues to be a major cause of morbidity and mortality in the tropics and

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subtropics, with about 40% of the world's population living in malaria-endemic areas which comprise more than half of the countries of the world (Sturchler, 1990).

Malaria is responsible for up to 500 million episodes of clinical infection and 2.7 million deaths every year, with more than one million of these deaths occurring in African children (WHO, 1996). Of these deaths, the overwhelming majority occur among children aged 5 years or younger, and 90% of the deaths each year are in rural sub-Saharan Africa. These deaths are unnecessary, since malaria is preventable and treatable, but ironically inevitable, because of lack of prevention and treatment due to poverty, war, and other economic and social instabilities in areas of malaria endemicity.

Malaria is a major cause and consequence of poverty in Africa. The African Summit on Roll Back Malaria (RBM), Abuja, Nigeria, April 2000, declared that the control of malaria would significantly increase Africa's economic productivity and the income of African families. The Group of the World's Eight Most Industrialized Nations (G8) Summit in Japan, July 2000, called for a 50% reduction in the malaria burden by the year 2010. Despite technological advances and global economic development, malaria is still the parasitic disease responsible for the greatest number of deaths worldwide. Malaria parasites infects between 300 to 500 million people, causing up to 2 million deaths globally per year (mostly children in sub-Saharan Africa) from complications of primarily cerebral malaria (CM) and severe malarial anaemia (SMA) [TDR., 1998].

P. falciparum malaria is endemic in Ghana and continues to be a major cause of morbidity and mortality in both the young and the old. It exacts a toll on human life grave enough to constitute a threat to economic and social development. It accounts for approximately 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 approximately 42% of all attendances (Ahmed, 1989).

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Morbidity and mortality figures may even be higher now. Characteristically, malaria is more prevalent in the rural areas, and children appear to be resistant to serious illness during the first 3 months of life (Afari *et al.*, 1995). Since the year 1999, Ghana has been implementing the RBM initiative with the objective of reducing malaria-related morbidity and mortality to a level that it will cease to be of public health importance. The initiative focuses on reducing incidence through the use of insecticide impregnated materials (ITMs), prompt diagnosis and proper treatment of all malaria cases, including improved quality of home-based clinical case management of uncomplicated malaria [Ministry of Health (MOH)/ Ghana Health Service (GHS), 2001].

CM is an important complication of *P. falciparum* infection, which is characterised by seizures and rapid deterioration in level of consciousness, leading to coma with a generally poor prognosis with mortality rate of 15-20% (Newton & Krishna, 1998). Despite this high mortality rate, the pathogenic mechanisms of CM have not been well elucidated. Little is known about the blood brain barrier (BBB) interactions in CM that result in the neurological disorder, and it is unclear how the intraerythrocytic parasite, which sequesters in the cerebral microvasculature but rarely enters the brain parenchyma, influence parenchymal function to induce coma and death. The unavailability of infected human specimens and suitable animal models has hindered a thorough understanding of the pathogenesis.

The *Plasmodium* sporozoites inoculated into the human host by infected female *Anopheles* mosquito, invade hepatocytes and become merozoites, which invade and reproduce in red blood cells as trophozoites. Ruptured red blood cells release merozoites that infect other red blood cells. *P. falciparum* strains induce rosetting and cytoadherence to erythrocytes leading to obstruction of blood flow in the microvasculature resulting in tissue ischaemia, reduced oxygen, nutrient deficiency and acute inflammation (Mazier *et al.*, 2000). These severe conditions occurring at the blood brain barrier (BBB) result in the diffuse, potentially reversible, brain pathology referred to as cerebral malaria (CM).

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The sequestration of parasitized erythrocytes (PEs) in the microvasculature of vital organs is central to the pathogenesis of severe *P. falciparum* malaria. It is unclear how the intraerythrocytic parasite, which sequesters in the cerebral microvasculature but does not enter the brain parenchyma, induce such a devastating neurological syndrome. Sequestration is mediated by specific interactions between parasite adherence ligands and host receptors on vascular endothelium, such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, cluster of differentiation (CD)-36, and endothelial (E)-Selectin.

Quantitatively, there are more PEs in the brain of human CM, than non-cerebral malaria (NCM), though it has been suggested that sequestration was not solely sufficient to cause CM (Turner *et al.,* 1994). Furthermore, they demonstrated a highly significant immunohistochemical (IHC) co-localization of sequestration with the expression of ICAM-1, CD36 and E-Selectin in human cerebral vessels, but no cellular inflammatory response, suggesting that these receptors have a role in sequestration in vivo and indicate that systemic endothelial activation is a feature of fatal malaria (Turner *et al.,* 1994).

Human studies have shown elevated plasma levels of proinflammatory cytokines, especially interleukin (IL)-1β and tumour necrosis factor (TNF)-α, in complicated malaria, with TNF-α appearing to play a central role. In African children, plasma concentrations of cytokines, particularly TNF-α, are higher in fatal than non-fatal CM and higher in children with CM than those with uncomplicated malaria (UM) [Kwiatkowski *et al.,* 1990]. Inhibition of TNF-α directly affects the disease outcome of severe malaria (Looareesuwan *et al.,* 1999).

However, recent evidence from mice indicates that it may be overproduction of lymphotoxin-α (LT-α) rather than TNF-α that leads to CM, since mice deficient in TNF-α were found to be just as susceptible to CM as controls whereas LT-α deficient mice were resistant to CM pathology, dying from hyperparasitaemia and severe anaemia instead (Engwerda *et al.,* 2002).

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In addition to systemic production, local cytokines release and their interaction with adhesion molecules have been suggested to be involved in organ-specific pathology in severe malaria, especially CM. There is compelling evidence that proinflammatory cytokines, including local IL-1β and TNF-α release, mediate the cerebral dysfunction in human CM (Artavanis *et al.*, 2003 ; Brown *et al.*, 1999b ; Udomsangpetch *et al.*, 1997 ; Porta *et al.*, 1993). However, the lack of a classical inflammatory response to the presence of PEs in the brain microvasculature indicates anti-inflammatory cytokine involvement.

TGF-β has been found in both haemorrhagic white-matter lesions of human CM (Brown *et al.*, 1999b), and in white-matter lesions of human immunodeficiency virus (HIV)-1 encephalitis brain samples (Johnson & Gold., 1996). This association of TGF-β with central nervous system (CNS) neurodegenerative lesions suggests an anti-inflammatory and neuroprotective role for TGF-β in the host defense mechanism against neuronal loss in neurodegenerative diseases (Brown *et al.*, 1999b ; Flanders *et al.*, 1998). Furthermore, in mice infected with lethal or non-lethal strains of malaria parasites, a strong and sustained TGF-β response, beginning on the 5th to 6th post-infection day when the peak parasite replication has been reached, was associated with abrogation of mortality and resolution of infection. Furthermore, neutralization of TGF-β leads to 100% mortality in BALB/c mice infected with normally non-lethal *P chabaudi* A/J (Omer & Riley., 1998).

Individuals vary in the amount of TNF-α produced when their peripheral blood mononuclear cells are stimulated in vitro, and family studies indicate that much of this variability is genetically determined (Knight & Kwiatkowski, 1999). It has also been suggested that the genotypes of the infecting *P. falciparum* may also contribute to this variability. Indeed, there may be a number of host and parasite parameters that influence the outcome of malaria infection in the Ghanaian child.

A study using *in-situ* hybridisation (ISH) and IHC showed TNF-α messenger ribonucleic acid (mRNA) and protein were expressed in microglia and astrocytes, monocytes

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in the spleen, and cerebral vascular endothelium in fatal murine cerebral malaria (FMCM) mice but not uninfected animals. Additionally, IL-1β mRNA was found in the brains of both uninfected and FMCM mice, however, IL-1β protein was expressed only in monocytes, the meningeal vascular endothelium, and neurons in the frontoparietal cortex in the FMCM brains (Medana *et al.*, 1997).

In another study using IHC and reverse transcriptase polymerase chain reaction (RT-PCR) on human brain postmortem tissues, TGF-β was expressed in normal brain, in CM, and in meningitis and encephalitis, whilst IL-1β was absent from normal brain but was detected in CM and other cerebral infections. Furthermore, TNF-α mRNA was expressed only in CM, although TNF-α protein was also seen in meningitis. But mRNA expression of these cytokines did not correlate with PE density as detected using RT-PCR for merozoite surface protein (MSP)-2 mRNA (Brown *et al.*, 1999b).

In-vitro studies have shown TNF-α mRNA in inflammatory infiltrates within the meninges of experimental rabbit pneumococcal meningitis (Bitsch *et al.*, 1997), and upregulation of neuronal TNF-α expression in response to bacterial lipopolysaccharide (Gahring *et al.*, 1996). IL-1β is not expressed in normal human brain, but induced and expressed intraparenchymally in human CM brain and in meningeal infiltrating leukocytes of human meningoencephalitides cases (Brown *et al.*, 1999b). In *in-vitro* studies, IL-1β is neurotoxic and rapidly induced in response to neuronal cell death, and therefore is suggested to play a causal role in ischaemic cell death and neurodegeneration in the brain (Rothwell & Strijbos, 1995).

Tongren and colleagues (2000) showed that the proinflammatory cytokines (IL-1β and TNF-α) and TH-1 cytokine [interferon (IFN)-γ] had the highest level of mRNA expression in the cerebellum during late *P. coatneyi* infection in rhesus monkeys, agreeing with histopathologic observations of the preferential sequestration of PE in the cerebellum in

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rhesus monkey (Tongren *et al.*, 2000 ; Smith *et al.*, 1996 ; Sein *et al.*, 1993b), and in human CM (Sein *et al.*, 1993a).

Recently, IHC studies on autopsy brain tissue from 8 cases of CM in Malawian children showed activation of endothelial cells and macrophages, and disruption of endothelial intercellular junctions in vessels containing sequestered PEs but no gross leakage of plasma proteins. However, the examination of the partition of albumin between circulating plasma and the cerebrospinal fluid (CSF) from 72 cases of CM showed subtle but measurable changes compatible with impaired BBB function in CM, suggesting that BBB breakdown occurs in areas of parasite sequestration in CM in the children. (Brown *et al.*, 2001).

1.2 HYPOTHESIS

As the mortality rate of 20-30% for severe falciparum malaria under even the best clinical conditions testifies, access to anti-malarial drugs is not sufficient to prevent an appreciable mortality from the disease (Clark & Schofield, 2000). Since changes to the management of malaria over the last 40 years have not significantly improved survival, a reduction in the mortality and morbidity may only come about by a better understanding of the pathophysiological processes that are responsible for severe disease and that determine the outcome before antimalarials have had time to work (Newton & Krishna, 1998).

Understanding the cause of death at a cellular level is essential, if additional rational treatments are to be developed. Studies indicate that there is increased production of proinflammatory cytokines (both systemically and locally) which subsequently upregulates endothelial adhesion molecules that facilitate sticking of PEs to endothelium (Ockenhouse *et al.*, 1992). Little is known about the BBB interactions in CM that result in the neurological disorder. This makes the study of the expression and distribution of cytokines and adhesion molecules in human brain tissue the logical next step in the bid to further elucidate the pathophysiology of CM.

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Increased expression of endothelial adhesion molecules resulting from raised host cytokine production (both systemically and locally) have been shown to occur in *in-vitro* animal models of CM, but these changes have been localized using immunohistochemical methods in only a handful of *in-vivo* human postmortem tissue studies of CM in African children, who bear the brunt of the disease, to compare and contrast findings. We therefore believe that by comparing cytokine and adhesion molecule expression in human postmortem brain tissue of CM, NCM and non-malaria (NM) cases using IHC, a better understanding of the cells responsible for the local production of these mediators can be ascertained.

Our hypothesis for this project is that the human CM postmortem brain tissue when compared to brain tissue from purulent bacterial meningitis (PBM), severe malarial anaemia (SMA), non-central nervous system infection (NCNSI) and non-infection (NI) cases, will show significant differences in the expression and distribution of cytokines and adhesion molecules.

1.3 AIM AND SPECIFIC OBJECTIVES

The main aim or overall objective of this project is to ascertain the role of cytokines and adhesion molecules in fatal CM.

The specific objectives of this project are;

(1) To compare the expression of ICAM-1, VCAM-1, E-Selectin, TNF-α, IL-1β and TGF- β in cerebral, cerebellar and brainstem postmortem sections of human CM;

(2) To compare the expression of these 6 antigens in postmortem brain sections of human CM to four other groups of diseases [namely severe malarial anaemia (SMA), purulent bacterial meningitis (PBM), non-central nervous system infections (NCNSI) and non-infections (NI)]; and

(3) To ascertain whether there is any significant co-localization of sequestration of PEs with the expression of ICAM-1, VCAM-1 and E-Selectin.

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1.4 OUTPUT OF THE STUDY

Although, the role of cytokines and adhesion molecules has been extensively studied in human malaria, the role of local cytokine release and associated up-regulation of adhesion molecules in human CM has not been adequately confirmed and substantiated using immunohistochemical (IHC) methods, especially in sub-Saharan African children who bear the brunt of malaria mortality.

This study is aimed at defining the role played by cytokines and adhesion molecules in the exacerbation of fatal CM in Ghanaian children, so as to complement the knowledge already obtained from Caucasian studies and animal models, since there are only a handful of immunohistological studies of postmortem tissue from African children with CM, and to compare and contrast findings.

1.5 BENEFICIARIES OF THE STUDY

This study will be of immense benefit to all the people in the world suffering from malaria by helping to develop novel strategies for the prevention of fatality from severe complicated clinical malaria. Malaria remains one of the most important health problems in sub-Saharan Africa and the development of a vaccine and effective treatment strategies against the disease, including immunotherapy, are among the main goals of the WHO.

Since immunotherapy with inhibitors or antibodies against immune mediators implicated in severe malarial disease are being developed for therapeutic use especially in CM in children to prevent fatality, it is clearly important to define the pattern of expression of these mediators in human postmortem brain tissue from Ghanaian children dying of CM. It is also the expectation that the Ministry of Health and Ghana as a whole will benefit immensely from an early development of immunotherapeutic agents, which will reduce the mortality from severe falciparum malaria.

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CHAPTER 2

LITERATURE REVIEW

2.1 THE CLINICAL MANIFESTATIONS OF MALARIA

The disease, malaria, is caused by protozoan parasites belonging to the genus *Plasmodium*. The term was originally restricted to plasmodial infections in man, but it now includes all infections caused by organisms belonging to the family *Plasmodiidae*, which are therefore commonly referred to as the malaria parasites. Malaria is as old as humankind, and cases are reportedly recorded in written documents dating from the sixth millennium B.C. Quinine, a toxic plant alkaloid made from the bark of the *Cinchona* tree in South America ("Peruvian bark"), was used to treat malaria more than 350 years ago, and Jesuit missionaries in South America introduced it into Europe by the 1630s and into India by 1657 (Desowitz, 1991).

The important determinants of the clinical pattern of malaria include species of parasite, age and immune status of infected person, degree of endemicity, vector population and inoculum size. The clinical manifestations of severe malaria are determined by the degree of immunity in the affected child, as well as genotypic predispositions. The parasite and host features that determine why a child who has been living in a malaria endemic area and who is likely to have had extensive previous exposure to infection, progresses to develop severe disease are still largely undefined (Newton & Krishna, 1998).

Initially, the children are protected by maternal factors (e.g. transplacental acquisition of maternal antibodies), intrinsic factors (e.g. foetal haemoglobin) and other factors such as milk diets and vector avoidance behaviour by mothers. The protective effect of these factors, however, begins to wane during the first 6 months, so that severe disease leading to death is maximal between the ages of 1 and 4 years and is more likely in younger children. Thereafter, the children acquire immunity, so that adults who have lived continuously in

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endemic areas rarely develop severe disease. In contrast, children and adults who have not been exposed to malaria previously can rapidly develop severe disease after exposure (Newton & Krishna, 1998).

The clinical manifestation of malaria ranges from the mild disease to severe, potentially fatal, syndromes such as CM, severe malarial anaemia (SMA) and multi-organ failure. Severe malaria is a spectrum of clinical syndromes unified by the single causative organism *P. falciparum*. It can be operationally defined as any malaria syndrome that is associated with a high mortality (>5%), even after appropriate hospital treatment (Newton & Krishna, 1998). In African children, the commonest presenting syndromes associated with significant mortality are CM, recurrent convulsions, metabolic dysfunction (manifesting as hypoglycaemia or lactic acidosis), and symptomatic anaemia. Children with the greatest risk of mortality can be identified as those with impaired consciousness or respiratory distress (Newton & Krishna, 1998).

Severe falciparum malaria is one of the most lethal parasitic infections in the world and is responsible for more than one million deaths in African children per year. Three overlapping clinical syndromes, metabolic acidosis manifesting as hyperpnea, cerebral malaria and severe anaemia, are responsible for nearly all the deaths in African children. In endemic areas, infants and young children are at higher risk for SMA, older children for CM, and primigravida women for infections that will result in anaemia and delivery of low birth weight babies, a consequence of placental malaria (Newton & Krishna, 1998).

Only the asexual erythrocytic stage of the malaria parasite lifecycle is associated with pathology. The incubation period of malaria is generally within 10 to 20 days. It is usually followed by the cold stage characterised by chills, intense cold and sometimes headache and digestive disturbances. The cold stage lasts for about an hour and is immediately followed by the hot stage with continuous fever, intense headache, anorexia, nausea and vomiting. The temperature commonly rises up to about 41°C or more and patient feels very thirsty. This

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stage can last for about 4 hours and has the tendency to recur periodically (Wernsdorfer, 1980).

The periodicity of these cardinal signs of malaria coincides with the synchronized rupture of PEs and the release of merozoites and toxins into the bloodstream. Characteristic names deriving from this periodicity are given to the type of malaria caused by the corresponding species of the parasite. Both *P. vivax* and *ovale* cause benign tertian malaria with an erythrocytic schizogony that lasts 48 hours. *P. malariae* causes benign quartan malaria with an erythrocytic schizogony that lasts 72 hours. *P falciparium* is the most virulent with an erythrocytic schizogony lasting 48 hours and hence causes malignant tertian malaria (Wernsdorfer, 1980).

Anaemia, and particularly severe anemia, is a common complication of malaria because completion of each erythrocytic cycle is associated with the rupture of PEs. It is very serious in persons at risk such as pregnant women, children and expatriates (Steketee *et al.*, 1996). Although severe anaemia is a common cause of admission, if blood transfusions are given quickly to those with cardiorespiratory distress, the mortality is low on its own, but much higher when associated with CM or acidosis (Marsh *et al.*, 1995).

Clinical syndromes of severe malaria exhibit significant overlap, and CM is frequently complicated by lactic acidosis and/or hypoglycaemia. Multi-organ involvement is relatively uncommon in children compared to adults with severe disease. The age distribution of these syndromes is different, so that SMA affects younger children than CM, with respiratory distress overlapping both syndromes (Marsh *et al.*, 1995). Most deaths from severe malaria occur within 24 hours of starting treatment (Marsh *et al.*, 1995 ; Waller *et al.*, 1995 ; Krishna *et al.*, 1994 ; Walker *et al.*, 1992 ; Newton *et al.*, 1991 ; Molyneux *et al.*, 1989), and most of those who survive make full recoveries within 48 hours of starting treatment (Waller *et al.*, 1995; Krishna *et al.*, 1994; Walker *et al.*, 1992; Gordeuk *et al.*, 1992; Molyneux *et al.*, 1989).

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The syndrome of CM is clinically discrete and relatively easily identified and has, therefore, been the subject of most of the published studies on severe malaria. However, it is important to appreciate that CM is one of the commonest manifestations of severe disease, but that severe malaria is not synonymous with CM. CM is a syndrome of impairment of consciousness associated with a falciparum malaria infection. Impairment of consciousness caused by other species of malaria has been described, but in these case reports, other causes of an encephalopathy have not been excluded and rigorous postmortem studies are lacking (Newton & Krishna, 1998).

CM generally has a poor prognosis with mortality of 15-20%, even with the use of anti-malarial drugs and active supportive measures (Newton & Krishna, 1998 ; Brewster *et al.*, 1990 ; Greenwood *et al.*, 1987). Careful follow-up studies have shown permanent neurological complications in some patients who recovered from the illness (Bondi, 1992). There are possible long-term consequences such as learning difficulties in children who recovered from the illness (Holding *et al.*, 1999 ; Brewster *et al.*, 1990).

2. 2 EPIDEMIOLOGY AND SOCIOECONOMIC SIGNIFICANCE OF MALARIA

Malaria is geographically confined to areas where the average summer temperature is above 16°C (Wernsdorfer, 1980), thus most of the world, except Canada and the northern parts of Europe and Asia, has sometime in the past been affected. The epidemiological situation has not always looked the same and dramatic changes have occurred during the centuries. Thus, it is thought that *P falciparum* malaria was not present in the Americas until the 15th century when it was probably introduced by the conquestadores and African slaves (Wernsdorfer, 1980). After this last major expansion of *P falciparum* malaria, the epidemiological situation remained mainly unchanged until the second half of the 19th century. Today, all Europe, the United States, most of the former Soviet Union and Australia

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are free of malaria. *P falciparum* infections are, thus, restricted to the subtropical and tropical areas of the World (Wernsdorfer, 1980).

The frequency of malaria infection vary dramatically between different regions. Malaria is called epidemic when the incidence rises rapidly above the usual level or when the infection occurs in an area where it was not previously present. Malaria has been called endemic in certain areas where there is a constant measurable incidence of natural transmission over several years. Palpable splenomegaly associated with intermittent fever and chills is usually considered as an index of chronic malaria. It is very common in areas with repeated infection and also when chemoprophylaxis with choloroquine is applied in drug resistant areas (Wernsdorfer, 1980).

The spleen size increases with the duration and the intensity of exposure to infection, and usually persists for several weeks after the clearance of the malaria infection. Malaria endemicity has been related to the spleen rate (percentage of individuals with clinically palpable spleen) in children (aged 2-9 years) and adults. The resulting endemicity classification is as follows: Hypoendemic (spleen rate in children of 0-10%); Mesoendemic (spleen rate in children of 11-49%); Hyperendemic (spleen rate in children of >50%, and also high spleen rate in adults); and Holoendemic (spleen rate in children of >75%, but low spleen rate in adults) [Wernsdorfer, 1980].

The prevalence of malaria has been increasing. Each year, up to 500 million people suffer from the disease, and as many as 2.7 million individuals, mostly African children, succumb and die. Severe falciparum malaria is one of the most lethal parasitic infections in the world and is responsible for more than one million deaths in African children per year (WHO, 1996). *P. falciparum* causes the most potentially dangerous malaria infection in man while *P. vivax* and *P. ovale* malarias are less dangerous although they cause significant morbidity. *P. malariae* is a rare infectious agent which sometimes induces severe complications like nephrotic syndrome (Wilcocks & Manson-Bahr, 1982).

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Falciparum malaria is the most lethal and frequently occuring form of the disease throughout the tropics and subtropics. Vivax malaria covers the widest geographic area, including temperate, tropical and subtropical zone, however, it does not occur in large areas of tropical Africa. On the other hand, ovale malaria is found mainly in tropical Africa, and malariae malaria is widely distributed, but it is not as common as vivax malaria. Significantly, *P. ovale* is still neither present in South nor in Central America (Wernsdorfer, 1980). *P. malariae* is the only human malaria parasite which can naturally infect non-human primates. Three other *Plasmodiidae* can infect man although extremely rarely (namely, *P cynomolgi, P. knowlesi,* and *P. simium),* and when occuring these are invariably laboratory infections (Wilcocks & Manson-Bahr, 1982 ; Wernsdorfer, 1980).

Malaria is one of the most common and important parasitic diseases of man worldwide. It continues to be a major cause of morbidity and mortality in the tropics and subtropics, with about 40% of the world's population living in malaria-endemic areas which include more than half of the countries of the world (Sturchler, 1990). Over 80% of all clinical cases and 90% of asymptomatic cases of malaria occur in countries in tropical Africa and it remains one of the major childhood killers in tropical Africa, taking the life of 1 out of every 20 children before the age of 5, putting children at the most risk (WHO, 1992). Other vulnerable groups, apart from children under five years, are pregnant women, particularly those in their first pregnancy and migratory populations including refugees and visitors.

Malarial infection probably results in 3.5 million low birth-weight infants every year (Steketee *et al.,* 1996), since an estimated 24 million pregnant women live in malaria-endemic areas. Sullivan and colleagues (1999) observed that cord blood parasitemia, placental parasitemia, and postdelivery maternal peripheral parasitemia were associated with preterm delivery (PTD). Additionally, parasitemia and/or clinically diagnosed malaria in the antenatal period were associated with intrauterine growth retardation (IUGR), and delivery parasitemia had borderline associations with IUGR (Sullivan *et al.*, 1999).

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Plasmodium falciparum is the principal cause of severe disease, since the other species of malaria rarely cause death or persistent sequelae. *P. falciparum* may infect humans at any time from conception to death. Children living in sub-Saharan Africa bear the brunt of the disease, as they are exposed to malaria frequently after birth, and either die from complications or experience clinical episodes of infection for many years until the slow and capricious development of antimalarial immunity (Edington, 1967).

Malaria is a major cause and consequence of poverty in Africa. The African Summit on RBM, Abuja, Nigeria, April 2000, declared that the control of malaria would significantly increase Africa's economic productivity and the income of African families. The G8 Summit in Japan, July 2000, called for a 50% reduction in the malaria burden by the year 2010. Taking into account factors such as tropical location, colonial history, and geographical isolation, countries with intensive malaria had income levels in 1995 of only 33% of those countries without malaria (Gallup & Sachs, 2001).

The situation is made considerably worse by the rapid spread of drug-resistant strains of the malaria parasite and insecticide-resistant mosquitoes. Because of its severe impact on the health of children, malaria is an impediment to education since school absenteeism due to malaria remains a detrimental factor in children's acquisition of the knowledge required to contribute to their country's development. Malaria is, therefore, of increasing public health importance in Ghana, where parasites are increasingly resistant to chloroquine, a relatively inexpensive, low-toxicity, and highly effective antimalarial drug [Ministry of Health (MOH)/ Ghana Health Service (GHS), 2001].

2. 3 THE MALARIA PARASITES

The pathogenic agent of malaria is a protozoan parasite belonging to the animal kingdom, the sub-phylum *Sporozoa*, the class *Telosporida* and sub-class *Haemosporidiida*, the order *Coccidida*, sub-order *Haemosporidiidea*, family *Plasmodiidae* and genus

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Plasmodium (Bruce-Chwatt, 1985). The zoological family of *Plasmodiidae* include the parasites which undergo two types of multiplication by asexual division (schizogony) in the vertebrate host and a single sexual multiplication (sporogony) in the mosquito host. The genus *Plasmodium* has been defined on the basis of one type of the asexual multiplication by division occuring in cells other than the erythrocytes of the vertebrate host (exoerythrocytic schizogony), and the other characteristic of this genus is that the mosquito hosts are various species of *Diptera* (Wernsdorfer, 1980).

There are 123 known species of *Plasmodium,* including at least 22 species found in primate hosts and 19 in rodents, bats or other mammals (Garnham, 1988). About 70 other plasmodial species have been described in birds and reptiles. Only 7 can infect man, and only 4 occur naturally in humans (namely *P. falciparum, P malariae, P. vivax, and P. ovale).* These parasites are transmitted by the female *Anopheles* mosquito which acts as the vector of the disease. Out of about 400 species of *Anopheles* mosquitoes, only 60 are known to be potential vectors of malaria under optimal and natural condition. The reasons why a species of *Anopheles* is able to be a malaria vector or vice versa are still unknown.

Three species of *Plasmodium* are peculiar to man, namely *P vivax, P. ovale* and *P falciparum.* One specie, *P malariae,* is common to man and African apes, and this same specie under the name of *P brasilianum* is probably the origin of the quartan parasite commonly present in many species of Latin American monkeys. Finally, several simian species of *Plasmodium* are found rarely in man - *P. knowlesi* and *P. simium* as zoonoses, *P. cynomolgi* and *P bastianellii* as accidental (laboratory-acquired) infections and *P schwetzi* and *P. inui* (and the subspecies *shortti)* as experimental infections. One simian species, *P. knowlesi,* was used extensively in the past for malaria therapy in patients suffering from general paralysis of the insane (GPI) [Wernsdorfer, 1980].

In 1847, Meckel observed black granules in the "protoplasmic masses" in the blood of severely ill malarious patients. However, *P. falciparum* parasites as a cause of malaria and

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exflagellation of the male gamete were first discovered in Constantine, Algeria in 1880 by the French Army Physician Alphonse Laveran, for which he received a belated Noble Prize in Medicine in 1907. It was later proven that malaria could be transmitted from one patient to another by infected blood inoculations, and mosquitoes suggested as the natural vehicles with the discovery of some of the developmental stages of the parasite in a mosquito (female *Anopheles)* that had previously fed on an Indian patient with *Plasmodium*, the causative agent, in his blood by Ronald Ross in 1898 (Noble Prize in Medicine laureate 1902).

Much knowledge of the malarial parasites and infections was gained from the 1920s onwards as not only *P vivax* but also *P malariae, P. knowlesi* and *P. falciparum* were used in the successful treatment of neurosyphilis. Although more than one hundred years have passed since Laveran's discovery, much is still unknown of the parasites entry into the red blood cells or fertilization in the gut of mosquitoes (Wernsdorfer, 1980).

2. 4 LIFE CYCLE OF MALARIA PARASITES

The vast majority of the infection of the human host is through the bite of an infected female *Anopheles* mosquito. In addition to this, three uncommon modes of transmission are also possible and are characterized by both a short incubation period and the absence of relapse. These are transmission through infected placenta which is responsible for congenital malaria, transmission through the inadvertent transfusion of infected blood, lastly and very rarely, accidental transmission by infected needles in the laboratory.

The parasites in the form of sporozoites are inoculated into the bloodstream of the host via the saliva before the mosquito takes its blood meal. The sporozoites reach and penetrate the parenchymal cells of the liver within 20 minutes, where within one to three weeks (depending on the specie) multiply mitotically and develop into exoerythrocytic schizonts each containing 10,000 to 30,000 merozoites. The infected liver cells rupture and release merozoites which invade erythrocytes to initiate the asexual erythrocytic cycle. Both

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mature and immature erythrocytes are invaded although the latter are invaded less efficiently. In *P. vivax* and *P. ovale,* some parasites (hypnozoites) may remain in the liver to cause relapses later (Bruce-Chwatt, 1985).

Merozoite penetration involves initial attachment to the erythrocyte and reorientation so that the apical end faces the erythrocyte membrane. A membrane junction and an invagination are formed and the junction moves along the surface of the parasite (Aikawa & Seed 1980). Finally the erythrocyte membrane is sealed after completion of merozoite entry (Aikawa & Seed 1980). Thus, when completely internalized, the parasite is surrounded by two membranes, the outer erythrocytic membrane and an inner membrane of probable erythrocytic origin. The invaginated membrane forms the parasitophorous vacuole where the ring stage parasite grows and develops into an amoeboid trophozoite, and through mitotic division into schizonts having up to 32 merozoites (asexual division). Within the parasitophorous vacuole, malarial pigment is continously formed and seen as black dots or rods by light microscopy. This pigment is at least in part composed of hematin, degraded haemoglobin (Sherman, 1979).

The schizont-infected cells burst and newly formed merozoites are released into the bloodstream, and this coincides with the fever in the host. These merozoites re-invade fresh erythrocytes and the cycle is repeated. In *P. falciparum* infection, at about 16 hours after erythrocytic penetration the trophozoite infected erythrocyte starts to expose knob-like excrescences on its outer surface. These knobs are believed to be involved in sequestration of late stage infected erythrocytes to the endothelial lining in postcapillary venules (Nakamura *et al.,* 1992), though adherence of PE to the vascular endothelium of brain, kidney, lung and circulating blood cells has been demonstrated, despite the absence of knobs on the surface of the PEs in fatal falciparum malaria in a splenectomized patient (Pongponratn et al., 2000).

Periodically, some of the merozoites/ring forms develop into sexual stage parasites, male and female gametocytes. These gametocytes cannot divide in humans but circulate in

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the peripheral blood and can be taken up by a mosquito during a blood meal. These gametocytes are infective to mosquitoes and inside the mosquito midgut they transform into gametes. The male gametes exflagellate and fertilize female gametes to form zygote, which develops into mature ookinetes. The ookinetes penetrates the gut epithelium and matures to form oocyst beneath the basal lamina (Wernsdorfer, 1980).

Each oocyst on maturation, releases up to 10,000 sporozoites which migrate to the salivary glands of the mosquito, ready to be transmitted to a new host at the next blood meal. It takes mature gametocytes about 10 days at 25°C to develop into infective sporozoites. The time increases with decreasing temperature. Shortly after the fertilization of parasites in the mosquito gut, meiosis takes place resulting in haploid parasites. All the stages of the parasite in both the mosquito and humans are haploid. Genetic recombination takes place during this meiosis, creating new variants of the parasite.

2.5 DEVELOPMENT OF MALARIA INFECTION

The clinical symptoms and signs of malaria occur when *P. falciparum* multiply asexually inside PEs (Marchiafava & Bignami, 1894), whilst the hepatic stages and gametocytes do not cause any symptoms or signs. *P. falciparum* can invade red cells of any age, and since more than one merozoite can infect a single erythrocyte, erythrocytes initially infected with multiple parasites are common in falciparum malaria. The cause of *P. falciparum's* virulence in comparison with other human parasites is unknown, but its multiplicative capacity and ability to sequester in the deep vascular beds are thought to be contributing factors (Newton & Krishna, 1998).

The median number of falciparum sporozoites initiating infection is 8–15, but this may reach up to 100 sporozoites (White & Ho, 1992). In contrast to the other malaria parasites, *P. falciparum* has a shorter pre-erythrocytic stage (5–7 days), shorter incubation period, and produces more merozoites from liver schizonts and after erythrocytic schizogony.

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Each infecting exoerythrocytic merozoite can yield up to 32 daughter merozoites once the erythrocytic stage of infection is initiated from the liver, and within a few days, a few thousand parasites liberated from the liver can progress to a total parasite burden of $>10^{12}$ parasites in adults (White *et al.*, 1992a ; White & Krishna, 1989).

The threshold of microscopic detection is reached after 3 to 4 asexual cycles, and a lethal parasite burden may be reached in another 3 to 4 cycles. The asexual phase of parasite development will begin in young children at a higher parasitaemia, as they have a smaller blood volume in which to dilute the merozoites liberated by hepatic merogony (White & Krishna, 1989). A lethal parasitaemia could therefore develop within 8 days from hepatic merogony in a young child with a blood volume of 500 mL (White *et al.*, 1992a).

During the first 24 hours of *P. falciparum*'s asexual life cycle, the PE is metabolically quiescent leading to only a modest increase in parasite size and the expression of very few parasite antigens on its surface. These PEs continue to circulate and are visible by microscopic examination of a patient's blood. By contrast, the second 24 hours of development is characterized by intense synthetic and metabolic activity (Sherman, 1979), leading to the expression of a recently identified family of parasite-encoded genes (the *var* genes), which are primarily responsible for increasing the adhesiveness of PEs to host ligands expressed on capillary and postcapillary venules (Su *et al.*, 1995).

The adhesion of erythrocytes infected with mature stages of parasites to capillary beds removes most of them from the circulation, thereby preventing their destruction by the spleen. It also allows asexual growth of the parasite and division to occur in a favourable hypoxic environment, and perhaps allows more efficient invasion of erythrocytes after schizogony (Marsh *et al.*, 1988).

During the second 24 hours of the asexual developmental cycle, there is a surge in the uptake of essential synthetic precursors, such as glucose (up to 25- to 50-fold increase compared with uninfected erythrocytes), amino acids, and nucleosides (Elford *et al.*, 1995) by

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the sequestered mature parasites which may compete directly with the needs of adjacent host tissue. This potential for metabolic diversion is an important additional consideration in the pathogenesis of severe malaria, particularly CM. These biochemical changes are associated with a rapid increase in parasite size, the visible deposition of haemozoin in the PE, and subsequently syncytial nuclear division, which produces daughter merozoites (Leete & Rubin, 1996).

The uptake of these metabolites, the disposal of lactate, the principal waste product resulting from the parasite's anaerobic glycolysis, and the factors that signal erythrocyte rupture when multiplication is complete are poorly understood (Elford *et al.,* 1995; Kanaani & Ginsburg, 1991), but the subsequent invasion of red cells takes place within a few minutes, and the developmental cycle is reinitiated.

A preponderance of more mature parasite forms on admission blood films (e.g. >20% trophozoites or schizonts) has been shown to be a prognostic indicator for fatality (Silamut & White, 1993). These circulating mature forms can be quantitated easily on peripheral blood films, but probably represent a fraction of the total sequestered parasite burden and these circulating parasites are not representative of the total parasite biomass in the patient. Indeed, the parasites visible on microscopy have not adhered to host tissues and are unlikely to be contributing directly to the severe symptoms of the disease (White & Krishna, 1989), and hence studies carried out on these circulating parasites, especially *ex vivo* studies, may not represent the pathogenic potential of sequestered parasites.

2. 6 BRAIN PATHOLOGY OF MALARIA INFECTION

2. 6. 1 Macroscopic Pathology

CM brains, packed with PEs, have a slaty-grey discolouration, which is evident on the cut surfaces of the brain in both adults (Aikawa *et al.,* 1980 ; Toro & Roman, 1978 ; Schmid, 1974 ; Khan & Durham, 1945 ; Dhayagude & Purandare, 1943 ; Thomson & Annecke, 1926)

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and children (Edington and Gilles, 1976 ; Thomas, 1971; Lemercier *et al.,* 1966). This colour reflects the presence of malarial pigment (haemazoin).

"Punctiform" or "Ring" haemorrhages are a common pathological feature of CM and are distributed throughout the brain, including the brainstem. They are more common in children who have had convulsions associated with CM than in other children (Thomas, 1971).

Macroscopic oedema, evidenced by an increase in brain weight and compression of cerebrospinal fluid (CSF) spaces (e.g., gyri, ventricles, and pericisternal spaces), is observed in CM, though some reports have downplayed its significance. The brains of African children (Walker *et al.,* 1992 ; Edington & Gilles, 1976 ; Thomas, 1971) and adults (Janota & Doshi, 1979 ; Rigdon and Fletcher, 1945) dying with CM frequently appear swollen with flattened gyri. However, Riganti and colleagues (1990) reported the incidence of cerebral oedema as similar in adult patients dying of CM and those dying without cerebral disease, and suggested that it did not contribute to cerebral disease.

2. 6. 2 Microscopic Pathology

Vascular congestion, evidenced by cerebral capillaries and venules distended with PEs, are the microscopic hallmark of severe falciparum malaria. In contrast to the peripheral blood, all mature stages of the parasite are seen within these vessels, both in adults (Silamut et al., 1999 ; MacPherson *et al.,* 1985 ; Aikawa *et al.,* 1980 ; Spitz, 1946 ; Cropper, 1908 ; Marchiafava & Bignami, 1894), as well as in African children (Lemercier *et al.,* 1966).

Ring haemorrhages, characterized by a blocked central capillary with an agglutinated mass of PEs surrounded by brain tissue that is necrotic and contains demyelinated fibres (Boonpucknavig & Boonpucknavig, 1988 ; Spitz, 1946) or a glial reaction, are seen in CM.

Small malarial granulomata (Dürck's nodules) are a distinctive pathological feature of malaria (Dhayagude & Purandare, 1943; Thomson and Annecke, 1926) associated with ring

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haemorrhages. They are not found in patients who die shortly after the onset of symptoms (Dhayagude & Purandare, 1943), and probably represent a more advanced stage of repair following haemorrhage in which necrotic tissue has been replaced by neuroglial cells and microglial tissue (Edington & Gilles, 1976 ; Spitz, 1946 ; Dhayagude & Purandare, 1943).

Malarial pigment or crystalline haemozoin is present in all erythrocytes that contain mature stages of parasites. Pigment is composed of sheets of β-haematin, which is derived from haem polymerisation after haemoglobin breakdown by parasites. Pigment also accumulates in monocytes and macrophages in those tissues where there is significant parasitaemia, including in the peripheral circulation where its presence is a prognostic indicator (Metzger *et al.*, 1995 ; Phu *et al.*, 1995). The presence of pigment in the brain of patients with CM when PEs are no longer present implies that coma was prolonged, and death ensued even after tissue parasitaemia had begun to clear.

Most pathologists have commented on the lack of inflammatory cells both in adults (Turner *et al.*, 1994 ; MacPherson *et al.*, 1985 ; Fitz-Hugh, 1944 ; Dudgeon and Clarke, 1917) and African children (Thomas, 1971). However, an accumulation of macrophages (with active phagocytosis of PEs and pigment), neutrophils, and plasma cells has been seen in areas of extravasated PEs (Boonpucknavig & Boonpucknavig, 1988), and has been interpreted as evidence of inflammation.

2. 6. 3 Endothelial Cell (EC) Involvement

Malaria is a haematogenous infection and the sequestration of PEs in the brain is an intravascular process with the entry of parasites into the brain parenchyma being very rare. Thus, the cerebral endothelial cell (EC) is a key interface between the blood space and the brain in cerebral malaria. However, little is known about the BBB interactions in CM that result in this devastating neurological disorder, whilst the parasite is still intraerythrocytic. The capillary ECs in fatal severe malaria have been described as swollen (Sein *et al.*, 1993b;

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Pongponratn *et al.*, 1991; Fitz-Hugh, 1944), necrotic, or desquamated (Aikawa *et al.*, 1980), but these changes appear only in samples taken more than 5 hr after death (Oo *et al.*, 1987), suggesting that these features are postmortem artefacts.

There is IHC evidence for EC activation during CM in humans (Silamut *et al.*, 1999 ; Turner *et al.*, 1998 ; Turner *et al.*, 1994 ; Ockenhouse *et al.*, 1992) and animal models of CM (Aikawa *et al.*, 1992). The ultrastructure of capillaries in fatal human malaria appears well preserved, with only a few vessels showing patchy degenerative changes and the EC showing morphological changes of activation with membrane ruffling, lipid droplet accumulation and formation of pseudopodia which are seen to interact with host leukocytes and PEs (Macpherson *et al.*, 1985). Simian models of CM have revealed the capacity of EC to phagocytose PEs (Robert *et al.*, 1996 ; Maeno *et al.*, 1993). The interaction between ECs and PEs, and their role in the transduction of stimuli to the neurons and/or neuroglia are very poorly understood.

2. 6. 4 Relationship between Elevated Cytokine Production and EC Activation

Human studies have shown elevated plasma levels of proinflammatory cytokines, particularly TNF-α , IL-1β and interferon (IFN)-γ, in severe malaria (reviewed by Newton & Krishna, 1998). In African children, plasma concentrations of cytokines, particularly TNF-α, are higher in fatal than non-fatal CM and higher in children with CM than those with uncomplicated malaria (Kwiatkowski *et al.*, 1990 ; Grau *et al.*, 1989a). TNF-α appears to play a central role, and treatment with anti-TNF-α antibodies significantly prolongs survival in CBA/Ca mice infected with *P berghei* and prevents the development of neurologic signs (Grau *et al.*, 1989b).

Mice deficient in type 2 TNF receptor (TNFR2) are protected against CM, while type 1 TNF receptor (TNFR1)-deficient mice exhibit normal susceptibility (Grau *et al.*, 1989b). The mechanism by which TNF triggers the neurological symptoms of CM is unclear,

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although experimental evidence suggest a critical role for TNFR2 expressed by brain microvascular endothelium in mediating inflammatory signals from membrane-bound TNF (Lucas *et al.*, 1997 ; Grau *et al.*, 1989b).

However, peripheral blood samples may not reflect the events taking place in the tissues and it will be extremely useful to study the interplay between chemokines, local cytokine production and cell adhesion molecules in tissue samples. In addition to systemic production, local cytokine release could contribute to organ-specific pathology. There is compelling evidence that the inflammatory response at the BBB involve adhesion molecules and proinflammatory cytokines (Brown *et al.*, 1999b ; Garcia *et al.*, 1999 ; Mazier & Iddrisa-Boubou., 1999 ; Rogerson *et al.*, 1999 ; Silamut *et al.*, 1999 ; Turner *et al.*, 1994 ; Ockenhouse *et al.*, 1991).

Recent studies have shown the expression of proinflammatory cytokine protein, including TNF-α, in postmortem brain tissue in human CM (Brown *et al.*, 1999b ; Udomsangpetch *et al.*, 1997 ; Porta *et al.*, 1993). TNF-α is overexpressed in cerebral microglia, astrocytes, monocytes and vascular endothelium in mice with CM, relative to controls (Medana *et al.*, 1997 ; Polder *et al.*, 1991a ; Polder *et al.*, 1991b ; Polder *et al.*, 1988).

Brown and colleagues (1999b), using IHC and RT-PCR, recently demonstrated increased induction and expression of the proinflammatory cytokines, TNF-α and IL-1β (both protein and mRNA), in human postmortem brain tissue in CM. TNF-α protein was detected in every cortex, brain stem, and cerebellum section of CM, whilst IL-1β protein immunostaining was limited to a weak positivity within some cerebral vessels in CM. TNF-α protein was also detected in brain sections from all meningitis cases whilst IL-1β protein was not detected , but IL-1β protein was seen by the use of immunoflourescence on infiltrating leukocytes in samples from meningitis cases (Brown *et al.*, 1999b). Furthermore, using immunofluorescence double-labeling, they co-localized TNF-α protein with synaptophysin in

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neuronal cell bodies (Brown *et al.*, 1999b). However, TGF-β protein was detected in a predominantly vascular distribution in brain tissue from all CM, meningitis, and cases without CNS infection. Double-labeling immunoflourescence with MHC class I (expressed on CNS endothelium) and astrocytic marker [glial fibrillary acidic protein (GFAP)] showed TGF-β in tissue surrounding larger vessels but no co-localization of TGF-β with astrocytes. Finally, TGF-β immunostaining was also seen in white-matter haemorrhagic lesions in samples from some CM cases (Brown *et al.*, 1999b).

Two *in-vitro* studies have shown TNF-α mRNA in inflammatory infiltrates within the meninges of experimental rabbit pneumococcal meningitis (Bitsch *et al.*, 1997), and upregulation of neuronal TNF-α expression in response to bacterial lipopolysaccharide (Gahring *et al.*, 1996). IL-1β is not expressed in normal human brain, but induced and expressed intraparenchymally in human CM brain and in meningeal infiltrating leukocytes of meningoencephalitides cases (Brown *et al.*, 1999b).

In *in-vitro* studies, IL-1β is neurotoxic and rapidly induced in response to neuronal cell death, and therefore suggested to play a causal role in ischaemic cell death and neurodegeneration in the brain (Rothwell & Strijbos, 1995). TGF-β has been found in both haemorrhagic white-matter lesions of human CM (Brown *et al.*, 1999b) and in white-matter lesions of human immunodeficiency virus (HIV)-1 encephalitis brain samples (Johnson & Gold, 1996). This association of TGF-β with central nervous system (CNS) neurodegenerative lesions suggests an anti-inflammatory and neuroprotective role for TGF-β in the host defense mechanism against neuronal cell loss (Flanders *et al.*, 1998).

Tongren and colleagues (2000) showed that the proinflammatory cytokines (IL-1β and TNF-α) and TH-1 cytokine [interferon (IFN)-γ] had the highest level of mRNA expression in the cerebellum during late *P. coatneyi* infection in rhesus monkeys, agreeing with histopathologic observations of the preferential sequestration of PE in the cerebellum in

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rhesus monkey (Tongren *et al.,* 2000 ; Smith *et al.*, 1996 ; Sein *et al.*, 1993b), and in human CM (Sein *et al.*, 1993a).

Damaged PEs release other factors, in addition to haemozoin, that inhibit macrophage phagocytosis and induce TNF-α production, which stimulates the expression of ICAM-1, VCAM-1, and E-Selectin that promotes CM (Ockenhouse *et al.,* 1992). One of these factors has now been confirmed to be *Plasmodium* glycosylphosphatidylinositol (GPI) toxin (Schofield & Hackett, 1993). TNF-α over-expression during CM also decreases the intergrity of microvessel endothelia (Molyneux *et al.,* 1991). Both falciparum and vivax infections are associated with increases in circulating serum markers of EC activation (sICAM-1 and sVCAM-1), and these markers were significantly higher in Gambian children with severe falciparum malaria compared with UM (Jakobsen *et al.*, 1994).

However, in a recent study in Gambian children, malaria was associated with elevations in circulating sICAM-1 levels (which correlated with TNF-α and IL-1β levels), but elevations were not related to disease severity (McGuire *et al.*, 1996). Thrombomodulin, another marker of endothelial cell damage, was also significantly higher in nonimmune adults with severe malaria compared with those with UM (Hemmer *et al.*, 1994), and elevations were positively correlated with peripheral parasitaemia and TNF-α levels. A role for tyrosine phosphorylation has been demonstrated in EC activation for two adhesion molecules (VCAM-1 and E-Selectin) induced by TNF-α (Weber *et al.*, 1995).

Plasmodium falciparum erythrocyte membrane protein (PfEMP)-1 has been shown to be the parasitized erythrocyte receptor for adherence to CD36, thrombospondin (TSP), and ICAM-1 (Baruch *et al.,* 1996). Recently, a particular region of PfEMP-1, called the cystine-rich interdomain region (CIDR)-1, has been demonstrated to be the PfEMP-1-binding domain for CD36 (Gamain *et al.,* 2001 ; Baruch *et al.*, 1997), and that modifications in the minimal CD36-binding region (M2 region) of CIDR-1 are responsible for the inability of CSA-selected or CSA-adherent parasites to bind to CD36 (Gamain *et al.*, 2001).

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ICAM-1, VCAM-1, CD36, CD31, E-Selectin, chondroitin-4-sulphate or chondroitin sulphate-A (CSA) and TSP expression are elevated during CM (Mazier *et al*., 2000 ; Silamut *et al.,* 1999 ; Rogerson *et al.,* 1999 ; Turner *et al.,* 1994 ; Ockenhouse *et al.,* 1991), and these adhesion factors are regulated by cytokines. CSA enhances the adhesion of PEs from pregnant donors to placental endothelia and mediates human placental malaria (Cooke *et al.,* 1996). ICAM-1 and CD36 are involved in inflammation as well as adherence of PEs to the BBB microvessels and rosetting of PEs to uninfected erythrocytes (Ockenhouse *et al.,* 1991), while VCAM-1 adheres to sickled reticulocytes in-vitro and is associated with inflammation of microvessel endothelia at the BBB in sickle cell anaemia (Gee & Platt, 1995). CD36 is expressed on microvessel endothelia, platelets as well as monocytes (Baruch *et al.,* 1996).

There is, therefore, overwhelming evidence that cytokines, in particular TNF-α, play a role in the pathogenesis of CM, and that TNF-α induces increased expression of the adhesion molecules involved in sequestration in human malaria. These findings begin to provide a mechanistic explanation for the development of severe malaria, as well as linking the mechanical hypothesis with the cytokine hypothesis.

2. 6. 5 Relationship between EC Activation, Sequestration, and CM

Shortly after the discovery of the malaria parasite by Laveran, malaria researchers in Rome noticed the discrepancy between the number and stages of parasite development in the peripheral blood and the brains of patients who died from CM (Marchiafava & Bignami, 1894). They observed that only the younger stages of the potentially lethal *P falciparum* parasite were seen in peripheral blood smears, whereas the more mature parasites were abundant in the cerebral vessels, and in contrast all stages of parasite development were represented in the peripheral blood of patients with the benign malarias.

Subsequently, Clark and Tomlinson (1949) observed intense congestion of the vessels throughout the brain and spinal cord with special prominence in the cortical layer of both the

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cerebrum and cerebellum, with these capillaries containing both parasitized and non-parasitized erythrocytes (NPEs), depending on the terminal degree of parasitaemia and localisation. They further remarked that frequently one area of the cortex would show scattered parasites while other areas will reveal almost complete parasitization of the erythrocytes. These findings gave rise to the mechanical hypothesis, which dominated medical thinking of the pathogenesis of severe falciparum malaria during the 20th century.

This process, known as sequestration, is not uniform and the intensity of sequestration differs between different organs (Nakazawa *et al.,* 1995 ; Riganti *et al.,* 1990 ; MacPherson *et al.,* 1985), and also differs between the different parts of the brain (Tongren *et al.,* 2000 ; Smith *et al.,* 1996 ; Sein *et al.,* 1993a ; Sein *et al.,* 1993b). This heterogeneity in the microvascular distribution of PEs in fatal malaria emphasizes the importance of the vascular endothelial receptors for parasitized erythrocyte cytoadherence in determining the extent and the distribution of sequestration (Silamut *et al.,* 1999).

Additionally, Silamut and colleagues (1999) found that increased receptor expression tended to be present throughout a "sequestered" vessel, and not just at the site of parasitized erythrocyte adherence, and thus not supporting the concept that the up-regulation of these receptors is solely a direct result of binding of PE to a particular endothelial cell but rather resulting from systemic processes such as the increased levels of proinflammatory cytokines. They, however, noted that this does not preclude the possibility that binding could induce phenotypic changes in an individual endothelial cell, which would increase its adhesive potential (Silamut *et al.,* 1999). Furthermore, there was no definite difference between patterns of endothelial receptor immunostaining in cerebral and non-cerebral malaria cases (Silamut *et al.,* 1999 ; Turner *et al.,* 1998 ; Turner *et al.,* 1994), thus confirming that endothelial activation in the brain is not specific to CM, and in support of the hypothesis that EC activation can occur independent of PE binding.

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Most pathologists have defined cerebral involvement as the presence of sequestered parasites in the brain (Silamut *et al.*, 1999 ; Turner *et al.*, 1994 ; Aikawa *et al.*, 1980 ; Edington & Gilles, 1976 ; Thomas, 1971; Lemercier *et al.*, 1966 ; Spitz, 1946). However, patients who die of non-cerebral complications, e.g., renal failure without clinical evidence of cerebral involvement, may also have cerebral sequestration (MacPherson *et al.*, 1985). Quantitatively, there are more PEs in the brain of human CM, than NCM, though it has been suggested that sequestration was not solely sufficient to cause CM (Turner *et al.*, 1994).

Silamut and colleagues (1999) recently observed that although CM was associated with intense sequestration within the brain, this was also seen in patients who did not lose consciousness, and suggested that this may reflect the time between admission and death, and does not disprove the concept that CM is associated specifically with cerebral sequestration. They further remarked that their relatively large study provides no support for the contention that CM can occur in the absence of cerebral sequestration, and that the absence of parasites from the brains of some fatal CM cases indicates effective antimalarial treatment and clearance of infected erythrocytes, but such treatment cannot always reverse malaria related or secondary pathological processes in severely ill patients (Silamut *et al.*, 1999).

Most postmortem studies [reviewed by Turner (1997)] have been conducted on adults, and in those series that include children, differences between adults and children have not been highlighted. The original autopsy studies in children were from Africa (Edington & Gilles, 1976 ; Thomas, 1971; Lemercier *et al.*, 1966), and few have been performed elsewhere. Detailed clinical data have not always been available to compare with pathological findings of fatal outcomes, and given the heterogeneity in clinical presentation this has hampered clinicopathological correlation (Jaffar *et al.*, 1997 ; Udomsangpetch *et al.*, 1997).

The central neuropathological feature of CM is the preferential sequestration of PEs in the cerebral microvasculature. Erythrocytes infected with the late maturing stages of the *P.*

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falciparum parasite (the trophozoite/ring stages and schizonts) disappear from the free circulation, causing a drop in the observed peripheral parasitemia, and become preferentially localised in deep vascular beds of vital organs. The sequestered PEs accumulate in the brain, lung, gut, liver, kidney and the heart. Several studies have shown adherence of PEs to the larger venules of the cerebral circulation (Silamut *et al.,* 1999 ; Turner *et al.,* 1998 ; Turner *et al.*, 1994 ; Walker *et al.,* 1992 ; Pongponratn *et al.,* 1991 ; Oo *et al.,* 1987 ; MacPherson *et al.,* 1985).

There is a great debate as to the specificity of cerebral sequestration in CM as opposed to non-cerebral cases. Pathological studies of tissues from cases of fatal malaria demonstrated that the process occurred preferentially in the brain in cases of cerebral malaria, and offered this putative explanation for the development of coma in these cases. Semi-quantitative and quantitative studies of different organs from fatal cases confirmed that sequestration of PEs in the cerebral microvasculature was significantly associated with clinical CM (Turner *et al.,* 1994 ; Pongponratn *et al.,* 1991 ; MacPherson *et al.,* 1985).

Turner and colleagues (1994) concluded that, quantitatively there are more PEs in the brain of CM, than NCM, though sequestration was not solely sufficient to cause CM. They also concluded that there was highly specific co-localization of sequestration with the expression of ICAM-1, CD36, and E-Selectin in cerebral vessels, but no cellular inflammatory response (Turner *et al.,* 1994). These suggest a link between EC activation, sequestration and CM. Unfortunately, there are few such postmortem tissue studies of malaria in Africans, especially among children with CM to compare and contrast findings.

The first semiquantitative assessment of the relationship between sequestration and human cerebral disease by MacPherson and colleagues (1985) used light and electron microscopic comparisons of samples obtained from Thai adults dying with strictly defined CM and those with multi-organ involvement without predominant cerebral symptoms. Statistically, significant differences were observed between the two clinical groups. Patients

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with CM had more PEs and more vessels affected than NCM patients. The degree of sequestration was greatest in the brain, followed by the heart and then the liver, lung, and kidney, all of which contained larger numbers of PEs than the blood. There was little evidence for haemorrhagic or inflammatory exudation in tissues, immune complex deposition, or widespread thrombosis. Knobs on PEs appeared to be the site of attachment to endothelial cells, some of which were swollen (MacPherson *et al.,* 1985).

Another Thai study of 39 adults confirmed that the percentage of PEs in organs was higher in CM patients than in NCM patients (Pongponratn *et al.,* 1991). Again in the CM group, the sequestration of PEs in the brain was significantly higher than in other organs, and there was a strong correlation between peripheral parasitaemia and PE sequestration in brain. This organ difference was not seen in the NCM group, although some degree of PE sequestration was visible in 50% of the NCM cases (Pongponratn *et al.,* 1991).

Subsequently, Turner and colleagues (1994) undertook a detailed quantitative analysis of the expression of EC markers in tissues from adult patients with fatal CM and compared with control patients. There was increased expression of ICAM-1 and E-selectin on cerebral vessels from malaria patients compared with controls. CD36 and TSP staining was sparse in all cerebral tissue. Parasites were highly co-localised to areas expressing ICAM-1, CD36, and E-selectin, supporting the notion that these molecules may be important vascular ligands *in vivo*. There was additional evidence of endothelial cell activation in malaria with increased expression of other markers such as major histocompatibility complex (MHC) Class II antigens, but these changes were not accompanied by increased local accumulation of inflammatory cells. The most marked sequestration of parasites was in the brain, confirming findings from previous studies, but there were also large accumulations of parasites and pigment in the spleen and liver (Turner *et al.,* 1994).

Turner and colleagues (1998) reported that systemic endothelial activation, represented by the up-regulation of inducible soluble cell adhesion molecules (sCAMs), were

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seen in both severe and uncomplicated malaria, and severe systemic sepsis when compared with uninfected controls. Furthermore, the plasma levels of sICAM-1, sVCAM-1 and sE-Selectin correlated positively with the severity of malaria, whereas TNF-α was raised nonspecifically in malaria and sepsis, and also that the IHC evidence of endothelial activation in skin biopsies did not correlate with sCAM levels or disease severity. They, therefore, concluded that a background of systemic endothelial activation occurs in both mild and severe malaria, and sepsis, and that the levels of sCAMs in malaria are not an accurate reflection of EC expression of CAMs in a particular vascular bed, as other factors might influence their levels during disease (Turner *et al.*, 1998).

A recent larger postmortem study of 50 Vietnamese and Thai adults (Silamut *et al.*, 1999) dying of severe and cerebral disease found heterogeneity in the distribution of parasites within brain tissue, and that within the same brain, different vessels had discrete but different populations of parasites, indicating that the adhesion characteristics of cerebrovascular endothelium change asynchronously during malaria and also that significant recirculation of PEs following sequestration is unlikely. They also observed that the cerebrovascular endothelial expression of the putative cytoadherence receptors ICAM-1, VCAM-1, E-Selectin and CSA, and also MHC class II was increased in fatal malaria. Additionally, they reported that some patients dying later in the course of infection did not show sequestration, although pigment was detectable, and that all developmental stages of *P. falciparum* are sequestered in the brain (Silamut *et al.*, 1999).

Other observers have reported that vessels sequestered with parasites are more prominent in gray rather than white matter in the rhesus monkey (Sein *et al.*, 1993b) and in humans (Nagatake *et al.*, 1992), and also that sequestration affected the cerebellum more than other parts of the CNS in the rhesus monkey (Tongren *et al.*, 2000 ; Smith *et al.*, 1996 ; Sein *et al.*, 1993b), and in human CM (Sein *et al.*, 1993a).

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Using RT-PCR to quantitate mRNA expression in four regions of the brain in *P. coatneyi*-infected rhesus monkeys, Tongren and colleagues (2000) reported that the expression level of TNF-α, IFN-γ, IL-1β, ICAM-1 and inducible nitric oxide synthase (iNOS) were highest in the cerebellum of infected animals, correlating well with pathologic observations of preferential sequestration of PEs in this region of the brain. They, further, observed that infected animals also had higher TNF-α and IL-1β expression levels in the cortex, white matter of cerebrum and midbrain, and concluded that the expression of proinflammatory and T-helper-1 (Th-1) cytokines, adhesion molecules and iNOS appears to predominate in the cerebellum of infected rhesus monkeys (Tongren *et al.,* 2000).

Several animal models have been developed to study the pathology of malaria in a more controlled setting. These are predominantly either murine or simian models, which use a number of different combinations of host and parasite species (Aikawa *et al.,* 1992 ; Gysin *et al.,* 1992 ; Neil & Hunt, 1992). However, there are some disagreements about the relevance of animal models when compared to the human situation. In murine models, it seems an autoimmune disease occurs and the sequestered cells are host leukocytes rather than infected erythrocytes.

The simian model is immunologically similar to human malaria in terms of cytokine and prostanglandin production (Yang et al., 1998). The PE sequestration patterns in the *P. coatneyi*-rhesus monkey model have been shown to mimic those of the *P. falciparum* infection in humans, including synchronized appearance of ring-stage parasites, the absence of late trophozoites and schizonts in the periphery, the temporal reduction in parasitemia following the appearance of ring-stage parasites, and the preferential binding of PEs to endothelium in the cerebellum (Smith et al., 1996 : Sein et al., 1993a ; Sein et al., 1993b). IHC has revealed the expression of antigens on cerebral microvessels in infected rhesus monkeys that cross react with antisera to human TSP, or platelet glycoprotein (CD36), ICAM-1 and E-Selectin (Nakumura et al., 1992).

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Additionally, the degree and distribution of sequestered parasites varies according to the species of malaria parasite and host. In the night monkey (*Aotus trivirgatus*), *P falciparum* sequestration affects in descending order myocardium, adipose tissue, and skeletal muscle, with no parasites visible in the brain (Miller, 1969). These differences in sequestration in animal models and humans is one of the key factors that complicates the extrapolation of findings in animal models to the understanding of human disease.

Finally, Turner (1997) reviewed the latest evidence from IHC and electron microscopic studies, and concluded that widespread cerebral EC activation and morphological changes occur in CM, as well as focal EC damage and necrosis, and that the immune cell response to intravascular sequestration appears to be limited, although activation of pigment-phagocytosing monocytes is a late feature.

2. 7 HOST FACTORS INVOLVED IN PATHOGENESIS OF MALARIA

2. 7. 1 The Mechanical Hypothesis (Involvement of Host EC Adhesion Molecules)

The finding of relatively mature parasites within the deep vascular bed at post mortem was made soon after the discovery of the parasite (Marchiafava & Bignami, 1894), and gave rise to the mechanical hypothesis for the pathogenesis of severe malaria, which dominated medical thinking of the pathogenesis of severe malaria during the 20th century. The mechanical hypothesis assumes that organs will be affected in proportion to the overall number of PEs sequestered in tissues, as well as their relative proportions within different tissue capillary beds. Thus, CM arises because of sequestration of PEs in cerebral capillaries and postcapillary venules, whereas patients who may have similar numbers of sequestered parasites in other tissues, but not the brain, would not be expected to develop a full-blown cerebral syndrome.

The two main objections to the mechanical hypothesis have been the lack of sequestration in some patients with cerebral symptoms associated with falciparum malaria,

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and the fact that most patients with CM recover without evidence of ischaemic damage. The lack of massive intracerebral sequestration of parasites in some patients with CM can be rationalised in a number of ways. Since CM is a diagnosis of exclusion, other diagnoses (e.g., encephalitis) may not have been excluded (White *et al.,* 1992b), and the coexistence of factors such as hypoglycaemia may also cause impairment of consciousness.

Furthermore, the mechanical hypothesis may be unable to explain the cause of death in all fatal CM cases, as some treated cases of CM have died without any parasites visualised on postmortem histological examination, although changes in host tissues may be similar to those seen in patients dying with parasite-engorged capillaries (Dudgeon & Clarke, 1917). An explanation for this finding is that parasites have responded successfully to treatment, but that the pathophysiological processes they had initiated have progressed to an irreversible stage, and resulted in a fatal outcome. Therefore, additional hypotheses that attempt to explain severe malaria, particularly CM, have been proposed.

2. 7. 2 The Cytokine Hypothesis

For all its dramatic manifestations of fever, metabolic acidosis, hypoglycaemia, seizures, coma and cerebral oedema, severe *P falciparum* infection in African children is remarkably similar to many other conditions, such as heat-stroke and chronic salicylate poisoning, that are not caused by infectious agents (Clark & Schofield, 2000). The coma exhibited by severe cases of falciparum malaria has traditionally been explained as resulting from mechanical blockage of blood vessels by sequestered PEs, causing local cerebral hypoxia. However, severe falciparum malaria is mimicked exactly by chronic salicylate poisoning, a condition accepted to be a complex metabolic dysfunction, devoid of PEs, sequestered or otherwise, and not remarkable for hypoxia (English *et al.,* 1996).

Salicylate has been shown to cause a systemic inflammatory state (English *et al.,* 1996), which is precisely the terminology used by Maegraith in 1948 to describe his then

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revolutionary view of severe falciparum malaria, which he likened functionally to systemic bacterial infections. Much later, it was proposed that Maegraith's observations could be explained by host mononuclear cells, under the influence of a long-proposed malaria toxin, releasing large amounts of the soluble mediators now called proinflammatory cytokines, particularly IL-1β and TNF-α, which are capable of inducing cerebral syndromes in patients with malaria (Clark & Rockett, 1994 ; Clark *et al.*, 1991 ; Clark *et al.*, 1981).

Evidence supporting this concept now includes the ability of experimental malaria to prime TNF-α production (Clark *et al.*, 1981), the close similarity to malaria of the side effects and biochemical changes observed when TNF-α is administered to tumour patients (Spiggs *et al.*, 1988), and clinical studies showing a correlation between disease severity and the circulating levels of cytokines (particularly TNF-α) in African (Krishna *et al.*, 1994 ; Nyakundi *et al.*, 1994 ; Shaffer *et al.*, 1991; Kwiatkowski *et al.*, 1990 ; Kern *et al.*, 1989 ; Grau et al., 1989a ; Grau *et al.*, 1989c ; Scuderi *et al.*,1986), and Melanesian (Butcher et al., 1990) children and European adults (Kern et al., 1989). In some of these studies, TNF-α levels are correlated with parasitaemia (Nyakundi *et al.*, 1994 ; Shaffer *et al.*, 1991), but most studies detected higher levels in children with CM rather than NCM.

P. falciparum-infected erythrocytes produce pyrogenic material that triggers the release of TNF-α and other cytokines from host mononuclear cells, and merogony (soon after which parasite material is liberated in largest quantities into the circulation *in vivo*) provokes the largest pulses of TNF-α release *in vitro* (Kwiatkowski, 1989 ; Kwiatkowski *et al.*, 1989). In the relatively benign vivax malaria (without cerebral symptoms), very high TNF-α levels have been measured transiently in serum (Karunaweera *et al.*, 1992), suggesting that such high systemic TNF levels do not themselves cause serious disease. Furthermore, elevations in TNF-α can exert antiparasitic effects by inhibiting parasite multiplication and synergising with other factors to produce gametocidal effects (de Naotunne *et al.*, 1993).

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Therefore, the timing and amounts of the different cytokines that are released, particularly TNF-α, may be important determinants of subsequent pathophysiological events and perhaps mortality. Thus, increased production of TNF-α early in malarial infection may be protective, whereas prolonged, high TNF-α levels may be detrimental. This temporal relationship has been difficult to identify in humans.

Parasites varied in their ability to induce TNF-α release (Allan *et al.*, 1993), and this variation correlated with the source of the parasites. Those parasites obtained from patients with CM induced more TNF-α release *ex vivo* than those from patients with UM (Allan *et al.*, 1995). Host factors are also important in determining the amount of TNF-α released in response to infection. TNF-α is a critical mediator of host defense against infection but may cause severe pathology when produced in excess. Individuals vary in the amount of TNF-α produced when their peripheral blood mononuclear cells are stimulated in vitro, and family studies indicate that much of this variability is genetically determined.

Since the TNF-α response to infection is partly regulated at the transcriptional level, TNF-α promoter polymorphisms have been the subject of intense interest as potential determinants of disease severity. A single nucleotide polymorphism at nucleotide –308 bp relative to the transcriptional start site has been associated with susceptibility to severe malaria, leishmaniasis, scarring trachoma, and lepromatous leprosy (Knight & Kwiatkowski, 1999). Gambian children with a homozygous polymorphism at -308 bp relative to the start of TNF-α transcription have an associated 7.7-fold increase in their relative risk of death or neurological sequelae in CM (McGuire *et al.*, 1994).

Knight and colleagues (1999) identified a single nucleotide polymorphism that causes the helix-turn-helix transcription factor OCT-1 to bind to a novel region of complex protein-DNA interactions and alters gene expression in human monocytes. This OCT-1 genotype, found in approximately 5% of Africans, is associated with fourfold increased susceptibility to CM in large case-control studies of West African and East African populations, after

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correction for other known TNF-α polymorphisms and linked human leukocyte antigen (HLA) alleles (Knight *et al.,* 1999).

Serum elevations of TNF-α result in pyrexia in both falciparum (Kwiatkowski *et al.,* 1989) and vivax malaria (Karunaweera *et al.,* 1992). A monoclonal antibody against TNF-α attenuates fever in children with CM, confirming the importance of TNF-α in causing fever (Kwiatkowski *et al.,* 1993). Using anti-TNF-α monoclonal antibodies as adjunctive therapy to either quinine or artemether in children with CM, van Hensbroek and colleagues (1996) reported that mortality was similar in the two groups, but residual neurological sequelae (assessed at 6 months) were detected in more survivors in the study group, compared with those receiving placebo. Additionally, the biological antipyretic effectiveness of the anti-TNF-α antibody was confirmed by the more rapid decline in rectal temperature in the treatment compared with the placebo group.

Thus, neutralisation of circulating TNF-α did not reduce mortality, but did produce an appropriate antipyretic effect. The explanation proposed for this finding is that the increased circulating TNF-α levels associated with CM is an epiphenomenon in the complex array of pathophysiological processes and does not itself increase mortality, and indeed, interfering with elevated TNF-α levels may be harmful. Alternative explanations have been suggested, including the possibility that antibody prolonged the action of TNF-α on endothelium by retaining it within the circulation. It may also be that the antibody was administered too late in the disease process, when secondary cascades of mediators had already begun exerting their biological effects.

More recently, polyclonal anti-TNF-α Fab used as an ancillary treatment for severe malaria reportedly resulted in a faster resolution of clinical manifestations and reduction of fever but also a tendency towards longer parasite clearance times. This Fab treatment reduced IFN-γ concentrations in a dose-related manner and unbound TNF-α was undetectable, but

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circulating concentrations of soluble E-Selectin, ICAM-1 and VCAM-1 were not affected (Looareesuwan *et al.*, 1999).

TNF-α is, therefore, recognised as being one of the most important of the proinflammatory cytokines, and elevations in TNF-α concentrations trigger many secondary cytokine cascades in severe malaria. There are associated increases in IL-1β and IFN-γ concentrations, as well as increases in circulating TNF receptors in acute infection. Circulating IL-6 (Molyneux *et al.*, 1991) and IL-8 levels (Friedland *et al.*, 1993) are increased in falciparum infection. Significantly, in uncomplicated malaria (UM), IL-8 levels remained elevated for up to 4 weeks after the acute infection had been cured (Friedland *et al.*, 1993).

The anti-inflammatory cytokine, IL-10 also plays a role in severe falciparum malaria (Akanmori *et al.*, 2000 ; Kurtzhals *et al.*, 1998), and indeed, IL-10 plasma levels were similar in CM and UM patients, whereas levels of TNF-α were higher in CM than in UM (Akanmori *et al.*, 2000 ; Kurtzhals *et al.*, 1998). IL-$10^{-/-}$ mice produce more TNF-α compared to the wild type in a mouse model of CM (Li *et al.*, 1999). The immune response to increasing *P. falciparum* parasitemia normally leads to increased production of TNF-α, which should be counterbalanced by anti-inflammatory cytokines, such as IL-10, and modulated by soluble TNF receptors (Akanmori *et al.*, 2000 ; Kurtzhals *et al.*, 1998 ; Ho *et al.*, 1998 ; Van Zee *et al.*, 1992 ; de Waal Malefyt *et al.*, 1991). These findings support the hypothesis that CM is the consequence of an excessive proinflammatory immune response that is insufficiently compensated for by a negative feedback response of anti-inflammatory cytokines or neutralising receptors.

In contrast, SMA is thought to be the consequence of the inability to produce optimal amounts of TNF-α in response to any given parasitemia, and that this suboptimal response may explain the corresponding uniformly low levels of IL-10 in SMA patients (Akanmori *et al.*, 2000 ; Othoro *et al.*, 1999 ; Kurtzhals *et al.*, 1998). At least 4 mechanisms have been implicated in the pathogenesis of malarial anaemia, namely erythrocyte lysis by mature

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intraerythrocytic schizonts, down-regulation of erythropoiesis (dyserythropoiesis) by cytokines such as TNF-α and IL-1 β, peripheral destruction of erythrocytes by the spleen and autoimmune haemolysis (reviewed by Newton & Krishna, 1998). The most obvious and most rapid of these is the lysis of erythrocytes by mature schizonts.

2. 7. 3 The Nitric Oxide Hypothesis

In recent years, work on the cytokine-based concept of malaria pathogenesis has focussed on the pathophysiological implications of the ability of these proinflammatory cytokines to generate the inducible form of nitric oxide synthase (iNOS), and thus produce a continuous, potentially large supply of nitric oxide (NO) in tissues that normally experience only low, tightly controlled levels of this ubiquitous cellular messenger. In critical locations, this iNOS could be functionally important, accounting for some of the reversible cerebral symptoms (Clark *et al.,* 1992).

It has, therefore, been suggested that the coma in some patients given a clinical diagnosis of CM is an integral part of a wider syndrome caused by systemic cytokine – iNOS excess rather than a local entity caused by mechanical blockage of blood vessels, and that it is part of a systemic change that has more in common with the metabolic encephalopathies than with simple hypoxia (Clark & Rockett, 1994).

Nevertheless, hypoxia has an important role in this view of malaria as systemic inflammation, in that, it synergizes with proinflammatory cytokines to enhance iNOS induction. Sequestration of PEs could, therefore, play a vital role in exacerbating the inflammatory role of these cytokines, thus making a given concentration of these mediators more potent in falciparum than in vivax malaria (Clark & Cowden, 1999). Therefore, the localisation of parasites to certain tissues by sequestration would result in higher local synthesis and release of these potent mediators, and cause more marked local derangements in function and metabolism of tissues than elevations in circulating cytokines. Clearly, in

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those patients in whom there is no significant sequestration, but cerebral symptoms are still present, other mechanisms would have to be invoked to explain coma. These have not been defined further in the cytokine-nitric oxide hypothesis.

Since the cytokine hypothesis was first elaborated, NO has been identified as a potential mediator for TNF-α action. Clark and colleagues (1992) proposed that TNF-α increased NO production, which caused the coma of CM. This NO is thought to be produced in cerebral ECs and to diffuse into brain tissue, interfering with neurotransmission (Clark *et al.,* 1992). It may also cause neurological damage and sequelae by forming peroxynitrite (Lipton *et al.,* 1993). An alternative suggestion for the role of NO in malaria is that it may be important in host defence, particularly in intracellular killing of parasites. This dual pathogenic-protective role for NO is reminiscent of similar roles suggested for cytokines, like TNF-α, and neither is mutually exclusive.

NO is a short-lived, highly reactive molecule that has a wide spectrum of biological activities. NO is involved in defence by killing intracellular microorganisms (Vouldoukis *et al.,* 1995; Wei *et.al.,* 1995), in maintaining circulatory status by its action on endothelial cells, and in neurotransmission (Vallance & Collier, 1994). It is produced both constitutively in certain tissues and in response to inflammatory stimuli through the action of cytokines that up-regulate the synthesis of iNOS. Nitrotyrosine is formed when peroxynitrite (a product of superoxide and NO) nitrosylates the benzene ring of the tyrosine residues of amino acids, and so its presence indicates the extent to which NO has been generated, and how far it has permeated (Clark & Schofield, 2000).

Maneerat and colleagues (2000), using IHC, recently reported that iNOS expression is increased in the brain in fatal CM, and that the iNOS expression was found in ECs, neurons, astrocytes and microglial cells in CM cases. There was also strong staining in macrophages surrounding ring haemorrhages, and iNOS staining was decreased in recovering malaria cases compared to acute CM, and was low in controls. Furthermore, quantification showed a

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significant association between the intensity and number of iNOS positive vessels with the severity of malaria related histopathological changes, although the total number of cells staining was not increased compared to recovering CM cases. They concluded that an acute induction of iNOS expression occurs in the brain during CM in a number of different cell types, and is increased in the acute phase of CM compared to cases recovering from coma, and therefore proposed that the induction of iNOS expression in CM may contribute to coma, seizures and death, as NO may activate a number of secondary neuropathological mechanisms in the brain, including modulators of synaptic function (Maneerat *et al.,* 2000).

2. 7. 4 The Malaria Toxin (*Plasmodium* GPI) Hypothesis

Following the demonstration that malarial fever is synchronous with the developmental cycle of the blood-stage parasite (Kwiatkowski, 1989 ; Kwiatkowski *et al.,* 1989), it was suggested that the proximal cause of the febrile paroxysm was a released toxin of parasite origin. Nonetheless, the identity of this malaria toxin has long remained obscure. More recently, studies are attempting to isolate and characterize (thus identity, biological properties, biosynthesis and structure) malarial material, possibly a glycosylphosphatidylinositol (GPI) moiety associated with malarial antigens.

GPI of *P. falciparum* was first shown by Schofield and Hackett (1993) to exert regulatory effects on host cells and act as a parasite toxin by its ability to induce TNF-α and IL-1β production by macrophages. GPI purified from the variant surface glycoprotein of *Trypanosoma (T.) brucei* had similar activities in macrophage activation (Tachado & Schofield, 1994), and could thus also account for the high level of IL-1β and TNF-α found in trypanosomiasis, which also mediate disease states in this infection.

The GPIs of both *P. falciparum* and *T. brucei*, when administered to mice, induce a transient pyrexia (Schofield & Hackett, 1993 ; Tachado & Schofield, 1994), and can cause the death of recipients through a TNF-mediated cachexia (Schofield & Hackett, 1993). These

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findings provided the first evidence for the proinflammatory activity of parasite-derived GPIs and served to identify a class of molecules now known to act as pathogenicity and virulence determinants in both African and American trypanosomiases, as well as malaria (Tachado *et al.*, 1999).

GPI from *Plasmodium* species directly, and in synergy with IFN-γ, increases iNOS gene expression and NO output (Tachado *et al.*, 1996), and also upregulates ICAM-1 and VCAM-1 expression in host cells (Schofield *et al.*, 1996). Significantly, monoclonal antibodies to malaria GPI neutralize iNOS (Tachado et al., 1996), ICAM-1 (Schofield et al., 1996) and TNF (Tachado et al., 1997 ; Schofield et al., 1993) induction by parasite extracts, demonstrating clearly that GPI is a necessary and major toxin of parasite origin responsible for these host responses. These studies have concluded that GPIs are likely to be the dominant agents of parasite origin responsible for the induction of bioinflammatory cytokines, and the NO that they subsequently generate.

2. 7. 5 The Permeability Hypothesis

Based on the cerebral oedema found at post mortem in human CM and Rhesus monkeys infected with *P. knowlesi*, Maegraith and Fletcher (1972) hypothesized that CM was caused by the stasis of blood secondary to an inflammatory state, and suggested that kinins increase the permeability of the BBB, causing an efflux of plasma out of the vessels, thereby concentrating the red blood cells within the cerebral vasculature and ultimately producing stasis of the blood.

Using the *P. knowlesi*-infected Rhesus monkey (*Macaca mulatta*) model, Migasena and Maegraith (1968b) showed an efflux of albumin from the blood into the CSF, and suggested that this would draw water into the brain interstitium resulting in cerebral oedema, since albumin is the main determinant of oncotic pressure. There was, however, no significant increase in CSF albumin, and they suggested that this was probably due to their

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earlier report that albumin was transported back into the blood at an almost equally fast rate (Migasena & Maegraith, 1968a). Additionally, they showed that chloroquine and hydrocorticosterone (both anti-inflammatory agents) reduced the flow of albumin in both directions (Migasena and Maegraith, 1968b; Maegraith and Fletcher, 1972), suggesting that the breakdown of the BBB was caused by inflammation.

This hypothesis led to the widespread use of steroids in human CM (Rees, 1982 ; Woodruff and Dickinson, 1968), an intervention subsequently shown to be inappropriate (Warrell *et al.*, 1982a ; Warrell *et al.*, 1982b), because there are important differences between monkeys dying of knowlesi malaria and human CM. *P. knowlesi* sequesters preferentially within the liver and the small intestine, with hardly any accumulation within the brain, hence despite rising parasite counts, infected monkeys retain consciousness until just before death (Anonymous, 1987).

In Zairian children with CM, the concentrations of CSF albumin, immunoglobulin (Ig) G, and IgM were not raised compared with normal European children, and were significantly lower than another group of Zairian children, most of whom had bacterial meningitis (Badibanga *et al.*, 1986). In nonimmune adult Thais with CM, the CSF levels of larger proteins such as α_2-macroglobin were not raised, but the CSF: serum ratio of albumin was significantly higher than in British controls, although there was no significant difference in the ratios during coma compared with recovery nor in fatal cases compared with those who survived (Warrell *et al.*, 1986).

Looareesuwan and colleagues (1983) reported normal lumbar puncture CSF pressures in most patients and the lack of cerebral oedema on computerized tomography (CT) scans of adults who survive CM. These findings suggested that the BBB is grossly intact in children (Badibanga *et al.*, 1986) and adults (Looareesuwan et al., 1983 ; Warrell *et al.*, 1986), but do not exclude the possibility that subtle focal BBB abnormality (permeable to only smaller

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molecules), subtle functional changes in BBB intergrity and mild cerebral oedema, not detectable by CT, may contribute to the pathophysiology of CM (Looareesuwan *et al.,* 1983).

Brown and colleagues (1999a) tested the hypothesis that PEs binding to receptors on cerebral ECs causes changes in the integrity of the BBB. They used IHC to examine the BBB in human CM, with antibodies to macrophages and endothelial activation markers, intercellular junction proteins, and plasma proteins. They found the distribution of the cell junction proteins occludin, vinculin and ZO-1 were altered in CM cases compared to controls, and that although fibrinogen was the only plasma protein detected in the perivascular space, there was widespread perivascular macrophage activation, suggesting that these macrophages had been exposed to plasma proteins. Hence, they drew the conclusion that functional changes to the BBB occur in CM, possibly as a result of the binding of PEs to cerebral Ecs (Brown *et al.,* 1999a).

Subsequently, using radial immunodiffusion assays, Brown and colleagues (2000) reported that albumin and immunoglobulin (Ig) G indices in matched plasma and cerebrospinal fluid (CSF) samples implied only minimal degree of BBB breakdown in a few cases of CM, with most remaining within the normal range. In contrast, cryptococcal, tubercular and acute bacterial meningitis produced significantly detectable changes in the composition of the CSF and evidence of BBB breakdown. They concluded that CM appears to involve only subtle functional changes in BBB integrity with minimal intraparenchymal inflammatory response compared with other neurologic infections, and therefore suggested that attention should be focused on local events within and around the cerebral microvasculature rather than a widespread parenchymal disease in CM.

More recently, IHC studies on autopsy brain tissue from 8 cases of CM in Malawian children showed activation of endothelial cells and macrophages, and disruption of endothelial intercellular junctions in vessels containing sequestered PEs, but no gross leakage of plasma proteins (Brown et al., 2001). The examination of the partition of albumin between

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circulating plasma and the CSF from 72 cases of CM showed subtle but measurable changes compatible with impaired BBB function in CM, and therefore suggested that BBB breakdown occurs in areas of parasite sequestration in CM in African children (Brown et al., 2001).

Finally, Adams and colleagues (2002) reviewed the latest evidence from postmortem, in vitro and animal studies highlighting the role of BBB breakdown in CM, and concluded that BBB integrity is disturbed during severe malaria causing leakage of cerebral vessels. They suggested that understanding how this happens and how it contributes to the pathogenesis of coma may provide new opportunities for the treatment of CM.

2. 7. 6 The Immunological Hypothesis

Experimental work demonstrated that golden hamsters that had a neonatal thymectomy (Wright, 1968) or those that were given antithymocyte serum (Wright *et al.*, 1971) were resistant to *P. berghei* infections. Grau and colleagues (1986), using *P. berghei*-infected mice, have showed that CD4+ T-cells were essential for the development of neurovascular lesions (Grau *et al.*, 1986). Subsequently, they demonstrate that T-cell-dependent macrophage activation led to the release of cytokines, including TNF-α, which mediated the development of these lesions (Grau *et al.*, 1987a), and that cyclosporin prevented the development of neurological complications in mice (Grau *et al.*, 1987b).

There are, however, fundamental differences between mouse and human CM, since in mice, monocytes are the principal cells that adhere to the endothelium, with little sequestration of PEs, and additionally Igs are consistently deposited on the cerebral endothelium in mice, but not in humans. Furthermore, coma is not a prominent feature of murine malaria, but rather the neurological manifestations are limb paralysis, deviation of the head, ataxia, and convulsions (Grau *et al.*, 1989c).

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Edington (1967) invoked immunological mechanisms to explain the low incidence of CM in malnourished African children, since it was thought that they were immunodeficient. Furthermore, Commey and colleagues (1980) suggested the involvement of immunological mechanisms in the relatively late peak incidence of CM in children living in endemic areas, despite the fact that these children have been exposed to malaria prior to the development of this complication (Commey *et al.,* 1980).

Malaria infections induce cellular and humoral immune responses in humans. An increase in circulating immune complexes and the depletion of complement occurs in severe falciparum malaria, and in some studies, is more common in human CM than NCM (Warrell, 1987), but immune complex disposition on the cerebral vessels is not a consistent feature of CM (Boonpucknavig & Boonpucknavig, 1988; MacPherson *et al.,* 1985). However, proliferative glomerulonephritis, a manifestation of immunological disease, has been reported in falciparum malaria (Boonpucknavig & Boonpucknavig, 1988). Significantly, anti-inflammatory agents like steroids (Hoffman *et al.,* 1988; Warrell *et al.,* 1982a), Ig (Taylor *et al.,* 1992), and the immunosuppressive agent cyclosporin A (Warrell *et al.,* 1990) have not been shown to be beneficial in human CM.

2. 7. 7 The Chemokine Hypothesis

Chemokines are lymphochemotactic factors that play a major role in the severity and outcome of many infectious and inflammatory diseases (Andjelkovic *et al.,* 1999 ; Mazier & Iddrisa-Boubou, 1999 ; Burgmann *et al.,* 1995 ; Sherry *et al.,* 1995), but their role in cerebral and non-cerebral malaria is not well understood.

The *Plasmodium* duffy antigen receptor for chemokines (DARC) promiscuously binds chemokines and enables PE adhesion (Horuk *et al.,* 1996 ; Ng *et al.,* 1999 ; Szabo *et al.,* 1995 ; Horuk *et al.,* 1993 ; Neote *et al.,* 1993). DARC also functions as a cell entry factor in *P. vivax* and *P. knowlesi* (Chitnis et al., 1996). Recent studies have shown that other

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chemokine receptor-like molecules on human astrocytes may function in the recruitment of both astrocytes and leukocytes to specified brain regions during cerebral malaria (Andjelkovic *et al.*, 1999).

2. 7. 8 The Reactive Oxygen Species (ROS) Hypothesis

In experimental *P berghei*-infected mice, ROS were produced during malaria infections (Stocker *et al.*, 1984), antioxidants prevented cerebral involvement (Thumwood *et al.*, 1989) and the breakdown of the BBB (Thumwood *et al.*, 1988), and also attenuated the fall in haemoglobin (Clark and Hunt, 1983). Clark and colleagues (1986) proposed that extravasated erythrocytes may lead to ROS generation, causing coma and damage to local tissues, including the brain. Monocyte generation of ROS was increased in adults with falciparum malaria, especially those with severe disease (Descamps Latscha *et al.*, 1987 ; Dubey *et al.*, 1991).

In African children, desferrioxamine, an ROS scavenger, appears to reduce the duration of deep coma (Gordeuk *et al.*, 1992), although it has not been shown to improve the outcome or decrease the incidence of neurological sequelae. Desferrioxamine may act by other mechanisms, therefore ROS appear to be generated in acute malarial infections, but their role in the pathogenesis of the complications of malaria is unclear.

2. 8 PARASITE FACTORS INVOLVED IN PATHOGENESIS OF MALARIA

Infection with *P falciparum* changes the host PEs in many profound ways, including altering the surface properties of infected cells to increase their adhesiveness to non-erythrocytic cells (cytoadherence) and non-parasitized erythrocytes (rosetting), and to decrease their deformability resulting in increased resistance to flow (rheology). Though all the above 3 parasite factors contribute to the development of microcirculatory obstruction, cytoadherence is the main contributor.

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2. 8. 1 Cytoadherence

Cytoadherence is the property of PEs to stick to non-erythrocytic cells or cell lines, and is the key pathophysiological process that distinguishes falciparum from other human malarias. PEs can bind to endothelium, in addition to a variety of host cells, such as monocytes, neutrophils and platelets, suggesting phenotypic heterogeneity of cytoadherence. Recent studies have reconciled the phenomenon of antigenic variation with the heterogeneity of parasite-encoded adhesion molecules expressed in the asexual stages of parasite development (Baruch *et al.*, 1995).

Cytoadherence depends on a high molecular mass family of proteins encoded by *var* genes (40 to 50 *var* genes on different parasite chromosomes have been identified) in *P falciparum*. These *var*-encoded proteins, PfEMP-1 family, are expressed on the surface of the PE and vary in molecular mass from 200 to 350 kDa (Baruch *et al.*, 1995), in parallel with parasite strain-specific antigenic variation and responsible for agglutination of infected cells when exposed to strain-specific antibodies.

The degenerately homologous domains of the *var* genes contain cystein-rich motifs similar to the duffy-binding protein of *P. vivax* responsible for red cell invasion and, therefore, have been called "duffy binding like (DBL)" regions (Su *et al.*, 1995). Thus, entry into red cells, antigenic variation, and evasion of host clearance by cytoadherence are probably all properties mediated by similar primary sequences.

PfEMP-1 has been localised primarily to parasite-induced "knobs" on the surface of infected red cells. These knobs are small (60–100 nm in diameter) electron-dense structures consisting of submembranous deposits of parasite proteins, such as histidine-rich protein (HRP)-1 and the mature parasite-infected erythrocyte surface antigen protein (Howard *et al.*, 1990). However, not all natural infections are associated with knobs, and parasite clones isolated *in vitro* can still bind without knobs, suggesting that knobs contribute to, but are not essential for, the process of cytoadherence (Petersen *et al.*, 1989).

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More recently, Pongponratn and colleagues (2000) noted the adherence of PEs to the vascular endothelium of brain, kidney, lung and circulating blood cells, despite the absence of knobs on the surface of the PEs in a splenectomized fatal falciparum malaria patient (Pongponratn *et al.*, 2000).

2. 8. 2 Rosetting

Rosetting is the phenomenon observed when PEs containing relatively mature stages of parasites become surrounded and bound to non-parasitized erythrocytes (NPEs) [Udomsangpetch *et al.*, 1989]. Rosettes commonly consist of up to 10 NPEs, but may be much larger ("giant rosettes"), and have been suggested to be a mechanism for increasing the chances of parasites multiplying by surrounding themselves with NPEs for easy re-invasion in the next replicative cycle (Wahlgren *et al.*, 1991), but this view is not shared by Pasvol and colleagues (1995).

The view that only sequestering species of malaria (*P falciparum, P. fragile,* and *P. chabaudi*) form rosettes has been challenged recently by the observations that *P. vivax* and *P. ovale* can also form rosettes (Angus *et al.*, 1996 ; Udomsangpetch *et al.*, 1995). Thus, the rosetting phenotype may be necessary, but is not sufficient, to cause severe malaria, and the relationship of this phenotype to cytoadherence is not clear.

Increased rosetting is a feature of severe malaria and is under-represented in patients with blood group O in contrast to blood groups A, B and AB (Rowe *et al.*, 1995), and this observation has been used to explain the relative protection of patients with blood group O from CM. Haemoglobinopathies, such as sickle cell disease and thalassaemia, may also impair rosetting ability, the latter probably because of microcytosis (Carlson *et al.*, 1994).

In *ex-vivo* assays, the mean number of parasites that formed rosettes was significantly higher in isolates from Gambian children with CM compared with those from UM cases, and

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the rosettes were much larger in the cerebral group (Treutiger *et al.*, 1992), but rosetting was not related to parasite density.

2. 7. 3 Rheology

The early studies of *P. knowlesi* PEs showed infection increased resistance to flow through 5μm polycarbonate sieves, obstructed pores at high parasitaemia and a decrease in red cell deformability, and these changes were suggested to contribute to the development of microcirculatory obstruction (Miller *et al.*, 1971).

Subsequently, Nash and colleagues (1989) reported a stage-dependent decrease in the deformability of red cells as *P. falciparum* matures, and that mature parasites require correspondingly larger pressures (4- to 6-fold, compared with controls) to allow entry of PEs into small (3μm) capillaries. These changes, they concluded, could reduce the circulatory flow in downstream postcapillary venules, and contribute to other pathophysiologically important processes such as cytoadherence (Nash *et al.*, 1989).

More recently, using ektacytometry, a reduction in red cell deformability has been strongly associated with a fatal outcome in severe malaria (Dondorp *et al.*, 1997).

2. 9 THE PROJECT BASE

It is evident from the above outline that, of the 8 pathophysiological mechanisms suggested to cause the diffuse reversible encephalopathy in CM, the linkage between the mechanical and cytokine hypothesis is the most substantiated. There is overwhelming evidence that the increased expression of cerebral endothelial adhesion molecules results from raised host cytokine production (both systemically and locally).

However, this has been shown to occur mainly in *in-vitro* studies and animal models of CM, but has been localized using immunohistological methods in only a handful of *in-vivo* postmortem tissue studies of human CM in African children, who certainly bear the brunt of

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the disease, to compare and contrast findings. It is, therefore, our view that by comparing cytokine and adhesion molecule expression in postmortem brain tissue of human CM, NCM and non-malaria (NM) cases using IHC, a better understanding of the cells responsible for the local production of these mediators can be ascertained.

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CHAPTER 3

MATERIALS AND METHODS

3. 1 RECRUITMENT OF PATIENTS AND COLLECTION OF BRAIN TISSUE

During the peak malaria season, from July to September 2001, all clinically certified deaths in children admitted to the Emergency Unit at the Department of Child Health, Korle-Bu Teaching Hospital, Accra, with detailed clinical and laboratory records and in whom duly signed written informed consent [Appendix I] had been obtained from parents or guardians after the death of their child were included in the study. After the death of a child, the parents or guardians were offered our condolence on the unfortunate death of their child and briefed on the aims and objectives of the project and their roles regarding the procedures involved in the participation of their child.

Each of the volunteering parents or guardians signed a consent form to attest that they fully understood the contents of an information sheet which explained the objectives of the project and an invitation to kindly permit us to perform a postmortem on their child to ascertain the exact cause of his/her death and to remove tiny brain tissue samples. Twenty-one (21) parents or guardians agreed to and signed the consent form for the participation of their children and to donate tiny brain tissue samples at autopsy for this study. Volunteered cadavers were immediately moved from the Emergency Unit to the morgue for cold storage at 4°C and a full autopsy with removal of brain tissue samples done within 12 hours of death.

In each volunteered case, brain tissue blocks of about 0.3-0.5cm^3 were surgically removed from 3 regions of the brain, namely cerebrum (C), cerebellum (CB) and brainstem (BS). Therefore, a total of sixty-three brain tissue samples were obtained and all were used for this IHC study. Brain smear cytology was done for each case at autopsy and stained with Giemsa [Appendix II] for the demonstration, or otherwise, of parasitized erythrocytes and/or malaria pigment in the cerebral microvasculature. These smears were done by placing the

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brain specimen between two glass microscope slides, pressing these together, and making thin smears– this method preserves long fragments of capillaries and venules (Raja, 1922).

Based on the WHO (2000) definition, a "severe malaria" case was defined as any level of parasitemia accompanied by one or more of the following signs;

1. Coma (Blantyre coma score*** $\leq$ 2).
2. One or more seizures.
3. Obtundation.
4. Prostration (clinical judgment; if child $\geq$ 7 months old, unable to sit unassisted).
5. Hyperparasitemia ($\geq$ 100,000 Pf/mm^3).
6. Severe anaemia (hemoglobin $\leq$ 5 g/dL).
7. Respiratory distress (respiratory rate $\geq$ 40/min with 2 of the following: nasal flaring, intercostal indrawing, subcostal recession, grunting).
8. Hypoglycemia (blood glucose $\leq$ 40 mg/dL).
9. Jaundice/Icterus.
10. Renal insufficiency (anuria for 24 hours or more).
11. Haemoglobinuria.
12. Shock (cold extremities, rapid heart rate, systolic blood pressure $\leq$ 50 mmHg).
13. Cessation of eating and drinking.
14. Repeated vomiting.
15. Hyperpyrexia (temperature $\geq$ 40°C).

***__Blantyre coma score__ = sum of Best Motor and Verbal Response, and Eye Movements.

Best Motor Response

2 Localizes painful stimulus (pressure on nailbed, sternum or supraorbital ridge).

1 Withdraws from painful stimulus.

0 No response, or extension posturing, to painful stimulus.

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Best Verbal Response

2 Appropriate cry or reply.

1 Inappropriate cry or reply.

0 Gasp, or no response at all

Eye movements (check to see if patient can see; blinks in response to visual threat)

1 Follows.

0 Doesn't follow.

The full autopsy gross findings, autopsy brain smear cytologic/microscopic findings, in addition to the detailed clinical and diagnostic laboratory records were used to classify the 21 cases into five groups, namely (1) Cerebral malaria [CM], (2) Malaria complicated by severe anaemia/severe malarial anaemia [SMA], (3) Purulent bacterial meningitis [PBM] (i.e. central nervous system infection other than cerebral malaria), (4) Non-central nervous system infection [NCNSI] (i.e. infection in an anatomic organ-system other than the central nervous system), and (5) Non-infection deaths [NI] (i.e. no focus of infection found clinically or at autopsy), based on the inclusion criteria below;

1. CM death:

(a) Fulfil WHO definition of severe malaria with Blantyre coma score ≤2, and (b) Presence of gross autopsy findings of slatey gray discolouration of brain and/or white matter petechial haemorrhages, and/or (c) Presence of brain smear cytologic findings of parasitised erythrocytes and/or malaria pigment in the cerebral microvasculature.

2. SMA death:

(a) Fulfil WHO definition of severe malaria with hemoglobin ≤ 5 g/dL, and (b) Absence of gross autopsy findings of slatey gray discolouration of brain and white matter petechial haemorrhages, but presence of moderate to severe pallor of all internal organs, and (c) Absence of brain smear cytologic findings of parasitised erythrocytes and/or malaria pigment in the cerebral microvasculature.

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3. PBM death:

(a) Does not fulfil WHO definition of severe malaria, and (b) Presence of gross autopsy findings of pus in meninges, and (c) Absence of gross autopsy findings of slatey gray discolouration of brain, moderate to severe pallor of all internal organs and white matter petechial haemorrhages, and (d) Absence of brain smear cytologic findings of parasitised erythrocytes and/or malaria pigment in the cerebral microvasculature.

4. NCNSI death:

(a) Does not fulfil WHO definition of severe malaria, and (b) Absence of gross autopsy findings of pus in meninges, and (c) Absence of gross autopsy findings of slatey gray discolouration of brain, moderate to severe pallor of all internal organs and white matter petechial haemorrhages, and (d) Presence of gross autopsy findings of infection in an anatomic organ-system apart from the central nervous system, and (e) Absence of brain smear cytologic findings of parasitised erythrocytes and/or malaria pigment in the cerebral microvasculature.

5. NI death:

(a) Does not fulfil WHO definition of severe malaria, and (b) Absence of gross autopsy findings of pus in meninges, and (c) Absence of gross autopsy findings of slatey gray discolouration of brain, moderate to severe pallor of all internal organs and white matter petechial haemorrhages, and (d) Absence of gross autopsy findings of infection in any anatomic organ-system, and (e) Absence of brain smear cytologic findings of parasitised erythrocytes and/or malaria pigment in the cerebral microvasculature.

Based on above criteria, the 21 volunteered cases were made up of 10 CM, 5 SMA, 1 PBM, 2 NCNSI, and 3 NI deaths.

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3. 2 FROZEN SECTION PROCESSING OF BRAIN TISSUE SAMPLES

All the sixty-three (63) brain tissue sample were preserved in liquid nitrogen by placing each block of tissue in a pre-labelled aluminium foil cup designed for this purpose and covered with tissue tek® freezing gel (OCT- SAKURA, The Netherlands). The contents of the cup was snap-frozen in chilled isopentane (Avonchem Limited, UK) until the tissue tek® gel froze (i.e. changed from colourless to white) around the tissue-block. Each cup was wrapped in aluminium foil and all the 3 resulting cups of brain tissue from each volunteer were wrapped in a larger aluminium foil and then immersed in liquid nitrogen. Snap-frozen tissue in liquid nitrogen was transported to the laboratory and quickly transferred into a -80°C freezer for storage until use.

Thirteen (13) cryostat sections of 5-7μ thickness each were cut from each of the 63 brain tissue samples using a cryotome (SAKURA, Japan), mounted unto SuperFrost® Plus (Menzel-Glazer, Germany) light microscope slides and 2 of each stained for the localization of the six antigenic sites (namely, ICAM-1, VCAM-1, E-Selectin, TNF-α, IL-1β, and TGF-β) and one negative control using indirect immunostaining microscopic technique with Universal DAKO Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) Kit™ System 40 for Monoclonal Mouse Antibody (DAKO Corporation, USA). Optimal antibody titres (with the highest specificity and sensitivity, and the least background staining) were obtained by serial dilutions in trial runs.

3. 3 MONOCLONAL ANTIBODIES USED IN STUDY

The panel of monoclonal antibodies used in the study is shown in Table 1. The endothelial markers used were 3 putative cytoadherence receptors, namely ICAM-1, VCAM-1, and E-Selectin. Antibodies to 2 proinflammatory cytokines (namely, TNF-α and IL-1β) and a third cytokine (TBF-β) with suggested anti-inflammatory and neuroprotective action (Flanders et al., 1998) were also used.

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Table 1 : Monoclonal Antibodies Used In Study

Antigen	Specificity of Monoclonal Antibodies	Source of Monoclonal Antuibodies	Optimal Titre or Dilution	Diluent Used
ICAM-1	Mouse Anti-Human	Immunotec, UK	1 : 500	Tris Buffered Saline (TBS, 0.005M, pH 7.6)
VCAM-1	Mouse Anti-Human	DAKO, UK	1 : 200	Tris Buffered Saline (TBS, 0.005M, pH 7.6)
E-Selectin	Mouse Anti-Human	DAKO, UK	1 : 200	Tris Buffered Saline (TBS, 0.005M, pH 7.6)
TNFα	Mouse Anti-Human	Serotec, UK	1 : 250	Tris Buffered Saline (TBS, 0.005M, pH 7.6)
TGFβ	Mouse Anti-Human	Serotec, UK	1 : 250	Tris Buffered Saline (TBS, 0.005M, pH 7.6)
IL-1β	Mouse Anti-Human	Serotec, UK	1 : 250	Tris Buffered Saline (TBS, 0.005M, pH 7.6)

3. 4 INDIRECT ALKALINE PHOSPHATASE ANTI-ALKALINE PHOSPHATASE IMMUNOSTAINING TECHNIQUE ON CRYOSTAT SECTIONS

5-7μm cryostat sections were cut unto SuperFrost Plus® light microscope slides and air-dried for 30 minutes at room temperature. Slides were labelled and the working area circled with PAP pen (Binding Site Company, UK.) to create a well around the sections. Sections were then fixed in 100% acetone for 10 minutes and air-dried for 30 minutes at room temperature. Sections were either immunostained immediately or stored at -40°C until use. Sections were washed with Tris Buffered Saline (TBS) pH 7.4 for 15 minutes (3 times, each for 5 minutes). Sections were then incubated with a 1:20 dilution of normal rabbit serum (DAKO, Denmark) in TBS, pH 7.4 for 30 minutes in a humidified chamber to reduce non-specific binding.

Excess serum was tipped off (NOT WASHED) and residual serum carefully mopped up with tissue paper without touching sections. Sections were then incubated for 60 minutes at room temperature or overnight at 4°C with primary monoclonal antibody (all 6 were mouse anti-human) diluted to the optimal titres arrived at in trial runs. Negative controls entailed the omission of the primary antibody and its replacement with mouse IgG (DAKO, Denmark)].

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Sections were then washed with TBS pH 7.4 for 15 minutes. Incubation for 30 minutes at room temperature with secondary antibody was then done. Sections were again washed with TBS pH 7.4 for 15 minutes. Sections were then incubated for 30 minutes with alkaline phosphatase anti-alkaline phosphatase antibody (DAKO APAAP Kit^{TM} System 40).

Sections were again washed with TBS pH 7.4 for 15 minutes (3 times, each for 5 minutes). Freshly prepared chromogenic substrate for alkaline phosphatase was then added to the sections for 8-10 minutes. The chromogenic substrate for alkaline phosphatase was prepared either by dissolving substrate tablet in substrate buffer (DAKO APAAP Kit^{TM} System 40) or as described in Appendix IV. Excess chromogenic substrate for alkaline phosphatase was washed off in running water for 5 minutes. Slides were counterstained with Meyer's Haematoxylin for 30 seconds to stain the nuclei, blue in running tap water to wash off excess stain, and mounted with glycergel (DAKO, Denmark), an acqueous mountant.

3. 5 EXAMINATION OF IMMUNOHISTOLOGICAL SECTIONS

Immunohistological assessment forms were designed for each brain tissue sample which has rows for microscopic location of the red reaction product and columns for antibody used [Appendix III]. The tissue sections immunostained for ICAM-1, VCAM-1 and E-Selectin were examined with an Olympus BH-2 microscope (with X40 objective and X10 eyepiece) and graded. The degree of staining and the percentage of vessels showing staining were assessed using a modified version of the semi-quantitative scoring system used by Turner and colleagues (1994), which is as follows;

	no endothelial cell staining ;
+/-	faint/low-level/mild scattered endothelial cell staining on < 25% of vessels ;
+	mild endothelial cell staining on > 25% and < 50% of vessels ;
++	moderate endothelial cell staining on > 50% and < 75% of vessels ; and
+++	strong positive endothelial cell staining on > 75% of vessels.

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The distribution or localization of staining was noted and the intensity of the red reaction product was graded during the examination (with X10 objective and X10 eyepiece) of the tissue sections immunostained for TNFα, TGFβ and IL-1β as follows;

no red colour ;

+/- faint/mild/low-level scattered staining in some fields ;

\+ moderate staining in some fields ;

++ moderate consistent staining in all fields ; and

+++ deep/strong consistent staining in all fields.

To examine the co-localization of sequestration with the expression of each of the 3 adhesion molecules, the following four categories of vessels were counted (with X40 objective and X10 eyepiece) in the 90 brain tissue sections, 2 each from the 3 regions of the brain of the 15 malaria cases (10 CM & 5 SMA): the number of vessels showing both receptor expression and sequestration (R^+S^+), the number of vessels showing receptor expression but no sequestration (R^+S^-), the number of vessels showing no receptor expression but sequestration (R^-S^+), and the number of vessels showing neither receptor expression nor sequestration (R^-S^-).

3. 6 STATISTICAL ANALYSIS

The percentage of immunostained cases and the intensity of staining were then compared between the 5 groups of cases and the 3 regions of the brain from which samples were obtained for this study. The co-localization of sequestration and receptor expression was analyzed using a chi-square (X^2) test on the 2x2 contingency tables constructed for each of the three adhesion molecules by comparing the expected and observed association in cerebral vessels for the malaria cases (both CM and SMA groups). The level of significance was 5% and all analyses were performed using Epi Info 2000 statistical software (CDC).

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CHAPTER 4

RESULTS

4.1 CLINICAL AND DIAGNOSTIC DETAILS OF STUDIED CASES

Table 2 shows the clinical and diagnostic details of the 21 volunteered cadavers.

Table 2 : Clinical and Diagnostic Details of the 21 Studied Cases

CN	Age mths	Sex	Ad To Dth hrs	Hb g/dl	BS	WBC $X10^9$	Parasite Count per µl of bld	Clin Diag	Dth To Aut hrs	Brain Smear Cytol	Aut Diag
1	56	M	28.5	8.1	2	6.8	14,654	CM	8	Pos	CM
2	96	M	13	12.2	2	6.9	54,392	CM	5.5	Pos	CM
3	50	F	4	6.0	0	17.9	21,413	CM	7	Pos	CM
4	48	F	3.5	4.7	4	15.8	88,796	SMA	3	Neg	SMA
5	108	F	12	7.4	2	13.3	14,486	CM	10	Pos	CM
6	132	M	31	9.2	1	15.6	78,624	CM	7	Pos	CM
7	48	F	14.5	6.9	2	10.3	74,092	CM	4.5	Pos	CM
8	18	M	8	8.1	5	14.1	0**	SBP	8	Neg	SBP
9	84	M	6	4.1	5	10.0	0	HSC	11	Neg	HSC
10	39	F	3	6.4	2	7.9	54,867	CM	6	Pos	CM
11	9	F	1	3.4	4	16.5	440,262	SMA	7.5	Neg	SMA
12	144	M	1.5	2.5	5	9.4	0	BDU	5	Neg	BDU
13	72	M	5	8.5	3	18.0	0	PBM	9	Neg	PBM
14	8	F	2.5	4.0	4	10.9	140,251	SMA	6.5	Neg	SMA
15	90	M	48	8.7	5	20.1	0*	TP&S	7	Neg	TP&S
16	18	M	2	3.0	4	13.1	231,175	SMA	6	Neg	SMA
17	18	F	3.5	2.8	3	11.4	91,782	SMA	5	Neg	SMA
18	48	F	31	8.0	2	9.0	80,640	CM	9	Pos	CM
19	72	F	2	7.2	2	11.1	17,789	CM	4	Pos	CM
20	42	F	1	6.6	1	12.8	40,115	CM	10	Pos	CM
21	36	M	37	10.4	5	7.5	0	NPS	8.5	Neg	NPS

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KEY TO TABLE 2

CN	Case Number
Age	Age in Months
Sex	M for Male, and F for Female
Ad To Dth	Duration in hours between Admission and Death
Hb	Haemoglobin Concentration in g/dl
BS	Blantyre Coma Scale
WBC	White Blood Cell Count ($X10^9/L$)
Parasite Count	Peak Parasite Count per microliter of blood
Clin Diag	Clinical Diagnosis
Dth To Aut	Duration in hours between Death and Autopsy
Brain Smear Cytol	Brain Smear Cytology (Pos for Positive Smear, Neg for Negative Smear)
Aut Diag	Autopsy Diagnosis
*	Blood Culture isolated *Salmonella typhi* after 48 hours of incubation
**	No Bacterial Growth after 7 days of incubation
CM	Cerebral Malaria
SMA	Severe Malaria Anaemia
PBM	Purulent Bacterial Meningitis
HSC	Hyperhaemolytic Sickle Cell Crisis
SBP	Severe Bronchopneumonia
BDU	Bleeding Duodenal Ulcer
TP&S	Typhoid Perforation and Septicaemia
NPS	Nephrotic Syndrome

Ten (10) died of CM, 5 of SMA, 1 of PBM, 2 of NCNSI and 3 of NI. The two NCNSI cases were Severe Bronchopneumonia, and Typhoid Perforation and Septicaemia, whilst the three NI cases were Nephrotic Syndrome, Bleeding Duodenal Ulcer and Hyperhaemolytic Sickle Cell (SC) Crisis. There were 10 males and 11 females.

The mean age of the CM deaths of 69.1 months (Standard Deviation of 32.1) was significantly higher than that of the SMA deaths of 20.2 months (Standard Deviation of

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16.25) [X^2 = 7.33, df = 1, P value = 0.0068]. 80% (12 out of 15) of the malaria deaths occurred within 24 hours of admission. The mean peak parasitemia of the CM deaths of 45,107/μL (Standard Deviation of 27,091) was significantly lower than that of the SMA deaths of 198,453/μL (Standard Deviation of 146,913) [X^2 = 14.68, df = 1, P value = 0.0001].

4. 2 IHC Staining For Adhesion Molecules ICAM-1, VCAM-1 & E-Selectin

Using an indirect immunostaining protocol with APAAP, the presence of ICAM-1, VCAM-1, and E-Selectin were evaluated in malaria cases (10 CM and 5 SMA) and non-malaria controls (1 PBM, 2 NCNSI, and 3 NI) brain tissue. Successive sections showed the vascular labeling for the three adhesion molecules studied (details are given below in Table 3).

4. 2. 1 Non-Malaria Cases, Except the Case of Typhoid Perforation and Septicaemia

Monoclonal antibodies to ICAM-1 showed low-level/mild/faint (average score of +/-) staining of the endothelia of few (< 25%) of the brain microvessels in all the non-malaria deaths, except the case of typhoid perforation and septicaemia (Figure 1).

VCAM-1 showed a low-level (average score of +/-) expression in few (< 25%) of the microvessels in some (highest being in the cerebellar sections of 4 out of 5) of the non-malaria deaths, except the case of typhoid perforation and septicaemia (Figure 2).

E-Selectin staining was consistently and uniformly negative (score of -) in all the cerebral microvessels in all the non-malaria controls, with the exception of the case of typhoid perforation and septicaemia (Figure 3).

The non-malaria control brain, except the case of typhoid perforation and septicaemia showed a reproducible cerebrovascular endothelial cell immunophenotype of ICAM-$1^{+/-}$, VCAM-$1^{+/-}$, and E-Selectin^{-} (Figures 1, 2 & 3).

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Figure 1 : A photomicrograph of a cerebellar section of a non-malaria case (Case Number 12 – BDU) stained with ICAM-1 showing low-level expression in < 25% of the microvessels (average score of +/-) in this high power field. The enzyme activity is the red stained areas (IHC X400).

Figure 2 : A photomicrograph of a cerebellar section from the same case as in Figure 1 above stained with VCAM-1 showing low-level expression in < 25% of the microvessels (average score of +/-) in the section (IHC X400).

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Figure 3 : A photomicrograph of a cerebellar section from the same case as in Figures 1 & 2 above stained with E-Selectin showing no expression (score of -) in this high power field, and all other fields of the section (IHC X400).

4. 2. 2 The Non-Malaria Case of Typhoid Perforation and Septicaemia (TP&S)

ICAM-1 showed mild staining (average score of +) in 25 to 50% of the microvessels in all the brain sections of the case of typhoid perforation and septicaemia (Figure 4).

VCAM-1 and E-Selectin showed mild staining (average score of +) in 25 to 50% of the microvessels in all the brain sections of the case of typhoid perforation and septicaemia (Figure 5).

The immunophenotype of the endothelial cells of the cerebral microvasculature of the case of typhoid perforation and septicaemia was ICAM-1^{+}, VCAM-1^{+}, and E-$Selectin^{+}$ (Figures 4 & 5).

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Figure 4 : A photomicrograph of a cerebellar section of the case of typhoid perforation and septicaemia (Case Number 15) stained with ICAM-1 showing mild expression in 25 to 50% of the microvessels (average score of +) in the section (IHC X400).

Figure 5 : A photomicrograph of a cerebellar section of the case of typhoid perforation and septicaemia (Case Number 15) stained with VCAM-1 showing mild expression in 25 to 50% of the microvessels (average score of +) in the section (IHC X400).

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Figure 7 : A photomicrograph of a cerebellar section from the same case as in Figure 6 above stained with mouse anti-human VCAM-1 monoclonal antibodies showing moderate expression in most (50 to 75%) of the microvessels (average score of ++) in the section. (IHC X400).

Figure 8 : A photomicrograph of a cerebellar section from the same case as in Figure 6 & 7 above stained with mouse anti-human E-Selectin monoclonal antibodies showing mild expression in 25 to 50% of the microvessels (average score of +) in the section. (IHC X400).

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There was no significant differences in endothelial staining between the 10 cases of fatal CM and the 5 cases of fatal SMA, since both groups had a similar increased intensity of ICAM-1, VCAM-1 and E-selectin expression of +++, ++ and + respectively (Figure 6 & 9). However, the increased adhesion molecule expression was more often not associated with sequestered PEs in the SMA cases, than for the CM cases. Thus, though heterogeneity between sequestration and receptor expression was observed in all the malaria cases, it was more extreme in the SMA cases (Figure 6 & 9).

The staining patterns of all the 3 adhesion molecules in brain sections from the case of typhoid perforation and septicaemia differed significantly from that of the other 5 non-malaria cases and the 15 malaria cases. The intensity of staining of all 3 adhesion molecules were significantly higher than that of the other 5 non-malaria cases, but not as intense as that of the 15 fatal malaria cases.

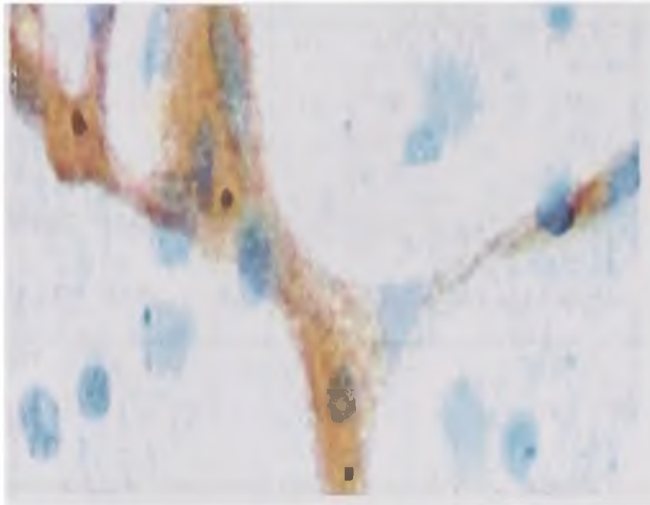

Figure 9 : A photomicrograph of a cerebellar section of a Malaria case (Cases Number 11 – SMA) stained with ICAM-1 showing a similar intensity of staining (average score of +++) as in the CM case in Figure 6 above. Note the more extreme heterogeneity between PE sequestration and receptor expression in this case compared to Figure 6 above (IHC X400).

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4. 2. 4 Comparison of Adhesion Molecule Staining in 3 Brain Regions

Generally, the maximal expression (in terms of increased percentage of cases showing expression and intensity of staining) of all the 3 adhesion molecules in the malaria cases was evident in the cerebellar sections (Figures 10 & 11, and Table 3).

Table 3 : Comparison of IHC Staining of Adhesion Molecules in 3 Brain Regions, and between Malaria, TP&S and Non-malaria Cases

Antigen	Non-malaria Cases, Except TP&S			TP&S Case			Malaria Cases		
	CB	C	BS	CB	C	BS	CB	C	BS
ICAM-1	5/5, 5+/-	5/5, 5+/-	5/5, 5+/-	1/1, 1+	1/1, 1+	1/1, 1+	15/15, 15+++	15/15, 14+++ & 1++	14/15, 11+++ & 3++
VCAM-1	4/5, 4+/-	3/5, 3+/-	2/5, 2+/-	1/1, 1+	1/1, 1+	1/1, 1+	15/15, 15++	15/15, 14++ & 1+	13/15, 13++
E-Selectin	0/5, 5-	0/5, 5-	0/5, 5-	1/1, 1+	1/1, 1+	1/1, 1+	15/15, 15+	14/15, 14+	12/15, 12+

KEY TO TABLE 3

5/5	Numerator for Number of Cases showing Expression, Denominator for Total Number of Cases.
CB	Cerebellum.
C	Cerebrum.
BS	Brainstem.
	no endothelial cell staining ;
+/-	faint/ low-level/ mild scattered endothelial cell staining on < 25% of vessels ;
+	mild endothelial cell staining on > 25% and < 50% of vessels ;
++	moderate endothelial cell staining on > 50% and < 75% of vessels ; and
+++	strong/ positive endothelial cell staining on > 75% of vessels.

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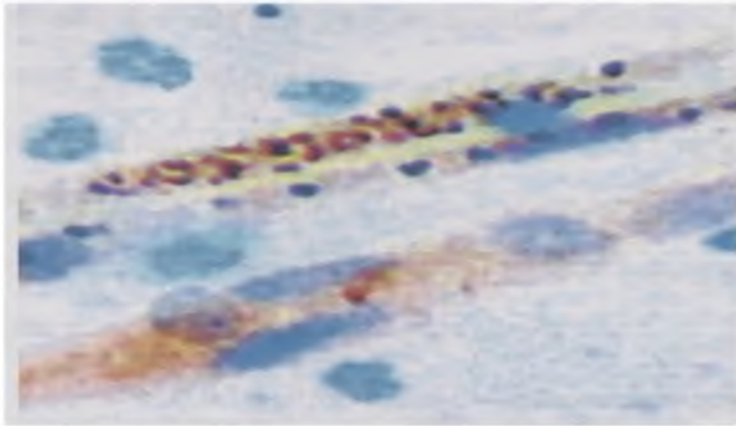

Figure 10 : A photomicrograph of a cerebellar section of a Malaria case (Cases Number 5 – CM) stained with ICAM-1 showing a strong intensity of staining of all the microvessels (average score of +++) in this high power field (IHC X400).

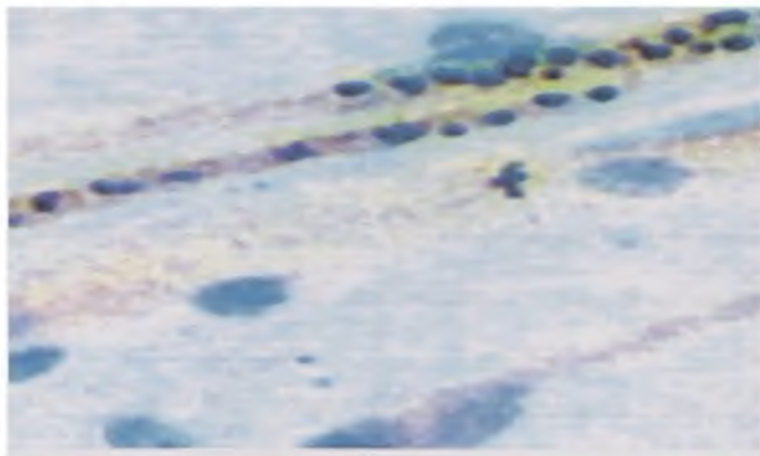

Figure 11 : A photomicrograph of a cerebral section of the same case as in Figure 10 above stained with ICAM-1 showing a strong intensity of staining (average score of +++), but note that not all the microvessels in this high power field are stained compared to Figure 10 above (IHC X400).

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4. 2. 5 Relationship Between Receptor Expression and Sequestration

We found that the presence of sequestered PEs was highly significantly associated with the expression of ICAM-1 ($P = 3.1 \times 10^{-16}$), VCAM-1 ($P = 1.2 \times 10^{-12}$) and E-selection ($P = 6.1 \times 10^{-16}$) [degrees of freedom (df) = 1] in the fatal malaria cerebral vessels [Table 4]. The relative risk of cerebral vessels expressing ICAM-1, VCAM-1 and E-Selectin showing sequestered PEs was 1.73, 1.53 and 1.67 respectively.

Table 4 : Quantitation of Cerebral Vessels and Co-localization of Sequestration with Expression of Receptors in the 15 Malaria Cases

Antigen	Total Number Of Vessels	R^+S^+	R^+S^-	R^-S^+	R^-S^-	X^2 Value	P Value (df = 1)	Relative Risk
ICAM-1	860	350	173	130	207	66.55	3.1×10^{-16}	1.73
VCAM-1	821	305	127	180	209	50.52	1.2×10^{-12}	1.53
E-Selectin	843	317	140	160	226	65.43	6.1×10^{-16}	1.67

KEY TO TABLE 4

R^+S^+ Number of vessels showing both receptor expression and sequestration

R^+S^- Number of vessels showing receptor expression but no sequetration

R^-S^+ Number of vessels showing no receptor expression but sequestration

R^-S^- Number of vessels showing neither receptor expression nor sequestration

There was no one particular receptor whose expression on the endothelial cell surface was consistently related to the presence of sequestered PEs in the cerebral microvessels. Generally, if a vessel showed positive staining for a marker of endothelial activation, this was present throughout its length, rather than being related to the presence of PEs in one segment or over one endothelial cell. Within vessels from the same patient and same region of the brain of the fatal malaria cases, there was heterogeneity between parasite sequestration and the presence of receptor staining. Hence, not uncommonly, there were vessels engorged with PEs without any evidence of adhesion molecule expression and vice versa.

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4.3 IHC Staining for Cytokines TNF-α, TGF-β and IL-1β

Successive sections from some non-malaria and malaria cases showed predominantly intravascular and perivascular distribution for TGF-β (Figures 12 & 13), whilst there was intravascular, perivascular and prominent brain parenchymal/ perinuclear staining for IL-1β (Figures 14, 15 & 16) and TNF-α (Figures 17, 18 & 19).

4.3.1 TGF-β Immunostaining

TGF-β was detected in a predominantly intravascular and perivascular distribution in brain tissue from all 5 groups studied, but expression was more intense in PBM and CM groups (Figures 12 & 13). TGF-β showed moderate intravascular and perivascular immunostaining in some low power fields (average score of +) of all the NCNSI sections (Figure 12), whilst its staining was strong in intravascular and perivascular locations in all low power fields (average score of +++) of all the CM and PBM sections (Figure 13).

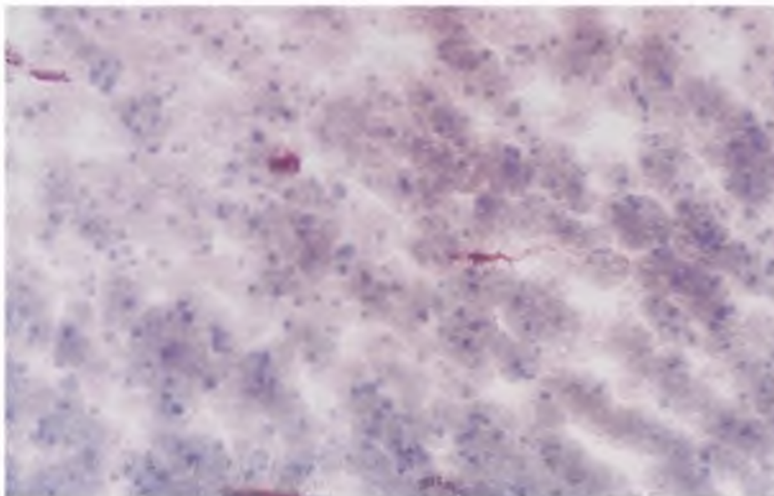

Figure 12 : A photomicrograph of a cerebellar section of a NCNSI case (Case Number 16 – SMA) stained with TGF-β showing moderate intravascular and perivascular immunostaining in some low power fields (average score of +) of the section (IHC X100).

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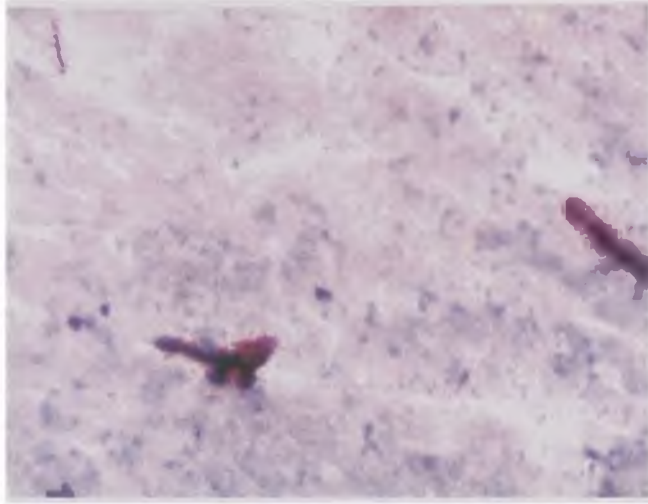

Figure 13 : A photomicrograph of a cerebellar section of a CM case (Case Number 18) stained with TGF-β showing strong intravascular and perivascular immunostaining in all low power fields (average score of +++) of the section (IHC X100).

4. 3. 2 IL-1β Immunostaining

IL-1β staining was limited to only some (none of the brainstem sections any IL-1β staining) of the PBM brain sections (Figure 14) and all of the CM brain sections (Figure 15) in a predominantly intravascular and perivascular pattern, but in none of the sections of the other three groups (Figure 16). Additionally, it was more intense in CM group than the PBM case. IL-1β showed moderate intravascular and perivascular immunostaining in some low power fields (average score of +) of some of the PBM sections (none of the PBM brainstem sections showed any IL-1β staining) [Figure 14]. IL-1β showed strong intravascular and perivascular immunostaining in all low power fields (average score of +++) of the CM sections (Figure 15), whilst it showed no staining (score of -) in none of the low power fields of all the NCNSI sections (Figure 16).

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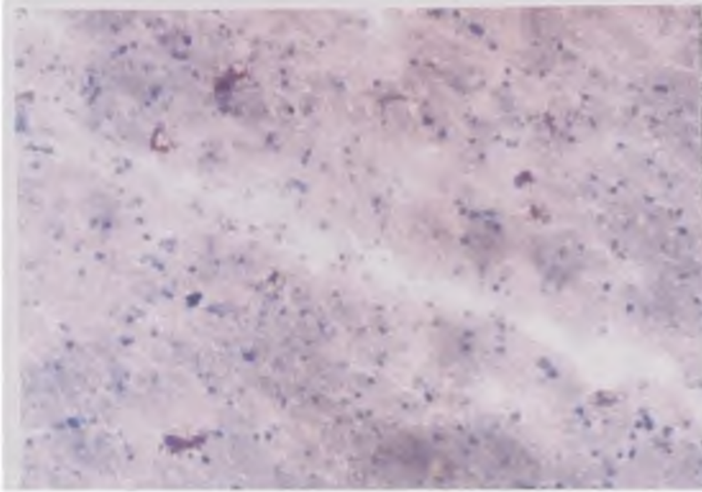

Figure 14 : A photomicrograph of a cerebellar section of the PBM case (Case Number 13) stained with IL-1β showing moderate intravascular and perivascular immunostaining in some low power fields (average score of +) of the section (IHC X100).

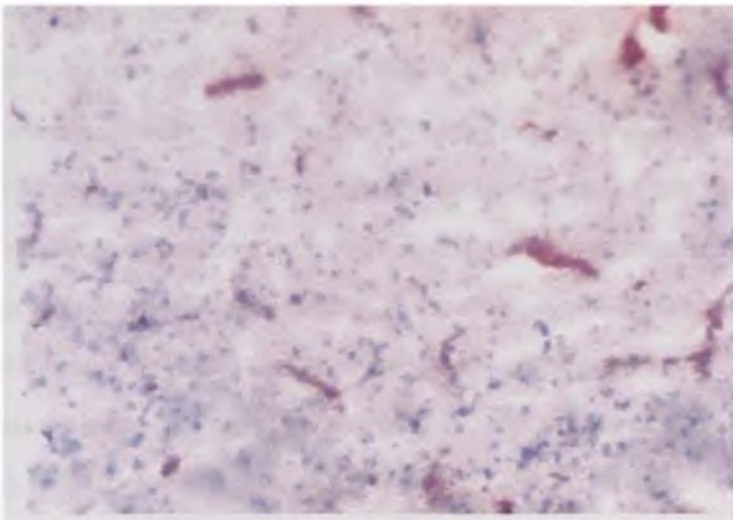

Figure 15 : A photomicrograph of a cerebellar section of the same CM case as in Figure 13 above (Case Number 18) stained with IL-1β showing strong intravascular and perivascular immunostaining in all low power fields (average score of +++) of the section (IHC X100).

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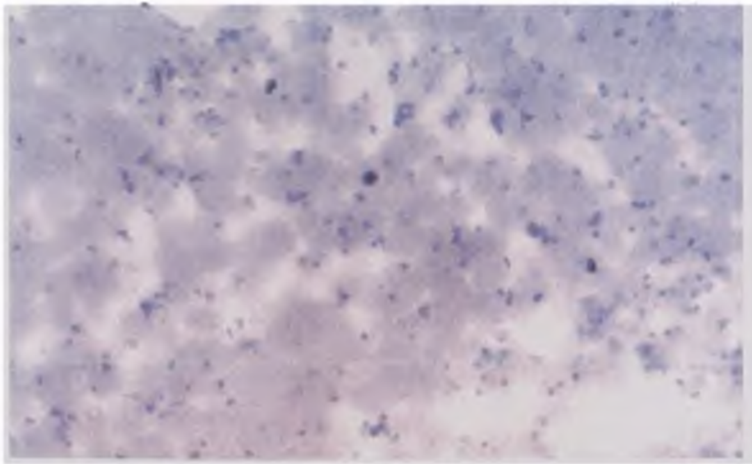

Figure 16 : A photomicrograph of a cerebellar section of the same NCNSI case as in Figure 12 above (Case Number 16 – SMA) stained with IL-1β showing no immunostaining in none of the low power fields (score of -) of all sections (IHC X100).

4. 3. 3 TNF-α Immunostaining

TNF-α was expressed in all the PBM (Figure 17) and CM (Figure 18) brain sections in intravascular, perivascular and intraparenchymal (perinuclear) pattern, but none of the sections of the other three groups (Figure 19). The intensity of staining was, generally, more intense in the CM group compared to the PBM case. TNF-α showed moderate intravascular, perivascular and parenchymal immunostaining in some low power fields (average score of +) of the PBM sections (Figure 17). TNF-α showed strong intravascular, perivascular and parenchymal staining in all low power fields (average score of +++) of all the CM sections (Figure 18), whilst it showed no immunostaining in none of the low power fields (score of -) of all the NCNSI sections (figure 19).

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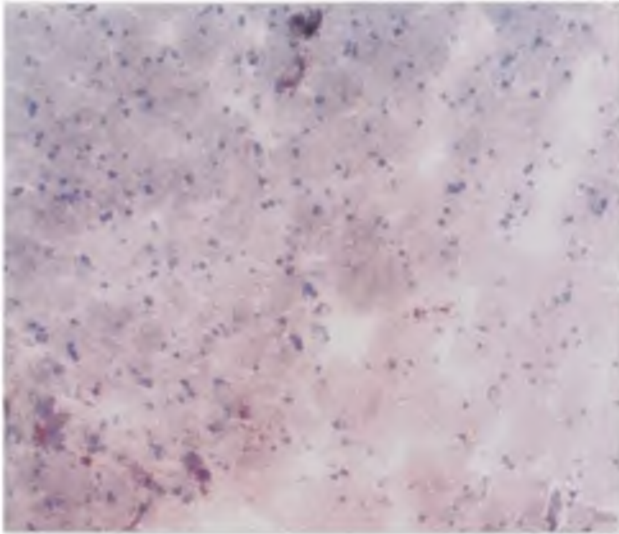

Figure 17 : A photomicrograph of a cerebellar section of the PBM case (Cases Number 13) stained with TNF-α showing moderate intravascular, perivascular and parenchymal immunostaining in some low power fields (average score of +) of the section (IHC X100).

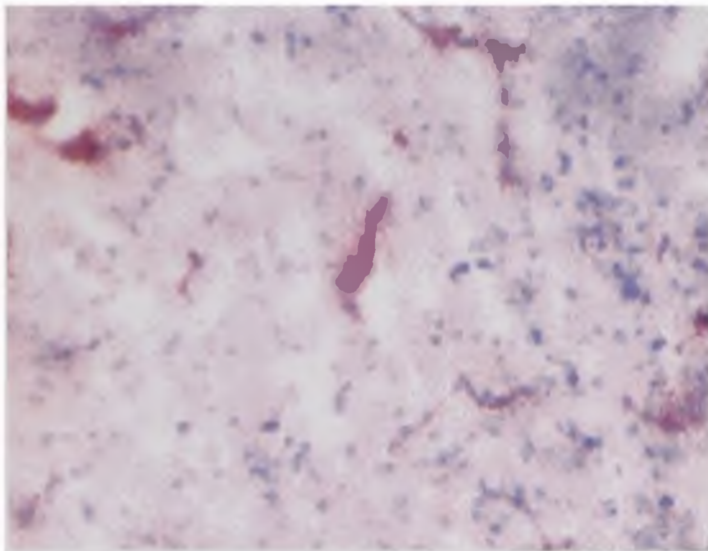

Figure 18 : A photomicrograph of a cerebellar section of the same CM case as in Figures 13 & 15 above stained with TNF-α showing strong intravascular, perivascular and parenchymal staining in all low power fields (average score +++) of the section (IHC X100).

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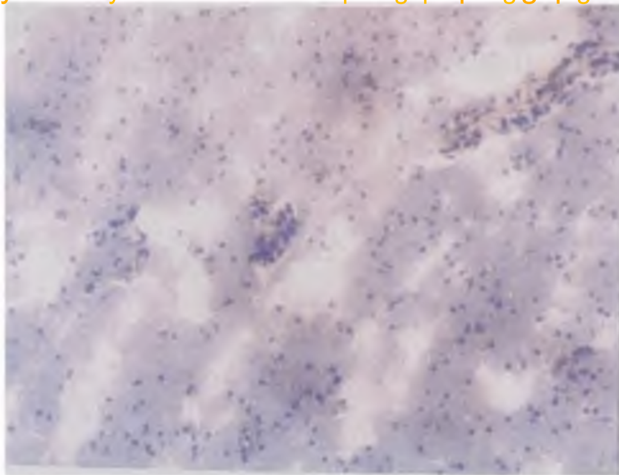

Figure 19 : A photomicrograph of a cerebellar section of the same NCNSI case as in Figures 12 & 16 above (Case Number 16 – SMA) stained with TNF-α showing no immunostaining in none of the low power fields (score of -) of all sections (IHC X100).

Generally, the expression of all the three cytokines studied was highest in the cerebellar sections of the studied cases showing expression (Table 5).

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Table 5 : Comparison of IHC Staining of Cytokines in 3 Brain Regions, and between CM, PBM and Non-CNS Pathology Cases

Antigen	CM Cases			PBM Case			Non-CNS Pathology Cases		
	CB	C	BS	CB	C	BS	CB	C	BS
TGFβ	10/10, 10+++	10/10, 8+++ & 2++	10/10, 7+++ & 3++	1/1, 1+++	1/1, 1+++	1/1, 1+++	10/10, 10+	10/10, 10+	10/10, 10+
IL-1β	10/10, 10+++	9/10, 9+++	9/10, 8+++ & 1++	1/1, 1+	1/1, 1+	0/1, 1-	0/10 10-	0/10 10-	0/10 10-
TNFα	10/10, 10+++	10/10, 10+++	10/10, 10+++	1/1, 1+	1/1, 1+	1/1, 1+	0/10 10-	0/10 10-	0/10 10-

KEY TO TABLE 5

5/5	Numerator for Number of Cases showing Expression, Denominator for Total Number of Cases.
CB	Cerebellum
C	Cerebrum
BS	Brainstem
	no red colour ;
+/-	faint/ mild/ low-level scattered staining in some fields ;
+	moderate staining in some fields ;
++	moderate consistent staining in all fields ; and
+++	deep/ strong consistent staining in all fields.

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CHAPTER 5

DISCUSSION

The pathology of fatal *Plasmodium falciparum* malaria has been extensively investigated, but many areas of controversy and inadequate knowledge still remain. Several hypotheses have been developed to explain the pathogenesis of CM. The release of *Plasmodium* GPI toxin, production of proinflammatory cytokines (both systematically and locally), up-regulation of cerebral endothelial adhesion molecule expression and associated sequestration of PEs and their downstream consequences, such as mechanical blockage, ischaemia, acidosis, haemorrhage, and nitric oxide production have been implicated in the pathogenesis (reviewed by Newton & Krishna, 1998).

Most studies, however, have focused on plasma and CSF levels of cytokines in clinical studies or have used animal models in tissue studies. There is a dearth of direct evidence for local cytokines release in human CM brains (Brown *et al.,* 1999b ; Udomsangpetch *et al.,* 1997 ; Porta *et al.,* 1993), mainly because of difficulties in obtaining human postmortem tissue from malaria cases. We, therefore, used IHC on human postmortem brain sections of CM, SMA, PBM, NCNSI and NI deaths to ensure a more extensive and exhaustive comparison in ascertaining the role of local production of cytokines and adhesion molecule expression in the brain in human CM.

We hypothesized that the human CM postmortem brain tissue would show significant differences, with respect to the distribution and amounts of cytokines and adhesion molecules, when compared to brain tissue form SMA, PBM, NCNSI and NI deaths. This project was, therefore, carried out to examine the role of cytokines and adhesion molecules in fatal cerebral malaria by comparing the expression of ICAM-1 VCAM-1, E-Selectin, TNF-α, TGF-β and IL-1β in cerebral, cerebellar and brainstem postmortem sections form 10 CM, 5 SMA, 1 PBM, 2 NCNSI and 3 NI deaths.

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Though the presence of these cytokines (Brown *et al.*, 1999b ; Udomsangpetch *et al.*, 1997 ; Porta *et al.*, 1993) and adhesion molecules (Silamut *et al.*, 1999 ; Turner *et al.*, 1998 ; Turner *et al.*, 1994) in human CM postmortem brain sections have been previously reported, the study described in this thesis was more extensive (involved more cases of CM) and attempted to compare the expression in CM between 3 brain regions and to four other groups (a wider spectrum) of diseases to enable a more exhaustive comparison.

5. 1 COMPARISON OF THE EXPRESSION OF ADHESION MOLECULES AND CYTOKINES BETWEEN THE 5 GROUPS OF DISEASES STUDIED

One specific objective of this study was to compare the expression of ICAM-1, VCAM-1, E-Selectin, TNF-α, IL-Iβ and TGF-β in postmortem brain tissue sections of human CM, PBM, SMA, NCNSI and NI deaths.

5. 1. 1 ICAM-1, VCAM-1 & E-Selectin Expression

The sequestration of PEs in the microvascultature of vital organs has been observed to be central to the pathogenesis of severe *P. falciparum* malaria. Studies have formally correlated the degree of sequestration in the brain with the presence or absence of cerebral symptoms, and have demonstrated that CM is quantitatively associated with cerebral sequestration (Pongponratn *et al.*, 1991 ; MacPherson *et al.*, 1985). Subsequently, studies have indicated that sequestration is mediated by specific interactions between parasite adherence ligands (PfEMP-1) and host receptors on vascular endothelium such as ICAM-1, VCAM-1, E-Selection, CD36, CSA, CD31 and TSP (Silamut *et al.*, 1999 : Turner *et al.*, 1994 ; Ockenhouse *et al.*, 1992).

Additionally, sequestration has been highly significantly co-localized with the expression of ICAM-1, CD36 and E-Selection, and thus suggesting that these receptors have a role in sequestration *in-vivo* (Turner *et al.*, 1994). Endothelial activation occurred not only

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in fatal CM, but also in the other fatal malaria syndromes, indicating that systemic endothelial activation is a feature of fatal malaria (Silamut *et al.,* 1999 ; Turner *et al.,* 1994). Turner and colleagues (1998), studying dermal microvascular endothelium, reported that endothelial activation occurred in both uncomplicated and severe malaria, and sepsis when compared with uninfected controls. Furthermore, the increased plasma levels of the soluble cell adhesion molecules (sCAMs), such as sICAM-1, sVCAM-1 and sE-Selectin, correlated positively with the severity of malaria (Turner *et al.*, 1998).

Endothelial activation is, therefore, a feature of both mild and severe malaria, and also severe systemic sepsis (Turner *et al.,* 1998), and is thought to be due to the widespread induction of endothelial adhesion molecule expression by raised levels of circulating proinflammatory cytokines, particularly TNF-α (Weber *et al.,* 1995).

In the present study, fatal malaria (both CM and SMA) and *Salmonela* septicaemia were associated with induction of endothelial activation markers, with significantly higher levels of ICAM-1, VCAM-1 and E-Selectin expression on vessels in the brain compared to non-malaria controls. ICAM-1 was most widely expressed and intense in the malaria cases, and hence may mediate the bulk of PE sequestration. Furthermore, we observed no significant differences between the endothelial receptor immunostaining in the CM and SMA cases, which is in agreement with previous reports (Silamut *et al.,* 1999 ; Turner *et al.,* 1998) and indicate that systemic endothelial activation is a feature of fatal malaria and sepsis. This finding confirms that endothelial activation in the brain is not specific to CM, and in support of the hypothesis that EC activation can occur independent of PE binding.

There was highly significant co-localization of sequestration with the expression of ICAM-1, VCAM-1 and E-Selectin in cerebral vessels of the malaria cases, which is in agreement with previous reports (Turner *et al.,* 1998) and further supports a role for these receptors in sequestration *in-vivo*.

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All the non-malaria (NM) controls, except the case of salmonella septicaemia, showed a low-level of ICAM-1 and VCAM-1 expression, but no E-Selectin expression consistently. There was no significant difference in the level of expression of these 3 adhesion molecules between the CM and SMA cases. Therefore, the systemic EC activation seen in fatal cases of malaria does not appear to be specific to this disease, but reflects a basic pathogenetic mechanism common to a number of conditions that cause systemic release of proinflammatory cytokines.

Significantly, the endothelial activation in the case of Salmonella septicaemia was not as intense as that in the fatal malaria sections, and unlike the CM sections was not associated with local proinflammatory cytokine release. The implication is that the local presence of malarial antigens by way of sequestered PEs in the fatal malaria sections contributes to the more intense endothelial activation compared to sepsis, and that the quantitatively more sequestered PEs in the cases of CM than SMA may account for the the observed local release of proinflammatory cytokines in CM but not in SMA. Similarly, the local presence of bacterial antigens in the PBM case may account for the observed local release of proinflammatory cytokines in the PBM case.

5. 1. 2 TNF-α, IL-Iβ & TGF-β Expression

Malaria disease severity has been found to correlate with serum levels of proinflammatory cytokines, particularly TNF-α level (Kwiatkowski *et al.*, 1990). There is recent evidence to support a role for local cytokine release, both proinflammatory (IL-1β and TNF-α) and anti-inflammatory (TGF-β), in human CM (Brown *et al.*, 1999b).

TGF-β was detected in an intravascular and perivascular distribution in the brain sections from all the 5 groups studied, but expression was most intense in the meningitis and CM groups (the cases with central nervous system infection). Serum leakage may be the most probable principal source of the low-level expression in the 3 groups with no central nervous

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system infection, whilst the more intense expression in the 2 groups with central nervous system infection may be the result of additional production by reactive glial responding to local tissue damage. This finding is similar to the report of Brown and colleagues (1999b), and further supports the suggested anti-inflammatory and neuroprotective role of TGF-β in host defense mechanism against neuronal cell loss (Flanders *et al.,* 1998).

TNF-α was detected within the brain parenchyma, in addition to intravascular and perivascular distribution, in only the PBM and CM groups, suggesting neuronal and/or glial up-regulation of TNF-α expression in response to bacterial and malarial antigens, respectively. The brain parenchymal expression of TNF-α in the CM brain sections of the Ghanaian children we studied collaborates a similar recent report in CM deaths in Malawian children (Brown *et al.,* 1999b). Two *in-vitro* studies had previously reported up-regulation of neuronal TNF-α expression (Gahring *et al.,* 1996) and TNF-α expression in infiltrating meningeal leukocytes (Bitsch *et al.,* 1997) in experimental animal models of meningitis, but we observed brain parenchymal TNF-α expression in the human PBM case studied.

IL-1β was detected in only the PBM and CM groups in an intravascular, perivascular and parenchymal pattern. It is therefore not expressed in cases without central nervous system infection, but is induced during central nervous system (CNS) disease, and hence may be neurotoxic and contribute to ischaemic cell death in the brain, as previously suggested (Rothwell & Strijbos, 1995). IL-1β, though present in CM, was not as high in intensity as TNF-α expression. Our observed IL-1β protein expression in both the PBM and CM brain sections, including parenchymal staining, contrasts the report of Brown and colleagues (1999) who observed no staining for IL-1β in brains without CM infection but IL-1β was only expressed using immunofluorescence on infiltrating leukocytes in PBM cases and not in brain parenchyma (Brown *et al.,* 1999).

Generally, the expression of the 3 cytokines studied (TNF-α, TGF-β and IL-1β) was higher in CM group compared to the PBM case, and in the cerebellar sections than the other

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two regions of the brain in the malaria brain sections studied. Interestingly, in this study the increased expression of the proinflammatory cytokines, IL-1β and TNF-α, was associated with increased adhesion molecule expression in the brain in CM, but in the SMA group, the similarly increased receptor expression was not associated with an elevation in the expression of these proinflammatory cytokines. Furthermore, in the meningitis case, the observed increase in the expression of these proinflammatory cytokine expression, including even intraperenchymal brain expression, was not associated with an increased receptor expression.

5. 2 COMPARISON OF THE EXPRESSION OF ADHESION MOLECULES AND CYTOKINE BETWEEN THE 3 REGIONS OF THE BRAIN STUDIED

Another of our specific objectives was to compare the expression of ICAM-1, VCAM-1, E-Selectin, TNF-α, TGF-β and IL-1β in cerebral, cerebellar and brainstem postmortem sections of human CM.

Generally, we found the expression of all the 6 antigens (ICAM-1 VCAM-1, E-Selectin, TNF-α, TGF-β and IL-1β) studied to be highest in the CM cerebellar sections. This observation in human CM is in agreement with the report of Tongren and colleagues (2000) and Smith and colleagues (1996), though they studied cytokines and adhesion molecule expression, respectively, in the *P. coatneyi*-infected Rhesus monkeys. Additionally, the highest expression of cytokines and adhesion molecules in the cerebellum in human CM correlates well with histopathologic observations of maximal sequestration of parasitized erythocytes in this region of the brain in both human CM (Sein *et al.*, 1993a) and Rhesus monkey model of CM (Tongren *et al.*, 2000 ; Smith *et al.*, 1996 ; Sein *et al.*, 1993b). Thus providing a supporting molecular basis for the previously reported histopathologic finding of maximal PE sequestration in cerebellar sections in both human CM and animal models of CM.

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The maximal proinflammatory cytokines (IL-1β and TNF-α) expression in the cerebellum of both human and animal CM was probably the direct result of increased local malarial antigen stimulation from the maximal PE sequestration in the cerebellum. The resulting maximal proinflammatory cytokine expression in the cerebellum, subsequently induced the maximal up-regulation of ICAM-1, VCAM-1 and E-Selectin expression observed in the cerebellum. This up-regulation of adhesion molecules leads to more sequestration which perpetuates a vicious cycle. These results, therefore, suggest that both PE sequestration and its associated proinflammatory cytokine production (both locally and systemically) probably play important roles that culminate in cerebral pathology.

In the current study, it appears that local proinflammatory cytokine release may be a major immune mediator during human CM pathogenesis, particularly in the cerebellum. The mechanisms by which induced proinflammatory cytokines in the brain in human CM mediate immunopathology is unclear. The maximal up-regulation of adhesion molecules and cytokines in human CM cerebellar sections, though the other brain regions in all probability should be equally exposed to the elevated circulating proinflammatory cytokines in plasma and CSF, suggests that the maximal sequestration of PEs in this region of the brain is the trigger event for local IL-1β and TNF-α expression. Most probably, parasite-derived factors resulting form sequestered PEs may have induced the local proinflammatory cytokine release. Our observed maximal cytokine and adhesion molecule induction and the previously reported maximal PE sequestration in human CM cerebellar sections (Sein *et al.*, 1993a) correlates well with the documented cognitive impairment in Kenyan, Senegalese and Zambian children surviving CM (Holding *et al.*, 1999 ; Brewster *et al.*, 1990), since the cerebellum controls co-ordinated movement and some forms of cognitive learning (Schmahmann.,).

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5. 3 CO-LOCALIZATION OF RECEPTOR EXPRESSION AND SEQUESTRATION

Another specific objective was to ascertain whether there is any significant co-localization of sequestration of PEs with expression of ICAM-1, VCAM-1 and E-Selectin. To implicate a host endothelial receptor as being important *in-vivo* for PE sequestration, it must be expressed where sequestration occurs and that PE adhesion should co-localize with receptor expression. We therefore used IHC techniques to identity receptor expression and quantitative visual methods to correlate the presence of sequestered PE with the expression of adhesion molecules:

We reasoned that if an antigen acts as a receptor, the number of vessels expressing the receptor should be concordant with the number of vessels with sequestered PEs and, conversely sequestered PEs should not be seen preferentially in vessels not expressing that receptor. We recognized that at a lower parasite burden, there may be vessels not showing sequestration but expressing the receptor, but the distribution of an antigen that is not acting as a receptor would show no relationship with that of sequestered PEs in vessels.

Our results also show that cerebral sequestration is not exclusive to CM, and although CM was associated with more intense sequestration within the brain, this was also seen to a lesser extent in some of the SMA brain sections, and hence the critical factor in developing cerebral symptoms of malaria is likely to be the quantity of sequestered PEs or parasite load in key areas of the brain. This may, therefore, reflect the time between admission and death, and does not disprove the concept that CM is associated specifically with cerebral sequestration. Probably, if these SMA cases had survived a little longer, there would have been more cerebral sequestration which would have resulted in cerebral symptoms in addition to that of severe anaemia.

The quantitative sequestration and receptor expression co-localization data demonstrated a highly significant association between ICAM-1, VCAM-1 and E-Selectin

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expression and PE sequestration in the cerebral vessels of the 15 malaria cases (both CM and SMA). This suggests (but does not prove) that these molecules may act as sequestration receptors *in-vivo* in malaria. There was no one particular receptor whose expression on the endothelial cell surface was consistently related to the presence of sequestered PEs in the cerebral microvessels, which was in agreement with previous reports (Silamut *et al.*, 1999 ; Turner *et al.*, 1994).

Whereas, generalized endothelial activation was present in many vessels as judged by increased expression of ICAM-1, VCAM-1 or E-selectin, this did not always equate to uniform parasite sequestration in all such vessels showing receptor expression. Additionally, the observation of vessels engorged with sequestered PEs without any evidence of adhesion molecule expression and vice versa was not a rarity. The heterogeneity observed in the distribution of sequestered PEs and receptor expression in fatal malaria emphasizes the importance of the vascular endothelial receptors for parasitized erythrocyte cytoadherence in determining the extent and the distribution of sequestration.

The changing expression of cytoadherence ligands [both host-derived adhesion molecules and parasite-derived parasitized cell adhesins (PfEMP-1)], and thus the changing vascular endothelial cell receptivity for parasitized cell adhesins, is the most likely explanation for the heterogeneity between receptor expression and sequestered PEs observed in this study. Receptor expression without sequestration may be due to a recent up-regulated adhesion molecule expression that has not yet encountered a passing parasitized erythrocyte expressing a complementary parasite-derived adhesin. The absence of the expression of adhesion molecules at a site of sequestration may not exclude its involvement in cytoadherence if that receptor is transiently up-regulated and turned-over rapidly.

Because each case studied reflects a single point in time during the disease process, which varies with each patient, differences in the temporal expression of receptors could be missed, and hence testing the above suggested hypothesis for the heterogeneity by relating

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the distribution of microvascular sequestration of PEs to the different endothelial receptors in autopsy specimens is not possible.

We observed that the increased receptor expression tended to be present throughout a "sequestered" vessel, and not just at the site of parasitized erythrocyte adherence, and thus not supporting the concept that the up-regulation of these receptors is solely a direct result of binding of PE to a particular endothelial cell but rather resulting from systemic processes such as the increased levels of proinflammatory cytokines. Furthermore, the finding of receptor expression in vessels showing no sequestration is at variance with the view that endothelial activation is caused by the binding of PEs to ECs, but as suggested by Silamut and colleagues (1999) this does not preclude the possibility that binding could induce phenotypic changes in an individual endothelial cell, which would increase its adhesive potential (Silamut *et al.*, 1999).

Attempts are being made to develop immunotherapeutic agents to reduce CM mortality and this present study, in children who bear the brunt of the disease, provides further details towards this noble and crucial quest. This study will enhance further our knowledge of the role of cytokines and adhesion molecules in the pathophysiology of CM and aid the efforts to target these mediators as novel immunotherapeutic agents for the prevention and/or treatment of CM.

Further studies to co-localize and determine the cells responsible for the local production of these immune mediators, their receptor interactions and the local responses to these mediators in the brain are the next logical steps forward.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Postmortem brain tissue from 10 CM cases and four other groups of diseases, 5 SMA, 1 PBM, 2 NCNSI and 3 NI cases, were examined for the expression of ICAM-1, VCAM-1, E-Selectin, TNF-α, TGF-β and IL-1β using IHC at light microscopic level.

We have confirmed that endothelial activation is a feature of fatal malaria and severe systemic sepsis, and that the expression of ICAM-1, VCAM-1 and E-Selectin are highly correlated with PE sequestration in the brain vessels of fatal malaria cases.

We have also shown that proinflammatory cytokines, IL-1β and TNF-α, are expressed in the brain in cases with CNS infection (such as PBM and CM), but not in cases without CNS infection. We found that these proinflammatory cytokines were induced in the brain during CM and PBM, and hence their induction in the brain is not specific or exclusive to CM. In contrast, TGF-β was expressed in the brain in all the 5 groups of disease studied, but was more intense in cases with neurodegenerative lesions, and hence supporting its suggested anti-inflammatory and neuroprotective role. In both PBM and CM, TNF-α was expressed intraparenehymally, in addition to intravascular and perivascular distribution.

In conclusion we have demonstrated an up-regulation of adhesion molecule expression in fatal malaria and *Salmonella* sepsis compared to non-malaria controls, but the difference in receptor expression between CM and SMA brain sections were negligible. Additionally, PE sequestration in cerebral microvasculature was highly co-localized with receptor expression. Finally, we believe this study to be the first to use IHC to demonstrate intraparenchymal TNF-α expression in human meningitis postmortem brain sections.

Further studies to ascertain and co-localized the cells (neurons and/or neuroglia) responsible for this local cytokine release and the local interactions and responses to these cytokines in the brain in human CM are required.

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APPENDIX

APPENDIX I : INFORMATION SHEET AND CONSENT FORM

INTRODUCTION

Currently, malaria claims the life of at least one million African Children every year and is the commonest cause of death in Ghanaian Children under 5 years of age. The first step in the development of new treatments to reduce malaria deaths is to determine what substances are involved in the worsening of malaria at the molecular level. You are being invited to grant us permission to perform an autopsy on your child to ascertain the exact cause of his/her death and to remove tiny brain tissue samples at autopsy for a project being organized by Dr. Henry Armah (M. Phil. Student) of the Department of Pathology of the University of Ghana Medical School, in collaboration with the Department of Child Health of the same institution to try and identify these substances.

PROCEDURE

If you agree to the participation of your child, you will be required to sign a consent form to attest that you have fully understood the contents of this information sheet and voluntarily decided on your child's participation.

WITHDRAWAL FROM STUDY

Your child's participation in this research is entirely voluntary. You can refuse participation or withdraw from the study at any stage without a penalty. The investigators may terminate your child's participation for reasons that will be explained to you by the healthcare professionals.

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CONFIDENTIALITY

All the information related to your child's participation in this study will be kept in the strictest confidentiality, and will not be revealed to anyone except where required by law and regulations. Any new information that may affect your willingness to participate will be made available to you.

BENEFIT

There will be no immediate direct personal benefit to your child, yourself or your family, but your participation in this study would lead to our better understanding of the processes that lead to the death of children with malaria. This could speed up the development of new treatment strategies to reduce the deaths associated with malaria.

QUESTIONS

Dr. Henry Armah (Project Investigator)/ Prof. E. K. Wiredu (Supervisor)/ Prof. A. A. Adjei (Supervisor) will be available to answer any questions, offer clarifications or give assistance in all matters relating to this project.

EXECUTION OF INFORMED CONSENT

I, ..., of ....

hereby certify that the contents of the above has been read by me/interpreted to me in the

.... language by ....

of ....

I have perfectly understood the same and thereby append my signature/ mark (Right Thumb Print) to this consent form as an evidence of my agreement to allow my child to participate in this project.

....

Signature/RTP   Date

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WITNESS

I, ..., of ....

hereby certify that I was present when the contents of the above was read by/ interpreted to

.... of ....in

the ....language by ....

of ....

The said .... seem to have perfectly understood the contents of the above before appending his/ her signature/ mark (Right Thumb Print) to this consent form in my presence as evidence of his/ her agreement to allow his/ her child to participate in this project.

....

Signature/RTP Date

Countersigned by a Project Investigator

Name .... Signature ....

Title .... Date ....

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APPENDIX II : GIEMSA STAINING OF BRAIN SMEAR CYTOLOGY

PURPOSE

For the demonstration of protozoa in tissue and other elements such as

- Chromosomes and
- Cytoplasmic granules

REAGENTS

1. Giemsa Stock solution

   Giemsa stain powder 4g

   Glycerol 25ml

   Methanol 25ml

   Dissolve the stain powder in the glycerol at 60 °C with regular shaking. Add methanol and shake well. Allow mixture to stand to ripen for 7 days. Filter solution before use.

2. Working Giemsa stain

   Giemsa stock solution 4mls

   Buffered distilled water (pH6.8) 96ml

3. 0.5% aqueous acetic acid.

PROCEDURE/METHOD

1. Bring section down to water
2. Rinse in distilled water (buffered pH 6.8)
3. Treat sections with working Giemsa stain .... Overnight
4. Rinse in distilled water
5. Rinse in 0.5% aqueous acetic acid until the section is pink (examine microscopically)

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6. Wash in tap water

7. Blot section dry

8. Dehydrate <u>very</u> rapidly through absolute alcohols

9. Clear in xylene

10. Mount in DPX

RUSULTS

Protozoon and some other micro-organisms .... Dark blue

Background .... Pink to pale Blue

Nuclei .... Blue

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APPENDIX III : IMMUNOHISTOCHEMISTRY ASSESSMENT FORM

Case Number ....

Region of Brain ....

Location Of Stain	ANTIGEN						
	ICAM-1	VCAM-1	E-Selectin	TNF-α	IL-1β	TGF-β	Negative Control
Vascular							
Intravascular							
Perivascular							
Parenchymal OR Perinuclear							

KEY

Scoring For Adhesion Molecules (Modified version of scoring of Turner et al., 1994).

	no endothelial cell staining
+/-	faint/ low-level/ mild scattered endothelial cell staining on < 25% of vessels
+	mild endothelial cell staining on > 25% and < 50% of vessels
++	moderate endothelial cell staining on > 50% and < 75% of vessels
+++	strong positive endothelial cell staining on > 75% of vessels

Scoring For Cytokines

	no red colour
+/-	faint/ mild/ low-level scattered staining in some fields
+	moderate staining in some fields
++	moderate consistent staining in all fields
+++	deep/ strong consistent staining in all fields.

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APPENDIX IV : MATERIALS, REAGENTS, SOLUTIONS, BUFFERS AND STAINS FOR IMMUNOHISTOCHEMISTRY USING APAAP

A : MATERIALS, REAGENTS AND SOLUTIONS

1. Liquid Nitrogen
2. Isopentane
3. " Tissue tek" OCT freezing gel
4. Cryotome and accessories
5. Superfrost Plus ™ Microscope slides and cover slips
6. Acetone (100% solution)
7. Tris [2-amino-2-(hydroxymethyl) propane-1,3-diol] salt
8. Sodium Chloride salt
9. 1M Hydrochloric acid (HCl) solution
10. Freshly prepared distilled water
11. Staining Baths
12. Glycergel acqueous mountant
13. Microscope

B : PREPARATION OF SOLUTIONS, BUFFERS AND STAINS

(i) Tris Buffered Saline (TBS) [0.05M Tris, 0.15M NaCl, pH 7.6)

0.5M TBS Stock Solution

Tris [2-amino-2-(hydroxymethyl) propane-1, 3-diol] 60.55g

NaCl 87.66g

Dissolve Tris and NaCl in approximately 500 ml of freshly prepared distilled water. Adjust pH to 7.6 with about 370 mls of 1M HCl. Then bring the total volume to 1000 ml with freshly prepared distilled water.

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0.05M TBS Working Solution

0.5M TBS Stock Solution .... 100 ml

Freshly prepared distilled water .... 900 ml

Make a 1 : 10 dilution of Stock Solution with freshly prepared distilled water as above before use. The diluted Working Buffer Solution is 0.005M Tris, 0.15M NaCl, pH 7.6.

(ii) Tris-HCl Buffer (0.1M Tris, pH 8.2)

Tris [2-amino-2-(hydroxymethyl) propane-1,3-diol] 1.21g

Dissolve Tris in 80 ml of freshly prepared distilled water. Adjust pH to 8.2 with about 4.8 mls of 1M HCl. Then bring the total volume to 100 mls with freshly prepared distilled water. Store at 4 °C.

(iii) Chromogenic Substrate for Alkaline Phosphatase

Naphthol-AS-MX phosphate, free acid 2mg

N, N- Dimethyl formamide 0.2ml

0.1M Tris-HCl buffer, pH 8.2 (see above) 9.8ml

Fast Red TR salt 10mg

Dissolve naphthol-AS-MX phosphate in N, N- dimethyl formamide. Add the Tris buffer. It is recommended that this naphthol-formamide-Tris buffer solution be made up fresh just before use, although it is thought to be stable at 4 °C for at least a week. Then, dissolve Fast Red TR in the naphthol-formamide-Tris buffer solution immediately before use on cryostat sections. Filter the solution.

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(iv) Mayer's Haematoxylin Stain

Haematoxylin 1g

Freshly prepared distilled water .... 1 litre

Dissolve with gentle heat

Add ammonium or Potassium alum 50g

Sodium iodate 0.2g

Stir solution to dissolve. Then add,

Citric acid 1g

Chloral hydrate 50g

The solution turns a reddish violet colour. Solution is ready for immediate use.

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APPENDIX V : MATERIALS AND PROCEDURE FOR SNAP FREEZING AND PRESERVATION OF POSTMORTEM BRAIN SAMPLES IN – 80^0 c FREEZER

A : MATERIALS

1. Scapel blade
2. Forceps
3. Petri dish
4. "Tissue Tek" OCT freezing gel
5. Isopentane
6. Aluminum foil made into small cups/troughs (specially designed for this project)
7. Liquid Nitrogen

B : PROCEDURE

1. Trim excess tissue using a scapel blade and forceps.
2. Transfer the trimmed tissue into aluminum foil cup/trough using a forcep.
3. Cover the tissue with "Tissue tek" OCT freezing gel (until tissue is completely submerged)
4. Pre-freeze the contents of the aluminum foil cup in chilled insopentane (chilled with liquid nitrogen)
5. When the gel is frozen (thus turned white) around the tissue, seal the open end of the aluminum foil cup and place in liquid nitrogen for transport to the laboratory where it is transferred into a -80^0c freezer until use.