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THE CHARACTERIZATION OF MONONUCLEAR PHAGOCYTES AND CYTOKINE POLYMORPHISM IN CEREBRAL TISSUE OF CHILDREN DYING FROM CEREBRAL MALARIA

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By

Roslyn Elizabeth Wofford McQueen

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ABSTRACT

THE CHARACTERIZATION OF MONONUCLEAR PHAGOCYTES AND CYTOKINE POLYMORPHISM IN CEREBRAL TISSUE OF CHILDREN DYING FROM CEREBRAL MALARIA

By

Roslyn Elizabeth Wofford McQueen

A life threatening consequence of malaria is the development of cerebral malaria (CM) which is a leading cause of death in children and non-immune infected adults. The main pathophysiological feature of CM is the sequestration of parasitized red blood cells (pRBC) in the brain which occlude blood vessels. Recently, monocytes and macrophages were detected in cerebral tissue of CM patients. Cytokines also play a major role in malaria by providing protection against infection, inflammation and injury. However, the overproduction of pro-inflammatory cytokines, specifically tumor necrosis factor-alpha (TNF-α) is associated with much of the pathology of CM. Consequentially, monocytes and macrophages are the predominant source of TNF-α.

These factors provided the basis for the development of the hypothesis and research objectives. The hypothesis was that activated macrophages in CM lesions are the source of local TNF-α production and patients with CM may have a genetic predisposition for an inappropriate production of TNF-α. The first research objective was to characterize the mononuclear phagocytes in the cerebral tissue and determine their state of activation. Immunohistochemical stains demonstrated the presence of activated macrophages in cerebral vessels. The second objective was to detect the local production of TNF-α in cerebral

tissue. *In situ* hybridization and an immunohistochemical stain revealed the local production of TNF-α messenger ribonucleic acid and protein in cerebral tissue by macrophages and some neuronal cells, suggesting that macrophages and cerebral cells produce cytokines in the local tissue. The third objective was to detect polymorphisms in the genes of pro- and anti-inflammatory cytokines. The deoxyribonucleic acid (DNA) was extracted from cerebral tissue and the genes of pro- and anti-inflammatory cytokines analyzed for polymorphisms in the promoter sequences, introns and leader sequences of TNF-α, interferon-gamma (IFN-γ), interleukin-six (IL-6), interleukin-ten (IL-10) and transforming growth factor-beta (TGF-β) by the polymerase chain reaction using sequence specific oligonucleotide primers. Polymorphisms in the genes of pro-inflammatory cytokines, TNF-α and IFN-γ were associated with the low production phenotype, while IL-6 and anti-inflammatory cytokines, IL-10 and TGF-β presented the high production phenotype.

In loving memory of my mother

In conclusion, the data demonstrated activated macrophages in cerebral tissue and the local production of TNF-α. Patients with CM may be genetically programmed to produce low amounts of pro-inflammatory cytokines, TNF-α and IFN-γ, high IL-6, and elevated amounts of anti-inflammatory cytokines IL-10 and TGF-β. This could have the effect of an anti-inflammatory cytokine predominance, resulting in dysfunctional macrophages and an immunologic imbalance. Further research in the field is warranted including therapeutic manipulation to reduce anti-inflammatory cytokine expression in patients predisposed to increased cytokine production.

DEDICATION

To my husband, Adolph,

To my sister, Yvette

and

In loving memory of my mother

ACKNOWLEDGMENTS

I would like to acknowledge, with gratitude and humility, the incessant support rendered to me by Drs. Charles Mackenzie and John Gerlach without whom this dissertation would not have been possible. My heartfelt thanks to Dr. Mackenzie who accepted me into the graduate program and provided continued support and encouragement throughout the program. To Dr. John Gerlach who directed and focused my research so that I was able to obtain meaningful data. I will be eternally grateful for the training, counseling, direction, support and encouragement rendered.

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KEY TO SYMBOLS OR ABBREVIATIONS

α	alpha
β	beta
γ	gamma
ATP	adenosine triphosphate
CS	circumsporozoite
°C	degrees Centigrade
DNA	deoxyribonucleic acid
DIG	digoxigenin
dH_20	distilled water
ddH_20	double distilled water
dsDNA	double stranded DNA
EDTA	ethylenediamine tetraacetate, di-sodium salt
>	greater than
<	less than
HSPG	heparin sulfate proteoglycans
IgG	immunoglobulin G
IFN-γ	interferon-gamma
IL-6	interleukin-six
IL-10	interleukin-ten
μl	microliters
μm	micrometer
μM	microMolar
ml	milliliter
mm	millimeter
mm^2	millimeters square
mM	millimolar
nm	nanometers
PET	paraffin embedded tissue
pRBC	parasitized red blood cells
PVM	parasitophorous vacuole membrane
%	percent
PLC	phospholipase C
PCR	polymerase chain reaction
RBCs	red blood cells
ssDNA	single stranded DNA
SDS	sodium dodecyl sulfate
SGF	sulfated glycoconjuage binding motif
TRAP	thrombospondin-related anonymous protein
TGF-β	transforming growth factor-beta
Tris-HCl	Tris (hydroxymethyl)aminomethane hydrochloric acid
TNF-α	tumor necrosis factor alpha
H_20	water

Chapter 1

INTRODUCTION AND OBJECTIVES

Malaria is a vector borne parasitic disease caused by obligate intracellular protozoa from the genus *Plasmodium*. Malaria infects 300-500 million people worldwide and causes 1-2 million deaths every year, mostly in children under the age of five (WHO 2000). A severe pathologic complication of malaria is the development of cerebral malaria (CM) which occurs in about 1% of *Plasmodium falciparum* infected patients. CM is a diffuse encephalopathy that presents as a state of an unrousable coma along with *P. falciparum* parasitemia (WHO 2000). The pathogenesis of CM is still poorly understood. The typical histopathologic presentation features engorgement of cerebral blood vessels with malaria infected erythrocytes and hemozoin, a monomic malarial pigment, hemozoin (MacPherson et al. 1985, Boonpucknavig et al 1990, Patnaik et al.1994, Newbold et al 1999). The mechanical blockage of the microvasculature is not thought to be the sole cause of the altered consciousness (Clark and Rockett 1994). Merozoite schizony in the parasitized red blood cells (pRBC) stimulates the local production of cytokines in the cerebral vasculature (Francis and Warrell 1993, Clark and Rockett 1994). The combination of occluded blood vessels containing sequestered pRBC and the overproduction of inflammatory cytokines have been implicated as essential factors for the pathogenesis of CM (Clark and Rockett 1994).

Recently, mononuclear phagocytes were detected in cerebral tissue of Malawian children dying of CM (Mackenzie et al.1999). These findings were

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Recently, mononuclear phagocytes were detected in cerebral tissue of Malawian children dying of CM (Mackenzie et al.1999). These findings were

unique, because, macrophage infiltration had previously been attributed only to the murine form of CM, and not to human CM. It was postulated that mononuclear phagocytes may play a role in the pathogenesis of CM.

Consequently, the detection of mononuclear phagocytes in cerebral tissue provided the basis for the fundamental study objective to characterize the mononuclear phagocytes in cerebral tissue and determine their role in the pathogenesis of CM. The overproduction of TNF-α is associated with much of the pathology of CM and monocytes/macrophages are the predominant source of TNF-α. These factors served as the impetus for development of the research hypothesis, that activated macrophages in CM lesions are the source of local TNF-α production and patients with CM may have a genetic predisposition for an inappropriate production of TNF-α.

Several studies provide evidence to support this hypothesis. Histological examinations of post mortem tissue describe the occlusion and cytoadherence of pRBC to the lumen of cerebral blood vessels. The endothelial cells were observed to exhibit moderate swelling and ballooning into the lumen from the sequestration (MacPherson et al.1985, Pongponratn et al.1985, 1991, Gopinathan et al.1986, Patnik et al.1994, Newbold et al. 1999). A number of molecules expressed on the surface of endothelial cells induce the adhesion of the parasite-infected RBCs including cluster of differentiation (CD)-36, intercellular adhesion molecule-1, vascular adhesion molecule-1, Endothelial-selectin (E-selectin), and thrombospondin, (Albelda et al. 1994, Igarashi et al. 1987, McCromick et al. 1997, Schofield et al. 1996, Newbold et al. 1999).

Changes occur on the surface of red blood cells within hours of parasitic invasion mitigating their adherence to endothelial cells. The pRBC express surface molecules which mediate cytoadherence include: *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), sequestrin, *P. falciparum* antigen 332 (Pf332), and modified RBC band three (Baruch et al. 1997, Newbold et al.1999, Thevenin et al. 1997).

Cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin (IL-6) upregulate the expression of cellular adhesion molecules (CAM) and mediate the binding of pRBCs to vascular endothelium (Urban et al. 1986, de Kossodo and Grau 1993, Grau and Behr 1995, Udomsangpetch et al. 1996, McCormick et al. 1997, Lou et al. 1998, Garcia et al. 1999, Wahlgren 1999, Yipp et al. 2000). The inappropriate expression of pro- and anti-inflammatory cytokines result in the prolonged CAM upregulation, sustained activation of phagocytes and failure to downregulate inflammatory cytokines. Elevated plasma levels of proinflammatory cytokines, TNF-α, IFN-γ and IL-6 have been detected in patients who died of falciparum malaria. The overexpression of cytokines correlated with parasite density (Grau et al. 1989, Day et al. 1999, Brown et al. 1999). Malarial antigens such as glycosylphosphatidylinositol enhance the production of IFN-γ by natural killer cells and T helper 1 (Th1) cells (Bate et al. 1991, Allan et al. 1995, de Souza et al. 1997, Tachado et al. 1997). The IFN-γ activates macrophages by stimulating the secretion of TNF-α, and the upregulation of CAMs and TNF-α receptors on brain endothelial cells (Grau et al. 1993, Grau and Behr 1994, Lou et al. 1998).

TNF-α has been implicated with the severe pathology of malaria because (1) elevated levels have been found in serum of patients dying from CM and (2) anti-TNF-α antibody injected in malaria infected mice prevented CM development (Grau et al. 1987, 1989). Additionally, IL-6 functions synergistically with TNF-α. Patients who died of malaria had significantly higher plasma TNF-α and IL-6 levels than those who survived (Kern et al. 1989, Molyneux et al. 1991).

There is compelling evidence in murine models that an imbalance between pro- and anti-inflammatory cytokines might contribute to the pathogenesis of CM (Day et al. 1999, Brown et al. 1999, Grau et al. 1987). Transforming growth factor-beta (TGF-β) and interleukin-ten (IL-10) are anti-inflammatory cytokines that suppress the production of pro-inflammatory cytokines, TNF-α, IL-6 and IFN-γ. Low levels of IL-10 and TGF-β levels were associated with a lethal outcome in murine malaria, whereas the higher expression prevented the development of malaria anemia by downregulating the pro-inflammatory cytokines (Omer and Riley 1998, Othoro et al. 1999, Day et al. 1999, Omer et al. 2000). Allelic polymorphism in the genes of TNF-α, TGF-β, IFN-γ, IL-6 and IL-10 may cause differences in their expression levels (McGuire et al. 1994, Turner et al. 1997, Awad et al. 1997, Wilson et al 1997, Pravica et al. 1998, Hutchinson 1999). Patients that produce low serum levels of IL-10 and who have the TNF-α high production genotype (-G308A, A/A) had a worse clinical outcome than those expressing normal cytokine levels. These observations raise the possibility that an inappropriate expression of cytokines provides the fundamental basis for the pathological changes observed in CM.

The investigations performed for this dissertation detected macrophages in cerebral tissue and evaluated their state of activation using immunohistochemical markers. The detection of TNF-α in cerebral tissue was determined using immunohistochemistry for local TNF-α protein production and *in situ* hybridization for TNF-α messenger RNA (mRNA). Cytokine genotyping was performed to investigate polymorphisms in the promoter regions, introns and leader sequences of the cytokines that may contribute to a fatal outcome in CM. Genotyping was performed by the polymerase chain reaction using sequence-specific oligonucleotide primers. The results are discussed in light of the pathogenesis of pediatric cerebral malaria.

Chapter 2

LITERATURE REVIEW

MALARIA

For centuries, malaria has been a formidable adversary in the never ending battle between man and microbe. An essential prefatory to this dissertation is a review of the etiology, transmission, prevalence, and pathology of malaria. The name malaria meaning "bad air" evolved from the Italian words *"mala"* which means bad, together with *"aria"* which is air. Malaria probably originated in Africa and is chronicled as far back as 30,000 years before Christ (B.C.)(Winslow and Connor 1967, Taylor and Strickland 2000).

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Throughout history, there has been fright air (miasma), the belief that disease was associated with vapors emanating from swamps or marshy regions. In the sixth century B.C., the association of periodic fevers with stagnant water and swamps led to the development of drainage systems by the Greeks and Romans to control the spread of disease (Taylor and Strickland 2000). The Greek physician, Hippocrates, was one of the first to describe the clinical manifestations and complications of malaria in the fifth century, B.C. He related the disease to the seasons of the year and the locations of patients' homes (Gilles 1993). The cause and treatment of malaria were unknown for hundreds of years. In 1880, Charles Laveran, a French Army surgeon, discovered the causative agent of malaria and was the first to describe the parasites in a patient's red blood cells. In 1886, Machiafava and Celli named the malarial parasite *Plasmodium* (Winslow and Connor 1967). The 1678 discovery that

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arthropod vectors could transmit various diseases provided the clue that mosquitoes might serve as a vector for malaria. In 1897, Ronald Ross had discovered a malaria parasite in the body of a mosquito that had previously fed on a *Plasmodium* infected patient. Using an avian malaria model, he experimentally proved that malaria was transmitted from mosquitoes. Later in 1898-99, the Italian scientists, Grassi, Bignami and Bastionelli, described the complete life cycle of the development of malaria in both hosts: female *Anopheles* mosquito and human (Winslow and Connor 1967, Gilles 1993, Taylor and Strickland 2000).

Phylogenetically, the animal kingdom is divided into two subkingdoms: multicellular *Metazoa* and unicellular *Protozoa*. Malaria is a vector borne parasitic disease caused by an obligate intracellular protozoan from the genus *Plasmodium*. The genus *Plasmodium* evolves from the class *Telosporidia*, Order Coccidiia, sub-order *Haemosporidia*, and family *Plasmodiiae*. Members of the Order *Plasmodiia*, form gametes in arthropod hosts, develop gametocytes in vertebrate erythrocytes, and deposit pigmented granules within the invaded red blood cells (Chandler and Read 1961). There are over 156 species of *Plasmodium*, but only four are known to infect humans, namely *P. malariae* (Laveran 1881), *P. vivax (*Grassi and Feletti 1890*), P. falciparum* (Welch 1897*),* and *P. ovale* (Stephens 1922, in Gilles 1993). It should be noted that some authors classify the *falciparum* species in the genus *Laverania*, i.e., *Laverania falciparum*, based on the development of the sexual form of the parasite in erythrocytes. The four species differ genetically, biologically, ecologically and

pathologically. Malaria infection is frequently referred to by such colloquial names as: benign or tertian malaria. The recommended nomenclature is to preface the disease (malaria) with the infecting species, i.e., vivax malaria, ovale malaria or falciparum malaria. The one exception is quartan malaria which is still acceptable for *P. malariae* infections.

A parasite is defined as *"an organism that lives on, or in, another and draws its nourishment therefrom"* (Stedman 1995, Giesecke 1994). The life cycle of the *Plasmodium* parasite is an alternation between invertebrate (*Anopheles* mosquito) and vertebrate (human) hosts followed by two intracellular stages and one extracellular developmental stage. The malaria parasite is transmitted to humans from the bite of female *Anopheles* mosquitoes. Albeit, the parasite can be acquired congenitally, or from blood transfusions. More than 400 *Anopheles* species have been detected. The major malarial vectors can be classified into approximately, 35 species grouped by geographical and biological characteristics (White 1982, Onori and Muir 1984, Service 1993). The *Anopheles* mosquito is widely distributed throughout the world, with a predominance in Africa, Asia, and Latin America (White 1982, Service 1993).

The life cycle of malaria is complex with significant differences among the four *Plasmodium* species. The following is an abbreviated overview of the transmission of malaria. Infection begins with a mosquito bite containing *Plasmodium* infected saliva. The malarial sporozoites travel through the bloodstream to the liver where they multiply, transform into merozoites rupturing the hepatocyte upon release. The merozoites invade red blood cells (RBCs) and

upon maturation rupture the cells quickly infecting other erythrocytes. The rupturing of RBCs by the merozoites produces the pathognomonic malaria symptoms of chills and fever which repeat every 48 hours in quartan malaria. The characterization of the genome of *P. falciparum* along with current molecular research have provided a greater understanding of the pathobiology of malaria although many aspects of the disease remain unknown.

Transmission of Malaria

Only the female *Anopheles* mosquito serves as a vector for the *Plasmodium* parasite. Male *Anopheles* mosquitoes feed on plant juices only and therefore do not serve as vectors for *Plasmodium.* Infection begins when a *Plasmodium* infected female *Anopheles* mosquito takes a blood meal to fertilize her eggs. The malarial sporozoites develop in the mosquito's midgut and attach to secretory cells of the salivary glands. The sporozoites are unique in that they can interact with both hosts: human and arthropod. The proboscis of the mosquito injects sporozoites from its salivary glands into the host. Morphologically, sporozoites are elongated, slightly curved, slender cells, covered with a 45 kilodalton (kDa) molecule known as the circumsporozoite (CS) protein (Menard et al.1997, Ying et al. 1997). CS plays a pleiotropic role in malaria. In mosquitoes, CS is essential for sporozoite development within oocysts, and in humans, it promotes the attachment of the sporozoite to the hepatocyte (Menard et al.1997). The infectivity of sporozoites correlates with the expression of CS and its distribution on the parasite's cell surface (Holder 1994).

It is not known whether the mosquito feeds directly from a capillary or from a blood pool formed from the inserted proboscis. Nevertheless, within minutes, the sporozoites disappear from the bloodstream migrating to the liver (Shortt and Garnham 1948, Frevert 1994, Holder 1994, Pradel and Frevert 2001). The injection of sporozoites induces a non specific immune response by the human host. Mononuclear phagocytes, natural killer cells and cytotoxic T cells destroy some of the sporozoites but many evade these cells and reach liver parenchymal cells (Rathore and McCutchan 2000). The inoculation of as little as one sporozoite is sufficient to initiate the malaria infection (Barnwell 2001).

Sporozoites Infect Hepatocytes

Sporozoites only bind to and invade hepatocytes. Minutes after infection, the malaria sporozoites enter hepatocytes where they multiply and develop into merozoites (Cerami et al.1992). The actual pathway from the inoculation site to the hepatocytes is a matter of speculation. Sporozoites seem to possess the ability to efficiently and rapidly home to the liver. The initial theory was that sporozoites infect hepatocytes directly, while current research supports the theory that the sporozoites are trapped by Kupffer cells (macrophages found on the luminal surface of the hepatic sinusoid) and transported to hepatocytes some time later (Sinden and Smith 1982).

Using time-lapse video images, *P. bergheri* sporozoites were observed to enter murine Kupffer cells and to exit in about one minute after parasitic infection (Mota et al. 2001). The sporozoites penetrated the Kupffer host cells by

breaching the plasma membrane, which rapidly repairs itself. Sporozoites could therefore migrate through the cytoplasm of several cells before invading the hepatocyte (Holder 1994, Mota et al. 2001). With this form of entry, phagocytic vacuoles were not formed and cytolytic enzymes remained inactive. As a result, the macrophages did not kill the probing sporozoites. In non-immune mice, Kupffer cells were observed transporting sporozoites to the liver cells, but in immunized animals, the antibody-coated parasites were immediately phagocytosized and killed by the macrophages (Danforth et al. 1980). Thus, malarial sporozoites can selectively recognize and actively invade Kupffer cells, avoid phagosomal acidification, and safely pass through phagocytes to the liver cells.

Two sporozoite surface proteins, CS and thrombospondin-related anonymous protein (TRAP), are essential for parasitic attachment to host cells. The parasites adhere to the hepatocytes by binding the CS protein to the thrombospondin surface receptors on the basolateral surface of the liver cells (Cerami et al. 1992). The CS protein provides a "velco-like" adhesive attachment to the host cell. CS protein from all *Plasmodium* species possess a conserved amino acid sequence (Trp-Ser-Pro-Cys-Ser-Val-Thr-Cys-Gly) in region II of the protein, which is homologous to the binding domain of thrombospondin (Cerami et al. 1992, Frevert 1994, Holder 1994, Ying et al.1997, Rathore and McCutchan 2000, Menard et al. 1997). TRAP is located in the sporozoite's micronemes (internal organelle in the anterior region of the cell) and inducibly translocated to the surface. TRAP is essential for gliding motility and

recognition of endothelial and hepatocyte receptors (Sinnis 1996, Hollingdale et al. 1998). Both sporozoite proteins (CS and TRAP) possess similar binding motifs and function in both the vector and human host (Menard et al.1997). After inoculation, sporozoites quickly and purposefully invade the liver. The speed and selectivity suggests that the liver tropism may be receptor-mediated. The conserved amino acid sequences of CS and TRAP play an integral role in hepatocyte recognition.

Hepatocytes, as well as various other host cells, have surface receptors that contain sulfated glycosaminoglycan chains of the proteoglycans (Frevert 1994, Holder 1994, Pinzon-Ortiz et al. 2001). To assess tissue tropism by sporozoites, proteoglycan receptor proteins of various tissue were incubated with CS and TRAP proteins. No binding was detected to spleen, brain, heart or lung tissue even though they possess proteoglycan receptors (Frevert 1994). The hepatocyte receptor appears to be a heparin sulfate proteoglycan (HSPG) (Frevert 1994, Holder 1994). One explanation for this tissue tropism is that HSPG may be post translationally modified in an organ specific manner. Liver HSPGs protrudes from hepatocytes directly into the sinusoid and mediates the clearance of substances from the blood circulation. Because of the heterogeneity of HSPG, the size and structure of the individual glycosaminoglycan chains remain unknown (Holder 1994, Frevert 1994, Ying et al. 1997). Thus, CS and TRAP bind to host cell receptors with a high degree of specificity. However, the molecular characterization of the binding sites of CS and TRAP to host cell receptors remain to be elucidated.

Upon entering the hepatocyte, the sporozoite becomes rounded and differentiates into a schizont and undergoes rapid multiplication. The hepatocytes swell during schizogony, flattening and displacing the nucleus to one side. In pre-erythrocytic schizogony/merogony, the parasitic nucleus undergoes repeated divisions followed by cytoplasmic divisions into as many parts as there are nuclei. This results in the formation of thousands of uninucleate merozoites (Frevert 1994, Holder 1994, Barnwell 2001). The swollen hepatocytes do not generate an inflammatory reaction and the host remains asymptomatic. This is because the infected hepatocytes do not display parasite derived antigens on their cell surface membrane, thereby protecting them from attack by antibodies (Ferreira et al. 1986). Within 40 to 48 hours of infection, tens of thousands of tiny (1-2 μm) malaria parasites can be observed within the hepatocytes (Barnwell 2001, Bray and Garnham 1982). In *P. falciparum* and *P. malariae* infections, the schizonts rupture and do not persist in the liver. In contrast, *P. vivax* and *P. ovale* develop two types of pre-erythrocyte forms. In the main type, merozoites develop normally, rupturing the RBC within six to nine days. An alternate stage, known as the hypnozoite, remains dormant in the liver for weeks, months or up to five years before initiating a relapse of the malaria infection (Bray and Garnham 1982, Gilles 1993).

Merozoites Invade Red Blood Cells

Pathologically, the merozoite is the most destructive stage in the life cycle of the parasite. The merozoite is a tiny (1-2 μm), pyramid-shaped, blood-

dependent organism that must find and enter an erythrocyte immediately upon release from the hepatocyte. RBC invasion is a complex, multi-step process. The invading parasites must recognize and attach to the RBC in a series of specific molecular interactions before entry takes place. Ultrastructural studies have divided the invasion of host RBCs into three stages: (1) attachment (2) invagination (3) and vacuole formation (Holder 1994, Bannister 2001). Attachment is mediated by specific receptors on both the parasite and RBC. The specificity of the interaction depends on the expression of RBC surface receptors for which the parasite has a ligand, as well as the age of the erythrocyte (Holder 1994). The merozoites attach to sialic acid residues expressed on the surface of integral membrane glycoproteins, glycophorin A and B (Perkins 1984, DeLuca et al. 1996, Chitnis 2001). Both *P. vivax* and *P. knowlesi* synthesize a 135 kDa protein in the micronemes that binds to the Duffy blood group antigen. The Duffy blood group antigen is a 35-46 kDa glycoprotein identified as a receptor for chemokines, interleukin-8 and melanoma growth stimulatory activity (Holder 1994). The epitopes Fy^a, Fy^b and Fy^6 have been implicated as receptors for both *P. vivax* and *P. knowlesi*. RBCs lacking the Duffy (Fy/Fy) blood group antigen are refractory to invasion by *vivax* merozoites in West Africa (Holder 1994, Hadley and Pelper 1997).

Electron microscopy reveals that invagination begins at the end opposite the apex of the parasite. Molecules on the surface of the merozoites mediate the initial recognition and attachment to the RBC. Both merozoite surface proteins (MSP) one and two are uniformly distributed on the parasite's surface.

MSP-1 is involved in the initial recognition of RBCs as serves as a ligand for sialic acid dependent RBC binding (DeLuca et al. 1996, Holder 1994). *P. falciparum* also express erythrocyte binding antigen (EBA)-175 which interacts with RBC sialic acid receptors (Holder 1994). The merozoite forms a pit on the surface of the RBC membrane (Pasvol and Wilson 1982). Internal parasitic organelles (i.e., rhoptries and micronemes) excrete multiple protease enzymes that allow the parasite to push itself into the membrane which eventually surrounds it. Three enzymes have been associated with merozoite cell entry, a serine protease and two phospholipases, i.e., a glycolipid-specific phospholipase C (PLC) and a glycolipid-degrading lipase (Bannister 2001). An ATP dependent cascade originates with the attachment of the merozoite to the RBC, followed by the translocation of the PLC to the membrane bound protease. Upon activation, the protease is secreted at the parasite-RBC junction resulting in entry of the parasite into the host cell. When the parasite enters the cell, it is surrounded by a surface coat from the plasma and enclosed by two membranes: the outer RBC membrane and an inner membrane known as the "parasitophorous vacuole membrane" (PVM). Within the PVM, the merozoite undergoes rapid asexual multiplication through successive stages: ring, trophozoite and schizont. The erythrocyte is a cell that performs little metabolism apart from glycolysis, therefore the parasite must have the ability to undergo all biosynthetic functions for its survival. The process of maturation and transformation by the parasite ultimately modifies the erythrocyte's surface, ultrastructure and deformability. The ring form, shaped like a signet ring, is the youngest trophozoite stage. This

form grows by increasing in cytoplasm until the vacuole disappears, becoming compact or amoeboid. Merozoites contain many proteases with different functions, including RBC invasion, merozoite escape and hemoglobin degradation. The parasite feeds on the RBC's hemoglobin which is hydrolyzed by secreted enzymes, i.e., heme polymerase and aspartate protease (Bray and Garnham 1982, Bannister 2001). The degraded hemoglobin accumulates in vacuoles which aggregate into large residual bodies of malaria pigment in the RBC cytoplasm. The breakdown of hemoglobin yields a yellow-brown to black malarial pigment known as hemozoin.

After maturation of the trophozoite, the parasite undergoes the process of multiplication called "erythrocytic schizogony/merogony" characterized by multiple nuclear divisions. At this stage the parasite occupies most of the RBC and the pigment tends to concentrate into a single mass. The nucleus divides three to five times generating 8-32 nuclei that become wrapped with cytoplasm to form individual merozoites (Bannister 2001). The parasites create an aperture through both the PVM and the RBC membrane to allow the merozoites to exit the erythrocyte in an orderly fashion (Bannister 2001). The RBC ruptures releasing the merozoites that then infect other RBCs. Erythrocytic schizogony/merogony is repeated until it is contained by the host's immune system, antimalarial drugs or a combination of both.

While most of the parasites within the RBCs develop into merozoites, rupture the cells, and then infect new RBCs, some parasites develop into sexual forms called gametocytes. The exact stimulus for gametocyte formation is

unknown. The gametocytes are round cells with a single nucleus and scattered pigment. When mature, they fill the entire host RBC. The nucleus becomes diploid in the macrogametocyte (female) and octaploid in the microgametocyte (male). The parasitic cycle is completed when a female *Anopheles* mosquito takes a bloodmeal from a person with mature gametocytes in the circulation. Gametocytes mature during the night which coincides with the nocturnal biting habits of *Anopheles* mosquitoes (Gilles 1993). Within the mosquito's gut, the male and female gametocytes fuse (sexual reproduction), forming ookinetes that cross the midgut wall and evolve from oocysts to sporozoites. The sporozoites mature in the salivary gland coated with the circumsporozoite protein. The inoculation of the sporozoites into a new human host perpetuates the malaria life cycle. The malaria infection can only exist where there are infected hosts and competent vectors to perpetuate the cycle.

In *P. vivax*, *P. ovale* and *P. malariae*, all morphological forms can be observed in the peripheral blood, while only ring forms and gametocytes are observed for *P. falciparum*. The latent period between the time the sporozoites enter the bloodstream and time trophozoites appear in the circulating RBC is about six to 12 days for *P. falciparum*, 10 to 12 days for *P. vivax*, 21 days for *P. malariae* and 14 to 15 days for *P. ovale* (Winslow and Connor 1967). There is an important relationship between the susceptibility of red cells and their metabolic age. *P. falciparum* invades all cells, *P. vivax* and *P.* ovale have a predilection for young cells, and *P. malariae* prefers mature cells. Nonetheless, *P. falciparum* invades a greater number of younger cells than older cells. The

younger cells have a greater density of appropriate receptor sites, increased metabolic activity and enhanced deformability (Weatherall and Abdalla 1982).

Clinical Features of Malaria

Malaria is an important cause of morbidity and mortality in holoendemic regions, but not everyone infected with the malaria parasite becomes seriously ill or dies (Blount 1969, Marsh 1993). Those most likely to die of malaria are persons without previous immunity, particularly children and persons from countries where the disease does not exist. The clinical features of malaria can vary depending on the infecting species, the level of parasitemia, and the immune status of the patient. The pre-erythrocyte stage (liver stage) of the infection is not associated with any detectable symptoms and minimal histopathological changes. Instead, the clinical manifestations are predominately associated with erythrocytic invasions by merozoites. The maturation of the schizont and the production of merozoites occurs at times characteristic of each *Plasmodium* species lasting 48 hours for vivax and falciparum malaria, while ovale malaria maintains a 50-hour cycle (Bray and Garnham 1982) and *P. malariae* ruptures at 72 hour intervals causing a quartan periodicity (Taylor and Strickland 2000). This dissertation will focus exclusively on *P. falciparum* and its associated pathology.

A malarial paroxysm is defined as "*the sudden onset of disease symptoms with recurrent manifestations such as chills and rigor*" (Stedman 1995). The rupturing of schizonts results in the outpouring of merozoites, malaria

pigment, and the contents of the ruptured RBC which stimulates the host's immune system. The general clinical presentation of malaria includes periodic paroxysms of shaking chills and high fever, which can be accompanied by headache, dizziness, backache, myalgia, arthralgia, weakness, vomiting, diarrhea, and total body pain (Taylor and Strickland 2000). Generally, the severity of illness is proportional to the number of pRBC and complications do not occur without significant parasitemia (MacPherson et al. 1985, Patnaik et al. 1994, Turner 1997, Silamut et al. 1999, Taylor and Strickland 2000). Acute *P. falciparum* infections produce high parasitemias, severe anemia, cerebral disturbances, renal failure, pulmonary edema, and death (Turner 1997, Taylor and Strickland 2000, Cotran et al. 1999). Malaria continues to be a significant cause of mortality and morbidity throughout the world (WHO 2000, CDC 2000).

Worldwide Prevalence of Malaria

Throughout history, malaria has plagued mankind and continues to be a formidable adversary that has alluded containment even in developed countries and made a mockery of the word "eradication." Perhaps the longevity of malaria is due to the genetic resilience and antigenic variability of the parasite (Biggs et al. 1991). Some 2.4 billion people, comprising 41% of the world's population, inhabit areas endemic for malaria in a total of 101 countries and territories (WHO 2000). Epidemiologic studies suggest that malaria kills one child every 30 seconds, and that 3,000 children less than five years of age die per day. In sub-Saharan Africa alone, there are more than 100 million cases of malaria each

year, with an estimated one million deaths, mostly of infants and young children (Taylor and Strickland 2000, WHO 2000, CDC 2000).

Prevalence of Malaria in the United States

An estimated 600,000 cases of malaria occurred in the continental United States (U.S.) in 1914 (CDC 2001). In 1955, the Department of Health, Education and Welfare initiated a malarial eradication program. The definition of *eradication implies both the complete interruption of transmission and the elimination of the parasites reservoir by a campaign limited in time and carried out so thoroughly that in the end there will be no resumption of transmission* (Russell 1957). A combination of improved socioeconomic conditions, water management, vector-control efforts and case management was implemented to resolved the transmission of malaria in the U.S. Malaria was declared eradicated by 1970 from the U.S., Canada, Europe, most of the Caribbean, South America, Australia, Japan, Korea and Taiwan (Gilles 1993).

However, despite these successes, the global eradication of malaria has not been attainable for a number of reasons, one being the development of mosquitoes resistant to DDT (dichlorodiphenyltrichloroethane, $C_4H_9Cl_5$) and Malathion ($C_{10}H_{19}0_{\ 6}PS_2$), also, *P. falciparum* parasites resistant to chloroquine and pyrimethamine, along with political and administrative difficulties. Malaria continues to be a major problem in endemic areas with little likelihood for any marked improvement in the immediate future. The extensive morbidity is associated with poverty, malnutrition and co-infection with other parasites.

Throughout the world, many countries are reporting an increasing number of cases of imported malaria due to the ease and affordability of international travel. Imported malaria is associated with the travel of a parasitemic person to a non-endemic disease area, or the introduction of an infective vector into a non-endemic area, e.g., "airport malaria" (Bruce-Chwatt 1982, CDC 1999, CDC 2001, Martens and Hall 2000, Lernery 2000). Infected persons may travel while incubating malaria and develop full-blown disease after arrival in normally malaria free communities (e.g., migrant workers from Mexico). Alternatively, mosquitoes inadvertently transported in an airplane from a malaria endemic area, can disembark the plane and bite a new population of people not normally at risk of malaria (Bruce-Chwatt 1982, CDC 1999, CDC 2001, Martens and Hall 2000).

According to the 1997 Malaria Surveillance CDC Report (CDC 2001), 1,544 cases of malaria were imported into the United States (698 from U.S. civilians, 592 cases in foreign civilian, 28 were U.S. military personnel and 226 unknown) representing a 10.9% increase from the 1,392 cases in 1996 (CDC 2001). Most of the imported malaria cases came from West Africa (46.2%), India (33.1%), South America and the Caribbean (16.2%). Of these cases, 48.9% were identified as *P. vivax,* while *P. falciparum* infected 36.7% of the patients. In contrast, in Africa 76.6% of the malaria infections that year were due to *P. falciparum* and 12.4% caused by *P. vivax*. The five areas in the U.S. reporting the largest number of malaria cases were California 374, New York City 303, Florida 120, New York State 84, Virginia and Illinois both with 68. For the

present year up to September 1, 2001, 22 provisional cases of imported malaria have been reported for the state of Michigan (CDC 2001).

There are two major species of the *Anopheles* mosquito in North America associated with the transmission of malaria: *A. quadrimaculatus* and *A. Freeborni* . In Michigan, there have been 124 documented cases of locally acquired malaria transmitted by *Plasmodium* infected mosquitos within the past fifteen years (CDC 1996, MacArthur et al. 2001). One case in Michigan was transmitted by malaria infected mosquitos at a campground in Southeast Michigan. On September 3, 1995, the CDC diagnosed a 31-year old male with vivax malaria. The patient had no history of malaria risk factors and no travel outside of the U.S. CDC light traps were placed at a campground and two malaria vectors *(A. quadrimaculatus* and *A. punctipennis*) were detected at the rural campground. This finding demonstrates that malaria can inadvertently be transported into a malaria-free area, thus reintroducing the disease. The relationship between transmission and disease acquisition is complex. Attempts to eradicate or control malaria will be futile if there is not a clear understanding of these links.

CEREBRAL MALARIA

The mortality rate associated with malaria has been as high as 50%. One million children, worldwide, die each year from the manifestations of malaria (WHO 2000). One of the most severe complication of malaria is the development of cerebral malaria (CM), and about 1% of *P. falciparum* infected

patients develop CM. CM is a diffuse encephalopathy caused by complications of *P. falciparum* malaria. It is characterized by with the sequestration of parasitized red blood cells (pRBC) which bind to endothelial cells within the brain. The full pathogenesis of CM is still poorly understood. The occlusion of cerebral microcirculation by the adhesion of pRBC to vascular endothelial cells is associated with cerebral hypoxia, metabolic disturbances, organ dysfunction and diminution of brain function (MacPherson et al. 1985, Boonpucknavig et al. 1990, White 1992, Patnaik et al. 1994, Turner 1997, Silamut et al. 1999, Taylor and Strickland 2000). The characteristic clinical presentation of CM includes: (1) unrousable coma persisting for at least 30 minutes after a generalized convulsion, (2) the demonstration of *P. falciparum* in the patient's blood smear, (3) the exclusion of other causes of encephalopathy, and (4) for fatal cases, histopathologic confirmation in cerebral tissue (MacPherson et al.1985, Patnik et al.1994, Warrell 1993).

CM can occurs most commonly at two to three years of age but can occur in children less than six months. Early symptoms include, fever and chills, accompanied by convulsions, irritability or apathy, cough, vomiting, and rarely diarrhea (Taylor and Strickland 2000). One or two days after the first symptoms, children become unrousable and lapse into a deep coma. CM is diagnosed upon the detection of *P. falciparum* parasitemia, no other obvious causes of altered consciousness, and a coma score of less than two. The level of consciousness can be assessed by evaluating and rating eye movements along with verbal and motor responses. The coma scale used in Blantyre, Malawi to

assess the levels of consciousness in children with malaria is shown in Table 1 (Taylor and Strickland 2000).

Table 1. Blantyre Coma Scale for Assessing Levels of Consciousness in Children with Malaria

		SCORE
Eye movements:	Direct	1
	Not direct	0
Verbal response	Appropriate cry or moan	2
	Inappropriate cry	1
	None	0
Motor response	Localizes painful stimuli	2
	Withdraws limb from pain	1
	Non-specific or absent response	0
TOTAL	Normal Coma	0-5
	Unrousable coma	≤ 2

The level of consciousness was assessed by rating the level of eye movement, verbal response and motor response. Children with cerebral malaria were more likely to have a coma score less than (≤) or equal to two.
Adapted from Taylor and Strickland 2000

The mortality rate for CM patients is typically between 15 to 25% with most deaths occurring within the first 24 hours of hospitalization (Taylor and Strickland 2000). In survivors, the duration of the coma is usually shorter and averages about 30 hours before the patient regains full consciousness. Most of these latter patients recover completely, but 10% develop neurologic sequelae, including hemiplegia, cortical blindness, epilepsy, inability to speak, cerebellar ataxia, extensor posturing, generalized spasticity or hypotonia, psychosis,

tremors, mental retardation, and behavioral disturbances (Warrell 1993).

There are two commonly held theories for the pathogenesis of CM: the sequestration theory and the endogenous mediator i.e. cytokine theory. According to the presence of infected RBC is an essential initial event. In the sequestration theory, the pRBC do not circulate but are sequestered in various organs where they adhere to vascular endothelial cells. Mechanical blockage of cerebral blood vessels by pRBC leads to cerebral anoxia, diminished brain function and coma (White 1982, MacPherson et al. 1985, Gopinathan et al 1986, Boonpucknavig et al. 1990, Marsh 1992, Turner et al. 1994). This theory purports an association between the presence of large numbers of pRBCs blocking vessels, the formation of rosettes (in which pRBC bind to non-parasitized RBCs), and the development of the syndrome (Bernedt et al.1994).

Ultimately, the sequestration theory attributes the manifestation of CM to mechanical blockage of cerebral capillaries which leads to anoxia and eventually coma. An unexplained phenomenon is the high percentage of CM patients who survive, in spite of a prolonged unrousable coma. One study reported 131 children with CM, in an unrousable coma for an average of 31 hours, who regained full consciousness without neurological sequelae (Molyneaux 1991). Many doubt that hypoxia, due to simple mechanical blockage, is the sole cause of malarial comas (Kwiatkowksi et al. 1990, Shaffer et al. 1991, Porta et al. 1993, Grau and de Kossodo 1994).

The endogenous mediator theory presupposes that CM is caused by the production of toxic molecules. Last century's toxin theory proposed that the

parasite itself produced toxic molecules that led to the development of CM. The current, cytokine theory suggests that the host, instead of the parasite, is the source of toxic molecules (Clark and Rockett 1994). The cytokine theory hypotheses that cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1 are toxic when overproduced, and could cause syndromes such as those seen in CM. The cytokine theory is consistent with the presence of sequestered parasites to cerebral tissue, but that sequestration alone is not essential for the development of the coma. Merozoite schizony can trigger the release of cytokines such as TNF-α and IL-1 and blocked parasitized vessels may concentrate the overproduction of cytokines to the local tissue. Cytokines can also induce the expression nitric oxide (NO) which can also alter brain function (Clark and Rockett 1994). Therefore, the cytokine theory proposes that sequestered parasites trigger an immune response stimulated by schizony peptides which stimulates a high local production of pro-inflammatory cytokines and NO (Clark and Rockett 1994).

The absence of sequestered pRBC in the brain has also been reported in fatal CM (Boonpucknavig 1990, Toro and Roman 1978). The debate about CM without sequestration is currently a hot topic. Many debaters attribute the discrepancy to poor sampling, using antiquated standards and misdiagnosis.

The examination of tissue from CM patients shows accumulations of pRBC in many tissue particularly brain, lungs, heart and gut. There is a significant association between the amount of parasitic sequestration in the brain and the development of CM. The typical histologic presentation of the brain

includes blood vessels plugged with pRBC containing the malarial pigment hemozoin, ring hemorrhages surrounding capillaries and venules, and cerebral edema. Endothelial cells are typically swollen, hypertrophied and prominent due to increased vascular permeability. Occasionally malarial granulomatous lesions (Durck granulomas) are observed. Hemorrhages and granulomas appear mainly in the white matter of both the cerebrum and cerebellum (MacPherson et al. 1985, Boonpucknavig et al. 1990, Patnaik et al.1994, Newbold et al. 1999).

Cytoadherence

The virulence of *P. falciparum* is associated with its ability to modify the surface of the infected RBC mediating its adherence to host tissue, a process called cytoadherence. During erythrocytic development, the merozoites produce parasite-encoded proteins which insert into the RBC membrane mediating cytoadherence. The binding of pRBC to uninfected red cells to form erythrocyte rosettes, and to vascular endothelium are important factors in the pathogenesis of the disease (Udomsangpetch et al. 1996, Turner et al. 1994, Rowe et al. 2000). The two processes, rosette formation and cytoadherence, function independently of each other. Rosette formation may contribute to microcirculatory obstruction of cerebral capillaries and was highest in fatal CM patients (Udomsangpetch et al. 1996).

Two types of parasite encoded, adhesion related, surface proteins have been detected in malaria infected RBC: sequestrin and *P. falciparum* erythrocyte membrane protein(PfEMP)-1(Baruch et al. 1997, 1999). Merozoites secrete

sequestrin which forms 100 nanometer bumps on the surface of RBC called *knobs,* allowing them to adhere to endothelial cells. Knob formation is associated with the disposition of a number of parasite derived proteins beneath the RBC membrane including, the knob-associated histidine-rich protein (HRP1) (Berendt et al. 1994). PfEMP-1 serves as the receptor for both CD36 and thrombospondin (Bauch et al. 1997). Both sequestrin and PfEMP-1 are conserved, antigenically invariant, parasite proteins encoded by the *var* genes (so called because they exhibit antigenic variation).

The adherence of pRBC to cerebral tissue is mediated by several endothelial cell receptors. The two main endothelial receptors are CD36 and intercellular adhesion molecule (ICAM)-1, however, adherence is also mediated by thrombospondin (TSP), vascular cell adhesion molecule (VCAM)-1 endothelial leukocyte adhesion molecule (ELAM)-1, E-selection, and to a lesser extent chondroitin sulfate (van Schravendijk et al. 1992, Turner et al. 1994, McCormick et al.1997, Bauch et al. 1997, Udomsangpetch et al. 1996). Endothelial cell activation is a feature of fatal malaria, and the distribution of CD36, ICAM-1 and E-selectin is highly correlated with sequestration in the brain (Turner et al. 1994). Analyzing receptor specificity of CD36, ICAM-1, VCAM-1 and E-selectin to pRBC demonstrated that CD36 and ICAM-1(10- fold lower expression) play major roles in cytoadherence. However, the binding of pRBC to purified TSP demonstrated it non essential for cytoadherence (Berendt et al. 1994, Turner et al. 1994).

CD36 is an 88-kDa glycoprotein scavenger receptor expressed on the

surface of various cells including platelets, adipocytes, monocytes, macrophages, erythroblasts, melanoma cells and endothelial cells (van Schravendijk et al. 1992, Baruch et al. 1999). Using recombinant molecules and *P. falciparum* isolates, the ability of various cell adhesion molecules to bind pRBC were investigated. There was a significant correlation between pRBC sequestration and the expression of CD36 in various organs and vascular endothelium (Baruch et al. 1997). CD36 supported stable stationary binding to pRBC using experimental blood flow conditions, while ICAM-1 and TSP appeared unstable (Baruch et al. 1999). CD36 and ICAM-1 function synergistically to mediate the adherence of pRBC to endothelial cells. The synergy between CD36 and ICAM-1 increased with time, was necessary for parasite survival, and associated with disease severity (McCormick et al. 1997).

The pathogenicity of *P. falciparum* is due to the occlusion of cerebral blood vessels by aggregates of pRBC attached to endothelial cells. This event involves the participation of receptors on host endothelial cells and the expression of parasite derived proteins that insert into the membranes of infected erythrocytes (Baruch et al. 1999).

Leukocytes in Cerebral Malaria

Initial histopathology reports of CM described cerebral vessels blocked by *P. falciparum* infected erythrocytes, but leukocytes were not detected in the cerebral tissue (Aikawa et al. 19980, Pongponratn et al. 1985, Aikawa 1988, MacPherson et al. 1985, Grau et al. 1987, Oo et al. 1992). In murine CM, the

typical histology included both pRBC and parasite laden macrophages. Thus, a significant difference between human CM and murine CM was the presence of leukocytes in cerebral tissue (Grau et al. 1987). Mononuclear phagocytes may play a role in the development of human CM. Mononuclear cells were detected but not characterized, in the cerebral tissue of six autopsy cases in Thailand (Boonpucknavig et al. 1990). Furthermore, CD68+ monocyte/macrophages were described in cerebral tissue in an HIV infected patient who died of CM (Porta et al.1993). Recently, mononuclear phagocytes were also seen to be a significant component of the cerebral tissue of 21 Malawian children with CM, but not that of NCM, or of those dying from coma of other causes (Mackenzie et al. 1999). These observations suggested that mononuclear phagocytes may play an important role in the pathogenesis of CM.

MACROPHAGES IN CEREBRAL MALARIA

Cerebral malaria (CM) is associated with the inability of the host's immune system to control parasitic proliferation, neutralize excessive secretions of proinflammatory cytokines such as TNF-alpha (TNF-α) and block the sequestration of parasitized red blood cells (pRBCs) in vital organs. Activated macrophages are cardinal elements in the host's defenses against facultative and obligate intracellular pathogens (Adams and Hamilton 1992). The impetus for the hypothesis of this dissertation was the need to detect and characterize mononuclear phagocytes in cerebral tissue. Since the expression of malaria pathology is fundamentally dependent upon the status of the patient's immune

system, a review of mononuclear phagocytes in malaria is warranted.

When parasites enter the bloodstream, the host launches a complex defense against them mediated by elements of the nonspecific immune system. Innate immunity is the nonspecific response that serves as the first-line of defense to microbial challenges. The hallmark of the innate system is the lack of specific antigen identification. Cells of the innate immune system include neutrophils, monocytes, macrophages, dendritic cells, natural killer (NK) cells and gamma-delta (γδ) T cells. These cells play central roles in the clearance of microorganisms and require no prior adaptive immunity (Abbas et al. 1997, Kuby 1997). A simplistic description of the professional phagocyte is that they are like commissioned officers with the ability to send out inflammatory signals, generate cytokines and deal with the more challenging intracellular microorganisms. While, neutrophils are like the "grunts" that throw themselves on the battlefield during any invasion. The phagocytosis of merozoites by neutrophils has been studied by phase microscopy in a malaria infected patient. The merozoites were observed to be highly motile parasites inside the patient's erythrocytes. The parasites produced no changes in the RBC surface. Neither the monocytes nor the polymorphonuclear (PMNs) leukocytes responded although they repeatedly bypassed the infected cells. When an erythrocyte ruptured releasing merozoites, a PMN that had passed the RBC moments before the rupture, rapidly reversed its direction and purposefully ingested the organisms (Trubowitz and Masek 1968).

Leukocyte precursors develop in the bone marrow from pluripotential,

Cluster Differentiation-34 positive ($CD34^+$) stem cells through a process of differentiation, replication and maturation. The monoblast is the earliest recognizable monocyte, and is morphologically indistinguishable from the myeloblast. The development of macrophages from peripheral blood monocytes was demonstrated using rabbit ear chambers. Monocytes transmigrated randomly from the peripheral circulation through capillaries to the tissue and subsequently transformation into macrophages (Kass and Schnitzer 1973). Tritiated thymidine labeled cells showed that after leaving the bone marrow, the monocytes circulated in the peripheral blood with a transit time of eight hours to three days before homing to specific tissue sites for differentiation (Carr 1975, Jandl 1996). These studies led to the conclusion that monocytes are derived from precursor cells in the bone marrow that differentiate into macrophages in a tissue specific manner (van Furth 1975, Carr 1975, Stossel and Babior 1995). Monocyte transformation into tissue macrophages is accompanied by morphologic, biochemical and functional changes such as increased lysosomal enzyme content, modulation of surface antigens, increased ability to undergo adhesion and phagocytosis, and enhanced ability to kill tumor cells and microorganisms (Radzun et al. 1988, Jandl 1996). Monocytes/macrophages possess distinct cell surface receptors such as, constitutively expressed crystallizeable fragment (Fc) of immunoglobulin (Ig) receptors for IgG1, IgG3, and to a lesser extent IgM and IgE. They also express receptors for activated complement components C3a, C4b, and Bb, from the alternative complement pathway (Jandl 1996).

Mononuclear phagocytes, neutrophils, CD4+ T cells and NK cells are an

essential first line of defense against malaria. Parasite induced membrane damage can be recognized by mediators of the innate immune system. Non activated macrophages can phagocytize and ingest pRBCs and have the potential to dispose of 40-80% of the total red cell mass in a few days (Arese et al. 1991). NK cells mediate the lysis of pRBC by the release of soluble NK cell lytic factors (Biron et al 1999, Choudhury et al. 2000). Malaria parasites exist in several forms amenable to phagocytosis including: circulating sporozoites, merozoites released after RBC destruction, and as obligate intracellular parasites in pRBC (the hepatocytic stage being essentially non inflammatory). The ingestion of pRBC is a receptor-mediated engulfment process that begins with multiple small areas of attachment resulting in a gradual envelopment of the cell (Jandl 1996). After infection circulating monocytes and macrophages undergo marked changes in their surface phenotype and secretory activity, a process known as activation.

Association of Monocyte/Macrophage Function with the Age of the Child

The neonatal period is a state of relative immunodeficiency during which newborns are particularly vulnerable to bacterial, protozoa, and viral infections (Frenkel and Bryson 1987, Mills 1983). A neonate is defined as a newborn less than or equal to one month of age (Stedman 1995, Cotran et al. 1999, Beutler et al. 2001). Properties of monocytes/macrophages relate to their phagocytic ability, chemotactic movement, and microbial activity. While phagocytosis seems normal at birth, monocytes and macrophages from neonates appear to present

antigens poorly, have decreased chemotaxis, and impaired ability to produce specific antibodies (Schuit and Powell 1980, Mills 1983). Monocytes must move to sites of infections, adhere to endothelial cells and transmigrate from circulation to tissue. Firm adhesion to endothelial cells is mediated by integrins, CD11 and CD18. During ontogeny, macrophages in the fetal liver express CD11b by 12 weeks gestation, however, CD11a, b, and c are expressed at lower densities on cord blood monocytes than on cells from adults which may affect their ability to adhere firmly (Mills 1983, Beutler et al. 2001)

Monocytes and macrophage of newborns and adults synthesize inflammatory mediators such as the components of complement, IL-1β, IL-6, IFN-α and TNF-α in similar concentrations, but produce lower levels of IFN-γ, IL-8, IL-10 and G-CSF (Sautois et al. 1997). Also, lower proportions of HLA-DR, DP, DQ are expressed on neonatal monocytes than on adult monocytes. However, there has been no discernable difference in peroxidase activity detected between monocytes of neonates and adults (Christensen 2000).

By two months of age, infants can limit infection with certain pathogen (Frenkel and Bryson 1987). The functional ability of monocytes and macrophages were grouped into four age categories: cord blood and neonates less than one week of age, infants age two to 12 months, and one year to 10 years. Cord blood and neonatal cells younger than one week had decreased function compared with adults, however, function increased to an intermediate activity level by two months of age (Frenkel and Bryson 1987). At eight months, a child's mononuclear phagocytes can demonstrate adult level activity (Sautois

et al. 1997, Christensen 2000).

Activation of Macrophages

Macrophage activation is a cytokine mediated process that converts the cell from resting into a state of activation. Macrophage activation can be amplified or subdued, depending on the effects of cytokines: tumor necrosis factor-alpha (TNF-α), interferon-alpha (IFN-α), interferon-beta (IFN-β), interferon-gamma (IFN-γ), interleukin-1(IL-1), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), transforming growth factor-β (TGF-β), macrophage-colony stimulating factor (M-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF) all can act alone or in combination on macrophages (Adams and Hamilton 1992, Auger and Ross 1992, Strossel and Babior 1995, Schwarzer 1998). Activated macrophages can be very destructive to host tissues. Signals that suppress macrophage activation are equally important. A partial list of suppressive agents includes: alpha-2 macroglobulin, IL-4, TNF-α, IFN-α, IFN-β, prostaglandin-E2, TGF-β, high oxygen content, high glucose and immune complexes (Jandl 1996).

When activated the macrophages acquire an enhanced capacity to perform complex functions attributable to their defense against obligate intracellular malarial parasites, such as (1) phagocytosis of pRBC (Trubowitz and Masek 1968), (2) innate defense against the various stages of the *Plasmodium* parasite (Bouharoun-Tayoun et al. 1995, Healer et al. 1999), (3) antigen processing and presentation to lymphocytes (Janeway and Travers 1997, Kuby 1997), and (4) secretion of cytokines (Adams and Hamilton 1992, Auger and

Ross 1992, Strossel and Babior 1995, Schwarzer 1998).

The erythrocytic stage in the life cycle of the malaria parasite is the principal stage for the pathologic effects of the disease. The number of phagocytes are usually too few to have any significant effect on the clearance of the thousands of emerging merozoites. Thus, most of the merozoites re-invade other RBC before they come in contact with phagocytes. The phagocytosis of pRBCs depends on the developmental stage of the parasite and parasitic metabolites that alters the integrity of the host cell. The efficiency of phagocytosizing pRBC is often dependent upon whether the cells are opsonized, altered or impaired. The pRBC may be modified/impaired due to the membrane insertion of parasite encoded proteins, such as, PfEMP-1 and sequestrin. Opsonization, the coating of RBCs with antibodies or complement, mediates the clearance of parasites by macrophages that possess Fc and complement receptors on their cell surface. The addition of malarial antibodies to culture medium resulted in increased phagocytosis and a reduction in the number of malaria infected RBCs by the monocytes (Bouharoun-Tayoun et al. 1995). Antigenically, surface proteins (MSP-1) of the merozoite are critical triggers for antibody production, albeit the merozoites are only in the circulation for brief periods of time before infecting other RBCs.

The phagocytosis and destruction of pRBCs/merozoites by macrophages is followed by antigen processing and presentation. Upon ingestion, macrophages degrade the parasites into immunogenic peptides that bind to major histocompatibility complex (MHC) Class II molecules. Macrophages, dendritic cells and B lymphocytes express MHC-II molecules and process

antigens that are presented to CD4+ T lymphocytes (Janeway and Travers 1997, Kuby 1997, Adams and Hamilton 1992). T cells only recognize an antigen associated with an MHC molecule (Kuby 1997). IFN-γ, produced by NK cells, γδ T cells and activated Th1 lymphocytes, is important for the activation of macrophages, as well as the subsequent activation of parasite specific T helper (Th) cells. It is important for the delivery of IFN-γ to be focused on the macrophage, also, that its production be shut off immediately when the T cell loses contact with the macrophage. Macrophages, upon activation, secrete increased amounts of tumor necrosis factor alpha (TNF-α). The control of parasitemia early in infection is associated with the expression of high levels of both TNF-α and IFN-γ. The antithesis of this observation is the fact that mice treated with anti-thy1.1 (monoclonal antibody against NK cells) exhibit high parasitemia, produce low levels of both TNF-α and IFN-γ, and die early in infection (Choudhury et al. 2000).

TNF-α has a pleiotropic anti-malarial effect. Exogenous TNF-α was evaluated for its ability to stimulate phagocytosis of pRBC by monocytes and the ability of monocytes and lymphocytes to inhibit parasitic growth. The addition of TNF-α to cultures containing antibodies against *P. falciparum*, human monocytes and falciparum infected RBCs resulted in a twofold increase in the phagocytic index. The incubation of TNF-α alone with the malaria parasites showed no inhibition of growth, but a combination of low dose TNF-α, lymphocytes and monocytes decreased parasitic growth three times more than with monocytes alone (Muniz-Junqueira et al. 2001). The protective effect of TNF-α depends on

the interplay of factors such as monocytes, lymphocytes and antibodies.

Macrophage Dysfunction

Macrophage dysfunction has been speculated as a cause of the immunologic incompetence of malaria. Malarial protozoa have evolved to either evade or resist the innate immune surveillance of mononuclear phagocytes. While the ingestion of a few parasitized RBC (up to 3 schizonts) stimulates phagocytosis, larger amounts or longer exposure periods may paralyze the entire phagocytic system. The inability to macrophages to actively phagocytosize malaria parasites prevents the specific and non-specific immune response of macrophages that serve as phagocytes and professional antigen presenting cells.

Defects of macrophages/monocytes phagocytic activity have been associated with exposure to malaria blood stage antigens. During the erythrocytic stage, the parasite feeds on and degrades the host's hemoglobin. The parasites utilize the globin but are unable to catabolize the heme, which aggregates to form hemozoin (Schwarzer et al. 1998, Gilles 1993). This pigment can be detected in either phagocytic cells following the ingestion of whole parasites, or in hemozoin laden pRBC. Hemozoin is thought to be a key factor in the induction of malaria-associated immunosuppression. Viable monocytes that have been fed hemozoin, may be functionally impaired. Macrophages were unable to digest hemozoin, perform repetitive phagocytosis, generate an oxidative burst upon appropriate stimulation, or kill ingested parasites. Most

importantly, hemozoin laden cells were found to release large amounts of TNF-α, nitric oxide, and decreased amounts of IL-6 (Taramelli et al. 2000).

Hemozoin impairs the ability of phagocytic cells to present antigen. Antigen presentation and phagocytosis were analyzed by flow cytometry using antibodies to MHC class II, ICAM-1, integrin (CD11c), CD32 (a low affinity Fc receptor for IgG), CD64 (a high affinity receptor for IgG), CD11b, CD35, CD36, iC3b, C3b, and C4b. Significant defects were observed in the antigen presentation molecules MHC-II, ICAM-1, and CD11c of hemozoin laden macrophages (Scorza et al.1999).

The ability of macrophages to undergo phagocytosis and ingestion of *P. falciparum* infected cells was found to be compromised due to glycosylphosphatidylinositol (GPI), a toxic phospholipid produced by *P. falciparum.* GPI has been implicated in the pathogenesis of malaria by inducing the secretion of proinflammatory cytokines TNF-α and IL-1 by macrophages (Vijaykumar et al. 2001, Naik et al. 2000, Schofield et al. 1993). However, anti-GPI was shown to provide protection against clinical malaria. Adults resistant to clinical malaria were shown to contain high levels of persistent anti-GPI antibodies, whereas susceptible children lack or had low antibody levels. The absence of a persistent anti-GPI antibody response correlated with malaria-specific anemia and fever (Naik et al. 2000).

Some of the clinicopathologic consequences of *Plasmodium* invasion are not due to the presence of the parasite themselves, but rather the excessive production of cytokines released from macrophages at the time of schizont

rupture (Li and Langhorne 2000). Mononuclear phagocytes have been described within the cerebral blood vessels of Malawian children with CM. Since macrophages secrete a wide range of cytokines implicated in the pathogenesis of CM, particularly, TNF-α, TGF-β, IL-6 and IL-10, it is important to review the characterization, functions, and interplay between pro- and anti-inflammatory cytokines involved in CM.

CYTOKINES

Cytokines are low molecular weight proteins that play a crucial role in cellular communication and regulation of the immune system (Janeway and Travers 1997, Kuby 1997, Abbas et al. 1997). They can turn off, turn on, or modulate immune responses and are produced by a variety of cell types. Cytokines are secreted as hormone-like peptides with the ability to affect the behavior of other cells. The cytokines can act on themselves in an autocrine fashion, affect cells in the vicinity in a paracrine manner, or act systemically as an endocrine. Signals delivered by one cytokine can influence the production of a second, e.g., interferon-gamma upregulates the expression of tumor necrosis-alpha. Cytokines mediate their effects by binding to specific high-affinity receptors on target cells. The variable expression of cytokines underlines the "thin line" between protection and pathology. Pro-inflammatory cytokines such as TNF-α, can produce fever, inflammation, tissue destruction, shock and even death (Abbas et al. 1997). Anti-inflammatory cytokines, i.e., interleukin-10, function to down regulate the activity of pro-inflammatory cytokines. Genetic

polymorphism is defined as the existence of multiple alleles at a specific genetic locus. Allelic polymorphisms in the genes of cytokines have been correlated with different levels of *in vitro* gene transcription, and protein production (Hutchinson et al. 1999, Turner et al. 1997, Wilson et al.1997, Pravica et al. 1998, Awad et al. 1997).

Cytokine overproduction has been associated with disease severity in severe malaria and fatal outcomes (Grau et al. 1989, Kwiatkowski et al. 1990, Kurtzhals et al. 1999). An objective of this dissertation is the evaluation of the genetic propensity for an inappropriate expression of pro-inflammatory cytokines: tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-six (IL-6) and anti-inflammatory cytokines: transforming growth factor-beta (TGF-β), and interleukin-ten (IL-10) in CM. An overview of the functions, activities and genetic polymorphisms of these cytokines follows to provide an understanding of their role and significance in CM.

TUMOR NECROSIS FACTOR-ALPHA

Tumor necrosis factor-alpha (TNF-α) is a potent pro-inflammatory cytokine and immunomodulator implicated in the pathogenesis of CM. Originally known as cachectin, TNF-α plays crucial roles in inflammation, septic shock syndrome, cachexia, tumor necrosis, immune modulation, anorexia, viral replication, hematopoiesis and the manifestations of severe malaria (Abbas et al. 1997, Tracey and Cerami 1994, Beyaert and Fiers 1998). TNF-α was discovered after observing that the serum from mice infected with *Bacillus Calmette-Guerin*

(BCG) or lipopolysaccharide (LPS) induced tumor necrosis in tumor bearing mice. The tumors developed extensive hemorrhaging, while the normal cells remained unaffected. It was hypothesized that the sera contained a substance that caused the death, (i.e., necrosis) of the tumor cells but did not affect normal tissue (Carswell et al. 1975, Beyaert and Fiers 1998). As a result, this substance was named "*tumor necrosis factor.*"

The gene for TNF-α is located on the short arm of chromosome six in band 6p21.3 within the class III region of the major histocompatibility complex (MHC). TNF-α is encoded by a single gene of 2762 bases (Aggarwal 1992, Nedwin et al. 1985, Wang et al. 1985). The mature TNF-α protein is secreted as a 157 amino acid, 26 kDa non-glycosylated protein preceded by a 76 amino acid signal protein. The pre-sequence serves as either a signal peptide or a hydrophobic anchor that attaches the peptide to the cell membrane (Fiers 1992, Tracey and Cerami 1994). When a bolus of TNF-α was tracked over time in cycloheximide-treated cells, it confirmed that uncleaved TNF-α was transported to the cell surface and subsequently cleaved by TNF-α converting enzyme, TACE, releasing a 17 kDa monomer (Fiers 1994). The membrane-bound species of TNF-α is biologically active and responsible for the cell mediated cytotoxicity in monocytes (Aggarwal 1992).

TNF-α is a highly pleiotropic protein produced predominantly by activated macrophages, although monocytes, lymphocytes, natural killer (NK) cells, eosinophils, endothelial cells, mast cells, glial cells, astrocytes, Kupffer cells, granulosa cells and smooth muscle cells can also secrete this protein (Aggarwal

1992, Tracey and Cerami 1994). Biologically, TNF-α functions in a dose dependent mechanism. The half-life of TNF-α is short, approximately 30 minutes (Grau et al. 1993). When produced in a small quantity, TNF-α acts locally as an autocrine or paracrine regulator of local cells/tissue. When expressed in high concentrations, it enters the blood stream where it functions in an endocrine mechanism (Abbas et al. 1997, Tracey and Cerami 1994). The hallmark of TNF-α is its cytotoxicity to tumor cells. Depending on the type of target cell, TNF-α can induce necrotic or apoptotic cell death. Necrosis is characterized by cell swelling, destruction of cell organelles, and cellular lysis. While during apoptosis, the cell shrinks, forms apoptotic bodies, and develops internucleosomal DNA fragmentation (Cotran et al. 1999).

TNF-α plays an important role in the activation of the host's defenses against infections, inflammation or injury. It activates leukocytes, enhances adherence of neutrophils and monocytes to endothelium, activates phagocytosis, enhances specific antibody-dependent cellular cytotoxicity, triggers the production of pro-inflammatory cytokines such as IL-1, IL-6, and IL-8, and induces the expression of MHC class I and class II antigens (Tracey and Cerami 1994, Kuby 1997, Abbas et al. 1997, Janeway and Travers 1997, Cotran et al. 1999). TNF-α induces the up regulation of cell adhesion molecules resulting in the binding of granulocytes, lymphocytes and monocytes to endothelial cells (Beyaert and Fiers 1998). The mechanism of the TNF-α induced adhesiveness is based on a conformational rearrangement of integrins CD11a/CD18 and CD11b/CD18 on the cell surface.

TNF-α produces a variety of biologic effects, both protective and pathological. The role of TNF-α in fever and malarial disease severity has been a topic of much investigation (Kwiatkowski et al. 1990, Grau and Piguet 1993). TNF-α induces fever by crossing into the area of the hypothalamic centers that regulates body temperature and appetite (Tracey and Cerami 1994). High TNF-α production has been associated with the development of fever and the suppression of parasitic growth in patients with severe malaria (Kwiatkowski et al. 1990, Grau et al. 1993, Beyaert and Fiers 1998). The expression of TNF-α was evaluated during paroxysms of non-immune patients infected with *P. vivax* in Sri Lanka. Elevated TNF-α levels preceded the paroxysms by 30 to 60 minutes. The changes in body temperature closely paralleled the increase in TNF-α levels. The elevated levels in vivax malaria were not associated with any other pathology, such as cerebral complications or death. Surprisingly, the TNF-α levels in vivax malaria were much higher than levels observed in falciparum malaria (Karunaweera et al. 1992).

The expression of TNF-α also functions as a protective mechanism to inhibit parasite proliferation. The role of TNF-α was studied for its efficacy in both direct and indirect parasite killing effects. Using BCG-LPS serum from mice infected with *Mycobacterium bovis*, varying concentrations were added to cultures of *falciparum*-infected RBCs. When the cultures were examined, morphological deteriorations of the parasites were observed within the pRBCs. The TNF-α in the serum mediated the non-antibody dependent killing of *P. falciparum*-infected cells (Haidaris et al.1983). Nonetheless, the direct

antimalarial effect of TNF-α has been refuted. The addition of increasing concentrations recombinant TNF-α to *P. falciparum* cell cultures produced no direct effects on the parasite. Also, various concentrations of proinflammatory cytokines TNF-α, TNF-β , or IFN-γ failed to suppress *P. falciparum* when added to cell cultures of the parasites (Kumaratilake et al. 1990). Instead, TNF-α indirectly augments the killing of *P. falciparum* indirectly by activating neutrophils, monocytes and macrophages. TNF-α enhances phagocytes by increasing the expression of Fc, complement and adhesion receptors for parasitic killing (Kumaratilake et al. 1990). It also increases the release of myeloperoxidase containing granules and reactive oxygen intermediates from neutrophils and monocytes that contribute to parasite killing. Thereby, TNF-α can enhance the efficiency of both cellular and humoral immune responses against parasitic infections. Unless stimulated, neutrophils and macrophages may be indifferent to morphologically normal appearing pRBCs (Trubowitz and Masek1968). The simultaneous addition of neutrophils and low dose TNF-α together, however, augmented antimalarial activity (Kumaratilake et al. 1990). Macrophages can kill blood stage parasites independent of opsonization but function less efficiently, while neutrophils require the presence of antibodies. Additionally, pre-treatment of neutrophils with a combination of GM-CSF and TNF-α results in a synergistic increase in phagocytosis and killing of the malarial parasites (Kumaratilake et al. 1990, Kumaratilake et al. 1997). TNF-α plays a pivotal role in malaria and can mediate both protection and pathology.

TNF-α and Malaria

The overproduction of TNF-α has been implicated as a mediator of disease pathology. When serum levels of endogenous TNF-α were evaluated in controls and patients with neoplastic and infectious diseases, highly elevated TNF-α levels were only detected in patients with malaria and kala azar (Shaffer et al. 1991). This discovery that TNF-α levels were only elevated in patients with parasitic diseases suggested that this cytokine might be important for host defenses against parasitic infections (Medana et al.1997, Day et al. 1999).

TNF-α production is a normal host response to falciparum malaria, but excessive levels predispose the patient to CM and fatal outcome. TNF-α serum concentrations were measured in Malawian children with severe falciparum malaria. Mortality was positively correlated with elevated TNF-α serum levels, hypoglycemia, hyperparasitema and age less than three years. Hypoglycemia was related to the effects of TNF-α on hepatic gluconeogenesis (Grau et al.1989). The hyperparasitemia was associated with bursts of schizonts ruptures that liberated thousands of infectious merozoites. However, TNF-α was not elevated in cerebrospinal fluid, even in patients with very high serum concentrations. There was a positive correlation between elevated TNF-α levels and parasite density (Shafer et al. 1991). It was conclusively determined that an association exists between disease severity and the TNF-α serum level at the time of hospital admission. When plasma TNF-α levels were measured in Gambian children with uncomplicated falciparum malaria, the levels were twice as high in CM survivors and ten times higher in fatal cases, compared with

controls (Kwiatkowski et al. 1990). TNF-α induces dyserythropoiesis and contributes to the pathogenesis of malarial anemia (Clark and Chaudhri 1988, McGuire et al. 1999).

The murine model of CM is not believed to be identical to the pathology observed in human CM. Yet, experimental CM research has provided compelling evidence for the role of TNF-α in the pathogenesis of the disease. It was hypothesized that TNF-α plays a central role in CM because (1) elevated levels were found in mice with CM, (2) anti-TNF-α antibody injected in malaria infected mice prevented CM, (3) injection of TNF-α precipitated brain damage in otherwise resistant mice [i.e., mice infected with a species of *Plasmodium* that does not produce neurological damage or cause CM], and (4) transgenic mice expressing high levels of soluble TNF-α receptors did not develop the neurologic syndrome of CM (Grau et al.1989).

The expression of TNF-α occurs early in the course of the disease, before any clinical symptoms. *Plasmodium* infected mice were analyzed for the presence of TNF-α messenger ribonucleic acid (mRNA). As early as three days post infection, TNF-α mRNA was detected several days before the onset of cerebral symptoms. The TNF-α protein was not detected, however, until five days after infection (Grau et al. 1989). The early upregulation of TNF-α suggested that the initiation of the host response within the central nervous system occurs early in the disease process, before the appearance of TNF-α protein.

Elevated plasma TNF-α levels need not fully account for the cerebral

complications observed in CM. Local production of TNF-α in the brain could be involved. It was hypothesized that cytokines might be produced locally within the central nervous system (CNS) during fatal murine cerebral malaria (FMCM) (Medana et al. 1997). Brain sections were analyzed for the mRNA of cytokines: TNF-α, IL-1β, IL-4 and IL-6 by *in situ* hybridization and monoclonal antibodies were used to detection TNF-α, IL-1β, and glial fibrillary acidic protein by immunohistochemistry. TNF-α protein and mRNA were detected in microglia, astrocytes, monocytes and on the cerebral vascular endothelium in FMCM, but not uninfected mice. Both normal and CM animals presented IL-1β mRNA, and neither demonstrated IL-4 and IL-6. Postmortem tissue was used to detect the induction of proinflammatory cytokines in the brains of six Malawian patients. The mRNA for TNF-α, TGF-β, and IL-1β were detected in cerebral tissue of CM patients, but not in controls by reverse transcriptase polymerase chain reaction (RT-PCR) (Brown et al. 1999).

The complications of falciparum malaria result from the adhesion of pRBCs to endothelial cells in cerebral tissue (Clark and Rockett 1994). TNF-α modulates the adhesive properties of leukocytes and endothelial cells. It induces the upregulation of cell adhesion molecules such as, endothelial leukocyte adhesion molecule 1 (ELAM-1), intercellular adhesion molecule 1(ICAM-1), vascular cellular adhesion molecule 1 (VCAM-1) (Pober and Cotran 1990, Tracey and Cerami 1994, Beyaert and Fiers 1998, Urban et al. 1986, Grau et al. 1991, Udomsangpetch et al. 1996, McCormick et al. 1997, Lou et al. 1998, Garcia et al. 1999, Wahlgren 1999, Yipp et al. 2000). The endothelial cells

undergoing morphological changes contribute to increased microvascular permeability and to extravasation of neutrophil, lymphocytes, monocytes and macrophages (Cotran et al. 1999). The pRBCs interact synergistically with multiple adhesion molecules on vascular endothelium. The cytoadherence of pRBCs to human dermal microvascular endothelial cells (HDMECs) was also examined. The pRBCs were observed to tether on, roll along, and adhere to resting HDMECs constitutively expressing CD36 and ICAM-1. Stimulation with TNF-α for five and 24 hours resulted in up-regulation of ICAM-1 and induction of VCAM-1. The addition of TNF-α significantly increased the percentage cells that adhered to the endothelial (Yipp et al. 2000).

The role of adhesion molecules in the pathogenesis of CM was evaluated in murine CM (Grau et al. 1991). Leukocyte function antigen-1 (LFA-1) is the leukocyte ligand expressed on neutrophils and monocytes. LFA-1 binds to ICAM-1 expressed on endothelial cells. Malaria infected mice were treated with the monoclonal antibody against LFA-1 on days six, seven and ten and the antibody was able to prevent the development of CM. Most important, malaria infected mice at the brink of death were also treated with an anti-LFA-1 monoclonal antibody, which dramatically protected them from dying. TNF-α stimulated endothelial cells to express high levels of ICAM-1 which mediates the adhesion of LFA-1 positive cells which is critical to the pathogenesis of murine CM (Grau et al. 1991).

Infections with *P. vivax* are very rarely fatal and do not mitigate the severe complications observed in *P. falciparum* infections. Serum levels of TNF-α were

higher in vivax malaria patients than mild falciparum malaria patients, but as high as patients with CM. The two malaria species differ in their ability to stimulate host production of TNF-α. The differences in TNF-α expression are due to the more synchronous schizont ruptures of *P. vivax* leading to higher TNF-α levels than asynchronous *P. falciparum*. They differ in the amount and length of TNF-α production. Vivax malaria generates brisk increases TNF-α levels, while in falciparum malaria produces sustained levels that remain higher for longer periods of time. The expression of TNF-α in falciparum malaria is also associated with the upregulation of cell adhesion molecules, release of mediators, e.g., nitric oxide, and endothelial cell lesions which are do not occur in vivax malaria. The expression of TNF-α is generated in response to other parasite-specific factors of *P. falciparum* not associated with *P. vivax*, e.g. merozoite surface protein and GPI variability (Grau and Piguet 1993).

In summary, TNF-α is a pivotal mediator of inflammation that activates leukocytes, upregulates cell adhesion molecules and enhances pro-inflammatory cytokines. Its role can be either protective or pathological and it has been shown that elevated serum levels of TNF-α are positively correlated with disease severity and poor outcome. Polymorphisms in the TNF-α gene have been associated with cytokine overproduction and the fatal outcome in CM (Wilson et al. 1997).

TNF-α Polymorphism

The localization of TNF-α within the MHC region, where multiple alleles

are the norm, might predispose the TNF-α gene to the same propensity for polymorphism. A genomic biallelic polymorphism at position -308 from the transcriptional start site of the TNF-α gene has been associated with cytokine overproduction and the clinical outcome of malaria (Wilson et al. 1992, McGuire et al. 1994, Turner et al.1997, Knight et al. 1999). The polymorphism at position -308 involves a nucleotide substitution of guanine (G) to adenine (A), i.e., -G308A. This polymorphism is also associated with autoimmune disorders such as, myasthenia gravis (Skeie et al 1999) and pediatric bone cancer (Patio-Garcia et al. 2000).

The genetic propensity to produce high levels of TNF-α has been attributed to nucleotide variations in the regulatory region of the gene. Elevated TNF-α levels were frequently observed in children with CM, with the highest levels detected in patients who died of CM shortly after hospital admission (Grau et al. 1989, McGuire et al. 1994, Knight et al. 1999).

Three polymorphism of the TNF-α promoter have been associated with susceptibility to, or protection from, severe malaria. The single nucleotide polymorphisms all involve guanine to adenine substitutions at positions -G238A, -G308A, and -G376A relative to the transcriptional start site. The substitution at position -238 has been associated with decreased susceptibility and protection from severe malaria. The -G238A allele is not known to have functional significance but has been implicated with susceptibility to certain autoimmune disorders, e.g., rheumatoid arthritis, juvenile psoriasis and psoriatic arthritis (Knight et al. 1999). No genotype-phenotype correlations have been reported for the -G238A allele suggesting that this polymorphism does not have a direct

effect on gene expression (Wilson et al. 1997).

The polymorphisms at position -376 has been associated with increased susceptibility to CM. Fortunately, the -G376A polymorphism is counterbalanced by tight linkage with -G238A which confers a strong protective effect. It has been speculated that the TNF-α -G376A polymorphism arose as a mutation of a haplotype bearing the -G238A allele (Knight et al. 1999, May et al. 2000). The nucleotide substitution at position -G376A affects the binding of the helix-turn-helix transcription factor octamer (OCT-1) to the TNF-α promoter region. A mutation that recruits an additional transcription factor to a regulatory region has the potential to increase the complexity and specificity of gene regulation. OCT-1 alters basal gene expression in transfected human monocytes (Knight et al. 1999, May et al. 2000, Lewin 1994).

While playing a pivotal role in malaria, TNF-α functions both to suppress parasitic growth and to initiate pathologic clinical symptoms. TNF-α serum concentration was frequently elevated in patients with falciparum malaria, particularly those with CM (Grau et al. 1989, Wilson et al. 1997). The -G308A polymorphism affects TNF-α production and serves as a central mediator of CM in mice (Grau et al. 1987) and humans (Grau et al. 1989, McGuire, et al. 1994, Stuber et al. 1996, McGuire et al. 1999). Children homozygous for the -308 (A/A) allele exhibit the highest risk for death or severe neurological sequelae due to CM (McGuire et al. 1994, Wahlgren 1999, Knight 1999, Shaffer et al. 1991, Wilson et al. 1997). The -G308A polymorphism affects transcriptional activity, gene regulation and is associated with high TNF-α production (Wilson et al.

1997, Knight 1999, McGuire et al.1994, 1999, May et al. 2000). The -G308A allele lies on the extended haplotype HLA-A1-B8-DR3-DQ2, which is associated with autoimmunity and high TNF-α production. Malaria patients homozygous for -308 (A/A) allele was associated with a seven fold increased risk of death due to CM. Despite the adverse effects of homozygosity in malaria, it has beneficial effects in other major infectious disease such as measles, meningococcal disease, leprosy and tuberculosis (Wilson et al. 1997).

In summary, TNF-α exhibits potent biological activity and its production is tightly regulated both at the transcriptional and post transcriptional levels (Wilson et al. 1997). Low levels of TNF-α prove antiparasitic effects, while elevated levels provide protection from severe malaria, but excessive TNF-α levels are associated with pathologic complications such as severe malaria and CM. There is clear evidence that excessive production of TNF-α is associated with the pathophysiologic features of severe malaria, but this elevation is not specific for CM. Certain cytokine genotypes may exist as a result of selective pressure from infectious diseases. The marked overproduction of TNF-α has been associated with allelic polymorphism in the TNF-α gene at position -308 (McGuire, et al. 1994, 1999, Stuber et al. 1996, Wahlgren 1999, Knight et al. 1999, May et al. 2000). The effects exerted by TNF-α emphasizes the complexity of malarial pathogenesis involving an elusive pathogen that continuously varies its antigenic presentation.

INTERFERON-GAMMA

Interferon-gamma (IFN-γ) is a crucial component of the cytokine cascade secreted to the pre-erythrocytic and erythrocytic antigens of falciparum malaria. The production of IFN-γ by natural killer (NK) cells and thymic dependent (T) lymphocytes, activates macrophages that in turn, secrete tumor necrosis factor-α (TNF-α). This initial response is critical for the stimulation of phagocytosis to control parasitemia. However, the overproduction of IFN-γ and TNF-α are implicated with the severe pathology of CM and an imbalance in Th1/Th2 cytokine expression (Grau et al. 1989, Wilson et al.1997).

Historically, an interferon-like substance was detected in the cerebral spinal fluid (CSF) of patients suffering from infectious and noninfectious diseases (Sen and Lengyel 1992). IFN-γ is a proinflammatory cytokine and a member of the interferon cytokine family. The interferons are a serologically distinct group of proteins recognized for their antiviral properties that interfere with viral replication and inhibit viral transmission to uninfected cells. The interferons were differentiated into IFN-alpha (α), beta (β) and gamma (γ) based on their antigenic differences, molecular weight, pH sensitivity, thermal stability and anti-viral activity. Interferons α and β, also known as type I interferons, were identified as acid stable proteins, while type II, IFN-γ was acid labile (Wheelock 1965). IFN-γ has been referred to as "type II interferon," "interferon-like substance," and "acid labile interferon" (DeMaeyer and DeMaeyer-Guignard 1998). Purification studies identified IFN-γ as a homodimeric glycoprotein consisting of two, 21 and 24 kDa subunits (Yip et al. 1982). The IFN-γ protein

was detected as a single copy gene located in the p12.05q region of chromosome 12. The cloning of the 4.5 kilobase IFN-γ gene detected four exons and three introns that translate into an 166 amino acid polypeptide, with a 23-residue signal sequence (Gray et al.1982).

T Lymphocyte Subset Differentiation

The purification of IFN-γ (Yip et al. 1982) ultimately led to the phenotypic categorization of T lymphocytes based on their ability to express the CD4 and CD8 surface antigens (Mossman and Coffman 1989). Monoclonal antibodies identified CD4 positive (CD4+) lymphocytes as helper cells for antibody formation and the mediation of delayed type hypersensitivity reaction, while CD8+ cells functioned as cytotoxic and antigen-specific suppressor cells (Salgame et al. 1991). The CD4+ helper cells were divided into two subsets, Th1 and Th2 cells, derived from a common precursor, Th0 cells. Murine T lymphocyte sub-populations were characterized based on their pattern of cytokine expression in response to antigen stimulation. Th1 cells, involved in cell mediated immunity, secreted IL-2, IL-3, TNF-β and IFN-γ. The Th2 cells that provide help for specific antibody production (humoral immunity), produced IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13 but little or no IFN-γ (Mossman and Coffman 1989, Fiorentino et al. 1989, Salgame et al. 1991, Winkler et al. 1999). IFN-γ inhibited the proliferation of Th2 cells, while IL-4 inhibited Th1 cells. Consequently, IFN-γ and IL-4 mutually inhibited each others function, and allowed for the preferential expansion of either Th1 or Th2 cells during an

immune response. Th1 and Th2 subsets were also detected in human lymphocytes, although with overlapping expression patterns (Salgame et al. 1991). Differences in the Th1/Th2 cytokine balance have been linked to their ability to control parasite multiplication in falciparum malaria (Luty et al. 1999). Th1 cells imparted a protective immune response in murine malaria, while Th2 cells were more often associated with pathology (de Kossodo and Grau 1993).

IFN-γ functions as an immunoregulatory and antiviral cytokine that enhances cell mediated and humoral immunity by regulating the differentiation, activation and function of a variety of cells (Sen and Lengyel 1992). IFN-γ plays an essential role in the elimination of malaria parasites, possibly from enhancement in phagocytosis of the macrophages (Yoneto et al. 1999), stimulation of antigen presentation through enhanced expression of major histocompatibility complex (MHC) molecules (Wong et al. 1983), activation of monocyte cytotoxicity (Pace et al. 1983, Nathan et al. 1983), induction of Fc receptors during the activation of monocytes and macrophages (Petroni et al. 1988, Sen and Lengyel 1992), priming macrophages for activation (Pace et al. 1983), augmenting the expression of TNF-α receptors (Tsujimoto et al.1986, Ruggiero et al. 1986, Alvaro-Gracia et al. 1993), enhancement of NK cell activity (Munoz et al. 1983. Biron et al. 1999) and stimulation of immunoglobulin subclass switching in B cells (Petroni et al. 1988).

NK cells function as innate immunologic mediators of infection and a major source of IFN-γ. NK cells comprise a special sub-population of lymphocytes, which are neither T nor B lymphocytes. They are unique cytotoxic

lymphocytes that lack immunologic memory, and through targeted cell lysis, they can kill without prior sensitization (Munoz et al. 1983, Yoneto et al. 1999). Since these effector cells are not antigen specific, NK cells represent the first line of defense against a variety of pathogens. Early in malaria infection, NK cells and gamma: delta T cells (γδ) are thought to be the most significant source of IFN-γ essential for the control of parasitemia (Choudhury et al. 2000). One mechanism is the activation of macrophages. In self resolving murine malaria *P. yoelii* and *P. chabaudi* models, IFN-γ levels were elevated within 24 hours of infection. NK cell depleted and athymic mice infected with self resolving murine malaria each exhibited significantly reduced IFN-γ levels. Thus, early IFN-γ production appears to be mediated by both NK and T cells (De Souza et al. 1997).

The immunologic mechanisms evoked after infection involves both innate and adaptive immune responses by macrophages, NK cells and T lymphocyte sub-populations. Malarial antigens stimulate macrophages to produce TNF-α and IL-12 which leads to NK cell activation and the enhanced production of IFN-γ in the early phase of the disease. T lymphocytes express T cell receptors (TCR), a heterodimer composed of two transmembrane proteins, either alpha and beta (αβ) or gamma and delta (γδ) on their cell surfaces (Janeway and Travers 1997, Kuby 1997). Both αβ and γδ T cell populations secrete cytokines. γδ T cells can recognize antigen directly without the requirement of MHC molecule presentation (Choudhury et al. 2000, Janeway and Travers 1997, Kuby 1997). Activated T cells demonstrated the production IFN-γ and TNF-α within eighteen hours of contact with *P. falciparum* infected erythrocytes

(Hensmann and Kwiatkowski 2001). After macrophage activation, a combination of predominantly γδT cells and some NK cells produce IFN-γ to prime the induction of antigen specific T helper cells for parasiticidal effector mechanisms. The activation of Th1 cells results in additional IFN-γ production and further activation of macrophages essential for controlling parasitemia (Choudhury et al. 2000).

IFN-γ demonstrated a protective role against pre-erythrocytic and erythrocytic stages in different malaria species models. Prophylactic treatment of rhesus monkeys with recombinant IFN-γ totally suppressed infection by *P. cynomolgi* (Schofield et al. 1987). Mice pre-treated with IFN-γ before sporozoite inoculation resulted in the inability of the parasites to infect murine hepatocytes (Grau and Behr 1994). In falciparum malaria, sensitized T cells released IFN-γ which thwarted the development of the intrahepatic stages of the parasite in cell culture studies (Ferreira et al. 1986, Mellouk et al. 1987). In contrast, the administration of anti-IFN-γ, a monoclonal antibody against IFN-γ, abrogated protective immunity and resulted in the development of fulminant infection (Schofield et al. 1987). The production of IFN-γ was associated with significantly lower rates of reinfection in a prospective study in Gabonese children with mild falciparum malaria (Luty et al. 1999). The mononuclear phagocytes produced IFN-γ in response to sporozoites and merozoite antigenic peptides resulting in a control of parasitemia.

The Pathology of IFN-γ Overproduction in Malaria

IFN-γ was determined to be a crucial mediator in the clearance of malaria and activation of TNF-α. However, the severe complications of falciparum malaria appeared to involve the dysregulation of the immune system mitigated by IFN-γ overproduction. In mice, the susceptibility to develop CM was associated with their capacity to produce high levels of IFN-γ with an upregulation of the IFN-γ gene in response to specific malarial antigens (Grau and Behr 1994). IFN-γ activated macrophages which produced TNF-α. At the onset of cerebral complications, significant accumulations of IFN-γ mRNA were detected in the cerebral tissue of mice with CM. The overproduction of IFN-γ was directly associated with the development CM. IFN-γ activated macrophages and microglial cells which secreted TNF-α and led to the upregulation of TNF-α receptors on cerebral endothelial cells (Ruggiero et al. 1986). The activation of endothelial cells led to upregulation of cell adhesion molecules, notably ICAM-1, to which the parasitized red blood cells (pRBCs) adhered (de Kossodo and Grau 1993, Grau and Behr 1994). The *in vivo* treatment of *P. berghei* infected mice with an anti-IFN-γ monoclonal antibody protected the mice from the development of CM and prevented TNF-α overproduction (Grau et al. 1989).

IFN-γ Polymorphism

IFN-γ plays an essential role in the regulation of the immune response and alterations in its production has been associated with several diseases. Polymorphisms in the IFN-γ gene have been associated with an increased

propensity for cytokine over-expression. The 4.5 Kb *IFNG* gene consists to four exons, 114, 69, 183 and 132 base pairs (bp) and three introns of 1238, 95 and 2422 bp, respectively (DeMaeyer and DeMaeyer-Guignard 1998). The first intron is the largest and consists of 1238 base pairs with cytosine:adenine (CA) repeat regions. A variable length dinucleotide-repeat polymorphism has been described in humans and lower primates within the first intron of the IFN-γ gene between positions +1349 and +1373 (Awata et al. 1994, Pravica et al. 1997). A single nucleotide polymorphism located at the five prime (5') end of a CA repeat region was detected at position +874 in the first intron in which a thymidine (T) was substituted by adenine (A) in the IFN-γ gene (Awata et al.1994, Pravica et al. 1997).

Polymorphisms in the IFN-γ gene have been associated with several diseases including type 1 diabetes (Jahromin et al. 2000), insulin-dependent diabetes mellitus (Awata et al. 1994), multiple sclerosis (Giedraitis 1999), rheumatoid arthritis (Ollier et al. 2000) and lung transplantation fibrosis (Awad et al. 1999). The over production of IFN-γ has been described in the pathogenesis of CM. However, an association been IFN-γ production and polymorphisms of the IFN-γ gene have not been reported in CM.

INTERLEUKIN- 6

Interleukin-six (IL-6) is a multifunctional cytokine involved in acute phase reactions, immune responses, and hematopoiesis. Together with IL-1 and TNF-α, these cytokines exhibit pleiotropic and redundant actions during

inflammation (Jakobsen et al. 1994, Kuby 1997). Elevated IL-6 serum levels correlate with the severity of malaria infections. However, IL-6 does not play a role in the pathogenesis of cerebral malaria (CM) (Grau et al. 1990, Molyneux et al 1991, Kern et al. 1989, Wenisch et al. 1999).

IL-6 is a pro-inflammatory cytokine transcribed from a five kilobase gene consisting of five exons and four introns located on the short arm of chromosome seven, 7p15-21 (Yasukawa et al. 1987). At least five forms of the protein have been identified ranging in size from 23 to 30 kilodaltons (kDa). The heterogeneity of IL-6 has been attributed to the different glycosylation sites in the peptide (May et al. 1988). IL-6 is secreted as a 212 amino acid peptide containing a 28-residue signal sequence which is cleaved, resulting in a 186 amino acid molecule (May et al. 1992). Before the cloning and purification of the protein, IL-6 was known as interferon-β2, B cell stimulatory factor, 26 kDa protein, cytotoxic T cell differentiation factor, hybridoma/plasmacytoma growth factor, hepatocyte stimulating factor, monocyte granulocyte inducing factor 2, and thrombopoietin (May et al. 1988).

IL-6 is a multifunctional cytokine secreted in an autocrine, paracrine or endocrine fashion. Many different types of cells have receptors for IL-6. The cytokine binds to the IL-6 receptor (IL-6R) or to gp130, a common receptor subunit of the IL-6 cytokine family. Members of this cytokine family are involved in a variety of biological activities, including the immune response, inflammation, hematopoiesis, and oncogenesis by regulating cell growth, survival, and differentiation. IL-6 is secreted by various cell types central to the pathogenesis of CM including monocytes, T cells (Richards 1998), bone marrow stromal cells

(Chiu et al. 1988), fibroblasts (Kohse et al. l986), endothelial cells (Shalaby et al. 1989), microglia and astrocytes (Ray et al. 1989, Richards 1998, Kasahara et al. 1990).

The hallmark of the acute inflammatory response is the generation of liver-derived, acute phase proteins. IL-6 initiates the release of acute phase reactants from hepatocytes (Richards 1998). Acute phase proteins, such as C reactive protein, fibrinogen, ceruloplasmin, etc., are used as indicators of inflammation or infection (Cotran et al. 1999). The demonstrated activities of IL-6 include the differentiation of B cells into plasma cells, stimulation of IgG secretion, activation of T helper cells, cytotoxic T cells and NK cells, induction of hepatocytes to synthesize acute phase proteins, and enhancement of hematopoietic stem cells (Ray et al. 1989, Richards 1998, Kasahara et al. 1990)

Monocytes and macrophages may be the first cells to release IL-6 during an inflammatory reaction (Waage et al. 1990). The role of IL-6 as a stimulator or inhibitor of disease may depend on its concentration and degree of exposure. The presence of elevated levels of IL-6 in many diseases implies its importance in the disease process or the body's response to disease (Richards 1998). Elevated IL-6 serum levels are associated with a variety of inflammatory conditions including systemic lupus erythematosus (Sun et al. 2000), type 2 diabetes, multiple myeloma, multiple sclerosis, juvenile rheumatoid arthritis and Crohn's disease (Richards 1998). IL-6 has inhibitory effects on the production of TNF-α and IL-1β induced by LPS or phytohemagglutinin at both the protein and mRNA levels (Aderka et al. 1989, Schindler et al. 1990). Since TNF-α is a potent inducer of IL-6, the inhibition of TNF-α production by IL-6 may serve as a

form of negative regulation (Schindler et al. 1990). The suppressive effect of IL-6 on IL-1 and TNF-α is particularly important for endothelial cells and fibroblasts that express large amounts of IL-6 (Schindler et al. 1990). Similarly, IL-4, IL-10 (de Waal Malefyt et al. 1996) and glucocorticosteroids can inhibit the production of IL-6 (Waage et al. 1990, May et al. 1988).

IL-6 and Malaria

The excessive production of pro-inflammatory cytokines has been associated with the pathogenesis of severe malaria. In a study of 290 Gambian children, the concentration of IL-6 was higher in children with CM than in patients with mild malaria, asymptomatic malaria, and uninfected controls (Jakobsen et al. 1994). Similarly, patients who died of malaria had significantly higher plasma TNF-α and IL-6 levels than those who survived (Kern et al. 1989, Molyneux et al. 1991). Since children who died of CM had the highest level of TNF-α, the association between TNF-α and disease severity may be related to its ability to induce the secretion of IL-6.

Murine experimental cerebral malaria (ECM) was used to detect the role of IL-6 in the pathogenesis of CM. In kinetic studies of *Plasmodium* infected mice, IL-6 serum levels were markedly elevated seven days after infection but gradually returned to normal by day 21 (Grau et al. 1990). The highest level was detected in mice with fulminant neurological syndromes. Mice treated with an anti-IL-6 monoclonal antibody had reduced levels of IL-6, but this reduction failed to prevent the development of ECM. This suggested that IL-6 does not play a role in the pathogenesis of ECM although large amounts were produced in

malaria infections (Grau et al.1990).

There was a significant correlation between parasite clearance time and IL-6 levels. Soluble forms of IL-6 receptors (sIL-6R) can inhibit the expression of IL-6. Plasma levels of IL-6 and sIL-6R were determined in malaria patients before and during therapy. The levels of sIL-6R were significantly elevated in all patient groups before therapy, and increased further after the decline of IL-6 levels and clinical improvement. Thus, the increase in sIL-6R functioned to control the excessive release of IL-6 (Wenisch et al. 1999).

Sera from falciparum and vivax malaria patients were analyzed for IL-6, IFN-γ and TNF-α levels before and during antiparasitic treatment. Before treatment, the levels of all three cytokines were markedly elevated in falciparum malaria patients, but only elevated in a few vivax malaria patients. IL-6 and TNF-α levels correlated significantly with parasite density, but no such correlation was detected with IFN-γ. After treatment, IL-6 and TNF-α remained markedly elevated in falciparum patients with complicated clinical courses. Thus, TNF-α and IL-6 levels might serve as markers of disease severity (Kern et al. 1989).

Peripheral blood mononuclear cells from falciparum malaria patients were stimulated with *P. falciparum* antigens and pro-inflammatory cytokines levels measured. Kinetic studies showed that IL-6, IL-1 and TNF-α levels were detected as early as two to four hours after stimulation and peaked at four to eight hours. The addition of IL-10 inhibited IL-6 production and reduced the accumulation of IL-6 mRNA. However, when IL-10 was neutralized, a markedly elevated IL-6 and TNF-α production was observed in patients with

uncomplicated infections compared with severe falciparum malaria patients. Thus, the pathogenesis of severe falciparum malaria may involve both an excessive production of proinflammatory cytokines and a defective negative feedback (IL-10) mechanism (Ho et al. 1998).

IL-6 Polymorphism

A dysregulated IL-6 expression has been implicated in development of various diseases. It is well documented that nucleotide polymorphism of cytokine genes is associated with altered expression rates and some are associated with the development of disease (Wilson et al. 1997). A bi-allelic guanine (G) to cytosine (C) polymorphism was identified in the promoter region of the IL-6 gene at position -174 relative to the transcriptional start site. Polymorphisms in the IL-6 gene have been correlated with the protein secretion levels of the cytokine. The -G174C polymorphism modulated the expression of the IL-6 gene in which the C/C genotype was associated with low serum concentrations, while the G/G genotype produced normal levels. The -G174C substitution has also been implicated in several diseases, including bone mineral density (Ota et al. 2001), systemic lupus erythematosus (Linker-Israeli et al. 1996), type I diabetes (Jahromi et al. 2000), rheumatoid arthritis (Fishman et al. 1998),and insulin sensitivity (Fernandez-Real et al. 2000). There have been no reports of IL-6 polymorphism in the literature associated with malaria.

INTERLEUKIN-10

Anti-inflammatory cytokines serve as modulators of pro-inflammatory

cytokines in response to acute malaria infection. Interleukin (IL)-10 plays a pivotal role in inhibiting the expression of pro-inflammatory cytokines, but, low IL-10 levels enhance their expression (Day et al. 1999). The balance between pro- and anti-inflammatory cytokines in falciparum malaria is poorly understood. TNF-α, IFN-γ, IL-6, TGF-β and IL-10 play important roles in the balanced regulation of malaria infections (Merchant et al. 1994, Day et al.1999, Omer et al. 2000). A failure of this mechanism to maintain an immunologic balance has been implicated in the pathogenesis of CM and the potential for a fatal outcome. TNF-α, IFN-γ, and IL-6 activate macrophages and enhance the production of nitric oxide which is crucial for the killing of intracellular parasites (Nathan et al.1985, Grau et al. 1995). IL-10 and TGF-β inhibits the secretion of TNF-α, IFN-γ and IL-6 thus limiting their pro-inflammatory response (Wanidworanun and Strober 1993, Ho et al. 1998, Merchant et al. 1994, Day et al. 1999, Omer et al. 2000).

IL-10 is a homodimeric, anti-inflammatory cytokine. Originally named "cytokine synthesis inhibitory factor," IL-10 blocks the synthesis of cytokines produced by Th1 helper cells, activated monocytes and NK cells (Fiorentino et al. 1989). After the discovery that IFN-γ inhibited Th2 cells, it was hypothesized that Th2 clones might also produce a factor that could inhibit the proliferation and cytokine production of Th1 cells (Mosmann et al.1986). The supernatant of activated Th2 cells inhibited the production of IFN-γ by Th1 cells, but did not affect Th2 cells. The "cytokine synthesis inhibitory factor" was purified (Fiorentino et al. 1989), cloned and renamed IL-10 (Vieira et al. 1991). The 4.7 kilobase human IL-10 gene is located on chromosome 1q consisting of five exons and

four introns (Kim et al.1992). The IL-10 gene encodes a 178-residue protein preceded by a 18 amino acid leader sequence which is cleaved before secretion (Vieira et al. 1991). Surprisingly, the overall structure of IL-10 closely, resembles that of IFN-γ (Vieira et al. 1991). In the mouse, IL-10 is produced by Th2 and Th0 subsets of T helper cells but not Th1 or CD8 positive cells (Fiorentino et al. 1989). The production of IL-10 in humans may not be limited to the same T cell subsets as in mice. Human IL-10 production has been detected in activated Th0 and Th2 subsets, activated CD4+ and $CD8^+$ T cells, $CD4^+$ $CD45R0^-$ (memory), CD4+ $CD45RA^+$ (naive) subsets, B cells (Benjamin et al. 1992), monocytes, macrophages and keratinocytes (Yssel et al. 1992, de Waal Malefyt and de Vries 1996).

The expression of IL-10 affects a variety of different cell types. IL-10 stimulates B cells resulting in increased major histocompatibility complex (MHC) class II antigen expression and proliferation (Rousset et al. 1992). In contrast, IL-10 blocks MHC class II expression on monocytes, macrophages, and cytotoxic T cells reducing their capacity to activate T cells or mediate cytolysis (Matsuda et al. 1994, de Waal Malefyt et al. 1991, Fiorentino et al. 1991). IL-10 inhibits the secretion of IFN-γ, IL-1α, IL-1β, IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF), TNF-α (de Waal Malefyt et al. 1991) and anti-inflammatory cytokines, IL-4, IL-13 and even IL-10 itself (Mosmann et al.1986). Thus, IL-10 may be autoregulatory (de Waal Malefyt et al. 1991, 1993). Ironically, TNF-α can induce and even enhance the expression of IL-10 (Wanidworanum and Strober 1993). The production sequence of pro- and anti-inflammatory cytokines is not clear. Peripheral blood mononuclear cells (PBMC)

stimulated by falciparum malaria antigen were analyzed for the kinetics of pro- and anti-inflammatory cytokine production. TNF-α, IL-6 and IL-1β levels were detected within two to four hours after malarial stimulation, while, IL-10 was undetectable until eight to 12 hours later. In a dose response curve, low concentrations of IL-10 were sufficient to inhibit all three pro-inflammatory cytokines (Ho et al. 1998).

IL-10 and Malaria

Although kinetic studies showed that low levels of IL-10 could inhibit pro-inflammatory cytokines, low IL-10 levels have been associated with a poor malaria outcome (Day et al. 1999). The key difference between anemic and non-anemic patients with falciparum malaria in Kenya was the quantity of IL-10 produced (Kurtzhals et al.1999). The development of severe anemia may be due to the suppression of erythropoiesis by prolonged exposure to TNF-α (Clark and Chaudhri 1988). Anemic falciparum malaria patients had higher levels of TNF-α than IL-10, while patients with mild disease had higher IL-10 levels than TNF-α. The higher expression of IL-10 prevented the development of malaria anemia by downregulating the pro-inflammatory cytokines, but it did not alter the course of the primary infection (Othoro et al. 1999). To study the effect of IL-10 in the course of the infection, wild type (WT) and IL-10 deficient mice were infected with *P. chabaudi*. IL-10 deficient mice produced excessive amounts of TNF-α, IFN-γ, NO which led to an exacerbation of pathology and even death in a proportion of the mice. While, the inactivation of IFN-γ by treatment of IL-10 deficient mice with anti-IFN-γ did not ameliorate malaria symptoms but it was

effective in decreasing morality. This suggested that IL-10 was not crucial for the development of an immune response, but that it played a role in controlling or downregulating the pathology associated with the infection (Li et al. 1999).

The imbalance and quantity of pro- and anti-inflammatory cytokine levels were the most predictive factors of a fatal outcome in falciparum malaria. TNF-α, IL-6 and IL-10 levels were strongly correlated with the eventuality of a fatal outcome (Day et al. 1999). IL-10 and IL-6 were the two strongest predictors suggesting that they function in a counterregulatory mechanism where the anti-inflammatory cytokine response was matched with the degree of activation of the pro-inflammatory cytokines (Day et al. 1999).

IL-10 Polymorphism

The level of IL-10 production was determined to be critical for controlling the balance between humoral and inflammatory responses. The possibility that a person may be genetically predisposed to produce high or low levels of IL-10 may have important consequences in the pathogenesis of malaria. Single nucleotide polymorphisms in the promoter sequence of the IL-10 gene has been associated with IL-10 production levels (Turner et al. 1997). Three single base pair substitutions in the IL-10 promoter region at positions -G1082A, -C819T, -C592A relative to the start site of transcription have been implicated in the production of the IL-10 (Turner et al. 1997). Three putative haplotypes are associated with IL-10 production these are ACC/ACC (A at position -1082, C at position -819, and C at position -592) and ATA/ATA (A at position -1082, T at position -819, and A at position -592) result in low production, while GCC/GCC

(G at position -1082, C at position -819, and C at position -592) is associated with high IL-10 production (Turner et al. 1997). Since decreased IL-10 production has been implicated in the exacerbation of malaria pathology, one can speculate whether the anti-inflammatory to pro-inflammatory cytokine balance might result from polymorphisms in the genes of these cytokines.

Monocytes/macrophages, B cells and T cells secrete the IL-10 soon after the pro-inflammatory cytokines which inhibit their secretion (de Waal Malefyt et al. 1991). It is conceivable that an upregulation of TNF-α and a relative downregulation of IL-10 could alter the TNF:IL-10 ratio. In severe malaria, the plasma levels of TNF-α exceeds the levels of IL-10. The substitution of adenine for guanine at position -308 of the TNF-α promoter has been associated with a six-fold production increase and an independent determinant for CM (Knight et al. 1999, McGuire et al. 1998). However, it is unclear whether the nucleotide polymorphism in the TNF-α promoter also influences the IL-10:TNF plasma ratio levels. TNF-α plasma levels were not associated with polymorphism in the promoter region at positions -238, -244, -308, and -376/-238 from Gabonese children with severe malaria. The IL-10:TNF-α ratio was highest in children with the wild type TNF-α genotype, compared with promoter polymorphisms. A mutation at position -238 of the promoter strongly correlated with a lower IL-10:TNF-α plasma ratio, which mediated susceptibility to severe complications of malaria (May et al. 2000).

Patients with the high TNF-α production genotype (-G308A) and low IL-10 production haplotype (-1082A, ACC/ACC) had a worse clinical outcome for acute rejection in Crohn's disease and in kidney and kidney-pancreas (Pelletier et al.

2000), and renal transplantation patients (Sankaran et al. 1997). It is conceivable that polymorphism in the IL-10 gene alone or in combination with polymorphism in the TNF-α gene might contribute to the pathogenesis of CM, however studies are absent on this hypothesis.

TRANSFORMING GROWTH FACTOR-BETA

Transforming growth factor-beta (TGF-β) can be characterized as the "thermostat" of the immune system. As a pivotal regulator of inflammation, it functions as a pro-inflammatory cytokine at low concentrations and an anti-inflammatory one at high concentrations (Omer et al. 2000). The pathology associated with CM is largely immune mediated in which overexpressed pro-inflammatory cytokines mediate the cytoadherence of pRBC to vascular endothelium. TGF-β can downregulate the pathogenic effects of these pro-inflammatory cytokines. As a pleiotropic cytokine, TGF-β mediates the control and clearance of malaria parasites and downregulates the immune mediators. Evidence from animal studies (Omer and Riley 1998, Tsutsui and Kamiyama 2000) indicates that TGF-β may play a critical role in the pathology of CM and therefore warrants this literature review.

TGF-β was initially discovered as a protein with the ability to cause malignant transformation in rat fibroblasts (Roberts et al. 1981). Sequence information established that differentiation inhibiting factor, cartilage-inducing factor, glioblastoma-derived T cell suppressor factor, myoblast differentiation inhibition factor, and epithelial growth inhibitor were identical to TGF-β (Ruscetti

et al. 1998). There are five different isoforms of TGF-β designated numerically as TGF-β1 to β5, with only the first three occurring in mammalian cells (Massague 1987). The TGF-β1, β2 and β3 isoforms are structurally and functionally related yet encoded by different genes located on separate chromosomes, 19q13.1, 1q41, and 14q23, respectively (Fujii et al. 1986). The TGF-β gene consists of seven exons and six introns that transcribe a 390 (TGF-β1) or 412 (TGF-β2 and TGF-β3) amino acid large precursor molecule (Derynck et al. 1985). The protein is synthesized as a biologically inactive pre-propeptide that is proteolytic cleaved by furin convertase to yield the 112 amino acid monomer (Dubois et al.1995, Johnson and Gold 1996). The active protein consists of two identical disulfide-linked polypeptide chains consisting of the 112 amino acids of the C terminal part of the precursor protein (Derynck et al. 1985). After cleavage, the 25 kDa protein is expressed as a biologically active homodimer that interacts with specific cell surface receptors (Dubois et al. 1995, Omer et al. 2000). The TGF-β superfamily currently consists of more than 25 proteins, grouped into four subfamilies that share structural and functional characteristics (Massague 1987). Cells of the immune system synthesize mainly TGF-β1, while TGF-β2 and β3 are expressed in specific anatomic sites, i.e. central nervous system, anterior chamber of the eye (Abbas et al. 1997).

As a multi-functional cytokine, TGF-β1 is expressed by most if not all normal cells including, monocytes, macrophages, neutrophils, lymphocytes, endothelial cells, astrocytes, fibroblasts, osteoblasts, myocytes, chondrocytes, vascular smooth muscle cells, epithelial cells and platelets (large concentrations

stored alpha granules) (Abbas et al. 1997, Ruscetti et al. 1998, Derynck 1998, Suthanthiran et al. 2000). Importantly, it can be delivered to the site of tissue injury by inflammatory cells and platelets where it binds to fibronectin (Ruscetti et al.1998, Cotran et al. 1999).

TGF-β is an immunomodulatory cytokine with pervasive effects on the immune system. Much of its biological action is expressed in an autocrine or paracrine fashion (Suthanthiran et al. 2000). It exhibits various biological effects that are often conflicting in nature. Moreover, the effects of TGF-β can be either positive or negative depending on the cell types, conditions or cellular differentiation (Li et al. 1999).

Stimulatory Activities

TGF-β can function as a growth stimulator or inhibitor. Although TGF-β is largely a negative regulator of the immune system, it can also express positive effects. TGF-β plays a major role in bone remodeling, and is stored in the bone matrix for release during bone resorption (de Waal Malefyt 1998). It enhances matrix protein synthesis by fibroblasts, osteoblasts and endothelial cells which is important in tissue repair and remodeling. A pivotal regulatory cytokine, TGF-β functions as a pro- or anti-inflammatory cytokine depending on its concentration and environment. As a pro-inflammatory cytokine, TGF-β stimulates cytokine secretion of IL-1, IL-6, TNF-α, and basic fibroblast growth factor (Derynck 1994, Johnson and Gold 1996, Abbas et al. 1997, Ruscetti et al. 1998). TGF-β has the ability to stimulate its own production two to three fold in normal and transformed

cells (Van Obberghen-Schilling et al. 1988). Early in an infection, TGF-β serves as a potent chemotactic factor for fibroblasts, monocytes, macrophages, neutrophils and T lymphocytes to the site of inflammation (Derynck 1994, Wahl et al. 1987). TGF-β activates neutrophils, yet deactivates macrophages. At high concentrations, TGF-β functions as an anti-inflammatory agent that down regulates the production of pro-inflammatory cytokines (Omer and Riley 1998).

Anti-proliferative Effect of TGF-β

TGF-β functions as a potent growth inhibitor of various cell types, especially hematopoietic cells, by interfering with the cell cycle and counteracting the effects of pro-inflammatory cytokines. TGF-β antagonizes many responses of lymphocytes. It suppresses the proliferation, development and activity of B cells, T cells, thymocytes, large granular lymphocytes, NK cells and lymphokine activated killer cells (Biron et al. 1999, Derynck 1998, Abbas et al. 1997). TGF-β inhibits the production of IFN-γ and TNF-α from NK cells, restricts the production of TNF-α and nitric oxide from macrophages, and suppresses IgG and IgM synthesis and secretion (Johnson and Gold 1996, Pelletier et al. 1998, Abbas et al. 1997, Derynck 1998). In low concentrations, it induces the synthesis and secretion of platelet derived growth factor (PDGF), but in high concentrations, it is inhibitory with the ability to block the expression of PDGF receptors (Derynck 1998). An alternative means by which TGF-β downregulates pro-inflammatory cytokines is by augmentation of the other anti-inflammatory cytokines such as IL-4 and IL-10 (Omer and Riley 1998).

Role of TGF-β in malaria

The immunological events associated with the clearance of the malaria parasite and the development of pathology was investigated. Three species (two nonlethal and one lethal) of *Plasmodium* stimulated the secretion of TGF-β in experimental murine malaria. The levels of TGF-β were inversely correlated with the severity of the malaria infections in the mice. Low levels of biologically active TGF-β were associated with a lethal outcome, whereas sustained production resulted in a resolution of the infection (Omer and Riley 1998).

The basis for the lethality observed with low TGF-β levels was examined with the hypothesis that TGF-β was required to prevent severe pathology. Mice treated with anti-TGF-β, a neutralizing antibody to all mouse TGF-β isoforms, before and during malaria infection resulted in rapid growth of the parasites, elevated plasma levels of TNF-α and IFN-γ, and a quicker death. Treatment with recombinant TGF-β resulted in a lower rate of parasitic replication and an extended survival period post infection for the lethal malaria species. This suggested that TGF-β plays two important roles in malaria. Early in infection (at low levels) it promotes Th1 mediated mechanisms (normal levels of TNF-α and IFN-γ) that controlled parasitic growth. Later in infection at high levels, it downregulates the Th1 response to the initial inflammation-associated pathology (Omer and Riley 1998).

Elevated TGF-β exacerbated *Plasmodium* infections and suppressed Th1 mediated parasite clearance. Malaria infected mice with normal serum concentrations of TGF-β resolved their infections, while high TGF-β levels early

in infection resulted in uncontrollable parasitemia. When mice were treated with the anti-TGF-β (monoclonal antibody to neutralize the activity of endogenous TGF-β), there was a significant increase in IFN-γ and nitric oxide concentrations, a decline in parasitemia and survival from the infection. In contrast, treatment with recombinant TGF-β completely prevented CD4+ T cell activation producing a markedly reduced IFN-γ production and lethal outcome (Tsutsui and Kamiyama 2000).

The serum levels of TGF-β were measured in patients with acute *P. falciparum* malaria prior to, during, and after therapy. The serum levels of TGF-β were decreased prior to treatment, increased during therapy and were within normal range 21 days after treatment. There was no correlation between parasitemia and TGF-β serum levels. The decreased serum levels might have occurred from either (1) a decreased production and release of TGF-β, (2) enhanced clearance or utilization, or (3) tissue accumulation of TGF-β during falciparum malaria (Wenisch et al. 1995).

TGF-β and IL-12 serum concentrations were significantly lower than normal in Gabonese children with severe malaria, whereas TNF-α and IL-10 were significantly higher than normal. These results suggest that inflammatory cascade in severe malaria is characterized by suppression of the protective effect of TGF-β and IL-12, and that the overproduction of TNF-α may promote pathologic effects such as severe anemia (Perkins et al. 2000).

Cerebrospinal fluid (CSF) was used as a pathologic indicator of blood-brain barrier (BBB) damage during CM. Cytokine and albumin levels were

measured in patients dying of CM and other central nervous system (CNS) infectious diseases (i.e., meningitis, encephalitis and cerebral hemorrhage). Only subtle changes in BBB integrity were detected in CM with minimal intra parenchymal inflammatory responses compared with other neurologic infections. All CM patients had neurologic syndromes involving loss of consciousness and/or seizures. Yet the CSF gave no indication of gross BBB breakdown, activation of endothelial cells, or a significant degree of inflammatory or immune response within the brain. One change seen exclusively in CM was an increase in TGF-β1 levels in the CSF. TGF-β is thought to exert neuroprotection, resulting in the lack of generalized leukocyte recruitment to the brain parenchyma in CM (Brown et al. 2000).

The focal accumulation of TGF-β1, β2 and β3 provided important evidence about their involvement in the reorganization process of the brain parenchyma, immunologic dysfunction, and endothelial cell activation in patients with CM. TGF-β1 induced apoptosis in microglial cells and inhibited the expression of vascular cell adhesion molecule 1 (VCAM), thereby controlling CNS inflammation (Winkler et al. 1998). TGF-β2 mediated immunosuppression, inhibited leukocyte transmigration across BBB in CNS inhibited inflammation and promoted intracerebral macrophage proliferation. TGF-β3 was expressed in endothelial cells and smooth muscle cells and was considered an important modulator of cellular activation and transformation (Winkler et al. 1998).

The expression of TGF-β1, β2 and β3 were analyzed in brains of patients who died with CM and in controls. There were unique expressions of TGF-β in

different areas of the brain. Compared with normal control brains without neuropathological alterations, the brains of patients who died of CM had increased expression of cell type specific accumulations of TGFβ1, β2 and β3. Significant amounts of TGF-β1 were detected in immunoreactive astrocytes adjacent to brain vessels, while TGF-β2 expressing macrophages/microglial cells were localized in ring hemorrhages and Durck's granulomas. TGF-β3 was expressed in smooth muscle cells and endothelial cells of brain vessels containing sequestered pRBC. The immunolocalization of TGF-β3 in the brain vasculature with deposits of malarial pigment and pRBC suggests it might be involved in sequestration and thrombosis (Deninger et al. 2000).

The role of TGF-β in the pathogenesis of malaria has been investigated. The different patterns of dysregulation in CM indicate that an appropriate response requires a delicate balance between of TGF-β expression which is dependent upon the concentration and environment.

TGF-β Gene Polymorphism

Nucleotide polymorphisms detected in the promoter region and signal sequence of the human TGF-β gene correlate to overexpression or deficiency in the levels of TGF-β in various diseases and conditions. TGF-β may play a major role in the immunomodulation of malaria. However, research is absent about the correlation of polymorphisms in the TGF-β gene with the pathogenesis of CM. The causal association between TGF-β gene polymorphisms and cytokine expression in CM warrants further investigation.

Several nucleotide substitutions have been identified in the TGF-β gene, with only a few being associated with cytokine production and disease pathology. Three nucleotide substitutions were identified in the region downstream of the transcriptional start site at positions -C988A, -G800A, and -C509T, and also, an insertion of cytosine, at position +72 in a nontranslated region (Yamada 2000). Two polymorphisms in the TGF-β gene at positions +869 and +915 relative to the start site of transcription have been associated with overproduction and disease outcome (Awad et al.1998, Cambien et al. 1996, Arkwright et al. 2000, Densem et al. 2000, Yamada 2000). The nucleotide substitutions in the TGF-β signal sequence change the amino acid coding sequences, and ultimately, the translation of the amino acid molecules. A bi-allelic polymorphism at position +T869C where thymine (T) is replaced by cytosine (C) changes the amino acid at codon 10 from leucine to proline. Similarly, a bi-allelic polymorphism, +G915C, results in a guanine (G) to cytosine (C) substitution at codon 25, changing from arginine to proline (Derynck et al.1987). Substitutions at codons 10 and 25 both generate a novel proline in the molecular structure. For both polymorphisms the allele encoding proline is associated with lower TGF-β synthesis (Arkwright et al. 2000, Derynck et al. 1987, Awad et al. 1998, Yamada 2000).

Codon 10 Polymorphisms

The codon 10 (leucine to proline) and codon 25 (arginine to proline) substitutions are located in the signal sequence that is cleaved from the TGF-β

precursor peptide. The signal sequence of a peptide sponsors the transfer of the newly synthesized protein to the membrane of the endoplasmic reticulum, after which it is cleaved and post-translationally processed (Benson et al. 1985, Stryer 1995, Lewin 1995). Signal peptides are comprised of three regions, a positively charged N-terminal region, a hydrophobic core, and a polar C terminal region that defines the cleavage site (Creighton 1993, Stryer 1995). Codon 10 is part of a α-helical structure in the signal sequence region which directs the transport of the TGF-β protein through the cell (Derynck et al. 1987, Cambien et al. 1996). The signal sequence peptide region influences translation, protein secretion and transport (Suthanthiran et al 2000). The polymorphism at codon 10 (leucine to proline) is located in the hydrophobic core of the signal sequence. Since both leucine and proline are nonpolar, hydrophobic molecules, the polymorphism in the hydrophobic core should not affect the function of the signal peptide (Cambien et al. 1996, Creighton 1993). Leucine promotes the formation of α-helices, while, proline, which has a cyclic structure, causes the introduction of breaks, turns, and kinks into the α-helix (Creighton 1993, Stryer 1995). The +T869C polymorphism is also in linkage disequilibrium with the -C509T polymorphism and the combination of the two haplotypes might have an effect on gene transcription (Cambien et al. 1996).

Regulated protein expression of the TGF-β gene can be phenotypically classified into three categories representing high, intermediate or low cytokine production based on the cytokine polymorphism. Persons homozygous for leucine (Leu/Leu) at codon 10 (+869) was associated with highest concentration

of TGF-β, while leucine/proline (Leu/Pro) heterozygotes produced intermediate levels and, proline (Pro/Pro) homozygotes expressed low concentrations (Yamada 2000, Arkwright et al. 2000, Hutchinson et al. 1998).

The biological consequences of the inheritance of bi-allelic polymorphisms in the TGF-β gene correlates with a number of diseases. The high producer genotype at codon 10 (Leu/Leu) has been associated with bone mineral density, osteoporosis, and spinal osteoarthritis (Yamada 2000). In lung tissue, TGF-β hyperexpression has been attributed with cystic fibrosis, severe lung fibrosis, and rapid deterioration of lung function (Arkwright et al. 2000).

Codon 25 Polymorphisms

The leader sequence of the TGF-β gene must be cleaved by an enzyme in order for the protein to be released from the cell. The localization of arginine at codon 25 is close to the cleavage site and the substitution of proline, alters the charge and structure of the enzyme cleavage site. The arginine to proline polymorphism results in the substitution of a large polar amino acid at a locus occupied by a small non polar one. Mutation in the signal sequence can affect peptide export efficiency and influence intracellular trafficking, especially when there is a change in charge in the hydrophobic core (Benson et al. 1985, Hutchinson 1999, Yamada 2000).

The high, intermediate and low producer phenotypes have been associated with TGF-β polymorphism at codon 25. Those homozygous for arginine at codon 25 (Arg/Arg) produced the highest concentration of TGF-β,

while heterozygotes, Arg/Pro produced intermediate levels and Pro/Pro homozygote expressed in low concentrations. Elevated TGF-β levels have been associated with graft dysfunction in lung, heart, kidney and liver transplant rejections (Hutchinson et al. 1999). The high producing phenotype (Arg/Arg at codon 25) has been associated with lung fibrosis (Awad 1 et al. 998), lung transplantation rejection (Awad et al. 1997, 1998), and hypertension (Cambien et al. 1996, Li et al.1999).

Recapitulation

The hypothesis of this dissertation is that activated macrophages in CM lesions are the source of local TNF-α production and that CM patients may have a genetic predisposition for an inappropriate production of TNF-α. The cytokine theory of malaria purports that during the sequestration of pRBC, merozoite schizony stimulates the local production of cytokines in the cerebral vasculature (Clark and Rockett 1994). A localized concentration of cytokines could ultimately generate the lethality and pathology associated with CM. Facts to support the hypothesis of this investigation include:

- TNF-α upregulates the expression of cellular adhesion molecules that mediate the binding of pRBCs to vascular endothelium (Urban et al. 1986, Grau et al. 1991, Udomsangpetch et al. 1996, McCormick et al. 1997, Lou et al. 1998, Lucas et al. 1996, Lucas et al, 1997, Garcia et al. 1999, Wahlgren 1999, Yipp et al. 2000).
- In murine CM, macrophages and pRBCs are sequestrated in cerebral

blood vessels. Mice treated with anti-TNF-α did not develop CM or exhibit those lesions (Grau et al. 1987, Grau et al. 1988, Grau et al. 1991, Grau et al. 1994, Lou et al. 1998).

- Malarial antigens stimulate the production of IFN-γ which activates macrophages stimulating TNF-α production. (de Kossodo and Grau 1993, Grau and Behr 1994)
- Significantly higher plasma levels of proinflammatory cytokines, TNF-α, IL-6 and IL-10 were detected in patients who died of falciparum malaria than in survivors (Clark and Rockett 1994, Day et al. 1999, Brown et al. 1999).
- TGF-β is an important pivotal regulator of inflammation. Low levels of TGF-β were associated with a lethal outcome in murine malaria, whereas sustained production was associated with resolving infections (Omer and Riley 1998, Omer et al. 2000).

Macrophages serve as a source of pro- and anti-inflammatory cytokines crucial to the hosts' immunologic defenses against malaria. There is compelling evidence that an imbalance between pro- and anti-inflammatory cytokines might contribute to the pathogenesis of CM (Day et al. 1999, Brown et al. 1999, Grau et al. 1987), and that allelic polymorphism in the genes of TNF-α, IFN-γ, IL-6, TGF-β, and IL-10 may cause differences in their expression levels. As a result, hypothesis driven, research investigations were conducted based on three major research objectives:

- To characterize the mononuclear phagocytes in the cerebral tissue and

determine their state of activation.

- To detect the local production of TNF-α in cerebral tissue.
- To detect polymorphisms in the genes of pro- and anti-inflammatory.

A prospective study was conducted using cerebral tissue from Malawian children dying of CM, NCM and COC.

Chapter 3

MATERIALS AND METHODS

Chapter 3

MATERIALS

The fundamental study objective of this dissertation was to characterize mononuclear phagocytes observed in cerebral tissue and determine their role in CM. Secondary objectives were to detect the local production of TNF-α and analyze cerebral tissue for polymorphisms in the genes of the pro- and anti-inflammatory cytokines. Cerebral tissue from children dying from cerebral malaria (CM), and control patients with non cerebral malaria (NCM), and coma of other causes (COC) were analyzed to answer the proposed study objectives.

This investigation was a sub-study of an international, five year prospective study of the "Clinicopathological Correlates of Cerebral Malaria" in Malawi, Africa. It was funded by grants from the Wellcome Trust (United Kingdom) and the National Institutes of Health (United States). The study was approved by the Health Science Research Committee, the local ethical body, in Blantyre, Malawi. The Malaria Research Project began in 1996, at the Queen Elizabeth Central Hospital in the city of Blantyre, in Malawi, Africa. The principal investigator of the project was Terrie Taylor, M.D., a professor at Michigan State University (MSU), Department of Internal Medicine, with Malcolm Molyneux, M.D., as the co-principal investigator, a professor at the University of Liverpool, and Charles Mackenzie, F.R.C.V.S, Ph.D., Collaborator/Consultant, MSU, Department of Veterinary Pathology.

The study period was from January 1996 to December 2000. The study population included forty children who were diagnosed with fatal cases of either CM, NCM or COC. Autopsies were performed on patients for which written

informed consent was granted by the parents in consultation with local physicians.

Diagnostic Criteria of Patients

At Queen Elizabeth Central Hospital, patients were diagnosed with CM, NCM or COC based of the diagnostic criteria shown in Table 2. Altered consciousness was assessed using the Blantyre Coma Score (Table 1, see cerebral malaria section of this dissertation). *P. falciparum* malaria parasitemia was assessed on thick blood films and graded empirically as 0, 1+, 2+, 3+, 4+, as a percentage of the pRBCs, or the number of cells per cubic millimeter (mm^3). A diagnosis of anemia was rendered with hematocrit readings less than or equal to ($\leq$)15% (Taylor et al. 1996).

The clinical diagnosis of CM was fulfilled when a child presented in an unrousable coma, i.e. a coma score of less two, that persisted for at least 30 minutes after a generalized convulsion, the detection of *P. falciparum* in the patient's blood smear and the exclusion of other causes of encephalopathy. A diagnosis of NCM was established for patients with coma scores greater then two, anemia (hematocrit $\leq$ 15 %) and *P. falciparum* parasitemia. Patients diagnosed with COC were not malaria infected but presented with a coma score greater than or equal to two.

Table 2. Diagnostic Criteria of Patients

	Blantyre Coma score	Parasitemia	Other
CM	less than two, ≤ 2	Parasitemia	CSF and blood cultures negative
NCM	no coma	Parasitemia	Severe anemia, admitting hematocrit ≤15%
COC	greater than two, >2	aparasitemic	CSF and blood cultures positive, viral encephalitis

Patients were diagnosed with CM, NCM or COC based on the level of the coma score, detection of parasitemia, and other causes of encephalopathy. > means greater than, ≤ means less than or equal to. CM: cerebral malaria, NCM: Non cerebral malaria, COC: Coma of other causes. Diagnostic criteria adapted from 1996 and 1997 Clinicopathology Reports, Taylor et al.

Study Population

The study population (Table 3) included 22 children diagnosed with CM, 11 with NCM and seven from COC. CM patients consisted of 11 females and 12 males, whose ages ranged from eight months to five years two months. The NCM controls were comprised of five females and six males, ranging in age from seven months to four years. The NCM group were *P. falciparum* infected and diagnosed with severe anemia, septicemia and meningitis. The COC control group consisted of six females and one male, ranging in age from 25 months to eight years. They were diagnosed with meningitis, rat poisoning toxicity, Reyes syndrome, viral hypertensive encephalopathy, human immunodeficiency virus (HIV) infection and coma of unknown etiology. The children in the COC were somewhat older than those in the other two groups and the gender was skewed to include a greater number of females.

Autopsies

Autopsies are legally sanctioned in Malawi. The post mortems were conducted by visiting pathologists collaborating on the study. An uniform autopsy sampling protocol was established and followed for each case. Tissue was collected within 15 hours of death. Autopsy records indicated the time of death after hospital admission ranged from death on admission to greater than 22 hours. The time interval between death and post mortem ranged from two to 14 hours (Table 3). The tissue specimens were labeled and coded according to the anatomical site (Appendix A). Formalin-fixed tissue sections and frozen specimens were collected in triplicate and disbursed to the collaborating investigators. A portion of the tissue was fixed in 10% neutral buffered formalin and paraffin embedded, while another was frozen in liquid nitrogen and transported to MSU for processing (Taylor 1996). The parietal lobe (B2) of the cerebral cortex was the only brain tissue used for all investigations of this dissertation. The parietal lobe is part of the somatosensory association area of the brain. Damage to the parietal lobe is associated with agnosia, the inability to recognize objects when using a given sense, and apaxiam, the inability to perform an action (Nolte 1993).

Images

Images in this dissertation are presented in color.

TABLE 3 - MALAWI PROJECT AUTOPSY DATA									
Patient #	SEX	AGE	Clinial Diagnosis	Parasites on Admission	HCT (%)	Time of Death (hrs after adm)	Death to Post Mortem Interval	Post Mortem Duration	Misc.
96-1	F	27 mo	CM	31% pRBC	18	18 hrs.	11.5 hs.	2.5 hrs.	
96-2	M	19 mo	anemia	None	9	DOA	5 hrs.	3 hrs.	
96-3	F	19 mo	anemia	3+	7	DOA	4 hrs.	2.25 hrs.	
96-4	M	20 mo	anemia	19% pRBC	8	5 hrs	12 hr. 20 min	3 hrs. 10 min	
96-5	M	14 mo	CM	3.5% pRBC	21	18 hrs.	2hr, 15 min	3 hrs. 50 min	
96-6	F	17 mo	CM & anemia	6% pRBC	15	DOA	4 hrs.	4 hrs.	
96-7	F	29 mo	anemia	4+	4	DOA	2 hrs.	2 hr. 15 min	
96-8	F	3 yrs. 5 mo	Coma unknown	0	30	8 hrs.	5 hrs	No data	HIV+
96-9	M	1yr. 4 mo	CM	635/mm3	23	23 hrs. 20 min	4 hrs. 30 min	No data	
96-10	F	5yrs. 6 mo	OP toxicity	330/mm3	37	4 hrs	14 hrs.	No data	
96-11	F	2yrs. 5 mo	CM, anemia, pneumonia	29.5% pRBC	12	27 hrs.	13 hrs	No data	*H. influenzae*
96-12	M	6 mo	anemia, septicemia	None	11	4 hrs.	12 hrs	No data	*S.enteriditis*
96-13	M	1yr. 10 mo	CM	31.3% pRBC	20	DOA	3 hrs. 51 min	No data	
96-14	F	9 mo	Anemia, septicemia	1+	7	6.5 hrs	14 hrs. 38 min	No data	
96-15	F	8 mo.	CM, anemia	11,300/mm3	10	10 hrs.	6 hrs. 10 min	No data	
96-16	F	4 yrs. 3 mo	CM	125,950/mm3	27	4 hrs. 30 min	3 hrs. 4 min	No data	
96-17	F	4 yrs. 3 mo	Reyes syndrome	none	36	9 hrs	14 hrs.	No data	
96-18	F	7 mo	Meningitis	none	27	14 hrs.	8 hrs. 35 min	No data	
96-19	M	2yr. 4 mo	Anemia	1+	7	4 hrs.	12 hrs.	No data	Viral
96-20	F	8 yrs.	COC	1+	49	22 hrs. 20 min	6 hrs. 29 min	1 hr. 53 min	hypertensive
97-21	F	2 yrs. 1 mo	CM	189/200 WBCs	29%	12 hrs.	7 hrs. 47 min	2 hrs. 35 min	

TABLE 3 - Continued.

Patient #	SEX	AGE	Clinial Diagnosis	Parasites on Admission	HCT (%)	Time of Death (hrs after adm)	Death to Post Mortem Interval	Post Mortem Duration	Misc.
97-22	M	18 mo.	CM, anemia	scant	16%	13.5 hrs.	2 hr. 40 min	3 hrs. 30 min	
98-23	F	2 yrs. 6 mo.	CM	719,846/mm3	30%	7 hrs.	14 hrs. 29 min	2 hrs. 50 min	
98-24	F	4 yrs.	Meningitis	0	30%	1 hr.			*S. pneumoniae*
98-25	F	3 yrs. 8 mo	CM	ND	29%	11 hrs. 20 min			
98-26	M	2 yrs. 6 mo	CM	442,705/mm3	23%	30 hrs.	6 hrs. 35 min.	2 hrs. 30 min.	coma score: 1
98-27	M	1yr. 8 mo.	CM	4+	22%				coma score: 0
99-28	F	5 yrs. 1 mo.	CM	424,000/mm3	17%	3 min.	3 hrs. 20 min	3 hrs. 40 min	Ascaris, intussuception
99-29	M	3 yrs. 7 mo	CM	3+	15%	12 hrs.	10 hrs. 35 min,	4 hrs. 15 min	coma score: 2
99-30	M	1 yr. 7 mo	Malaria, severe anemia	302,000/mm3	8%	2 hrs.			*Salmonella typimurium*
99-31	M	3yr. 3 mo	CM	234,000/mm3	32%	21.5 hrs	18 hrs. 20 min	3 hrs. 10 min	
99-40	F	2 yrs. 2 mo	Brain necrosis, HIV	2+	40%	3 days	5 hr. 10 min	4 hr. 5 min	Probable HIV, coma score 1
00-41	F	25 mo.	meningitis	0	15%	3 days	9 hrs. 15 min	3 hrs. 15 min	Salmonella typhimurium
00-42	F	3 yrs. 1 mo	CM	275,000/mm3	14%	4 hrs.	3 hrs. 5 min	hrs. 45 min co	Coma score: 0
00-43	M	4 yrs. 9 mo	CM			24 hrs.	4 hrs. 40 min	2 hrs.	Coma score:<2
00-44	M	2 yrs. 10 mo	COC	+	26%	2 hrs. 40 min	17 hrs. 10 min	3 hrs. 35 min	salicylate toxicity
00-45	M	29 mo	CM	+++	40%	23 hrs. 10 min	9 hrs.	2 hrs. 50 min.	
00-46	M	3 yrs	Severe anemia	0	8%	10 hrs.	10 hrs. 40 min	2 hrs. 38 min	
00-47	F	22 mo	CM	++++	30%	5 hrs. 45 min	4 hrs. 10 min	2 hrs. 25 min	
00-48	M	4 yrs.	CM	+	15%	20 hrs.	2 hrs. 5 min	3 hrs.	
00-49	M	17 mo	CM	+	22%	DOA	10 hrs. 15 min	2 hrs. 45 min	

M: male, F: female, hrs: hours, hct: hematocrit, DOA: death on admission, yrs: years, mo: months,

Laboratory Reagents, Supplies and Equipment

All supplies, reagents and chemicals were purchased from the Biochemistry Store at Michigan State University. Analyses were performed at the following laboratories: Clinical Center, Life Science and West Fee Hall of Michigan State University, or Pediatric Research Laboratory at Hurley Medical Center, Flint, MI.

The laboratory plastic supplies were disposable, sterile, RNase/DNase treated by the manufacturer. Glassware and re-usable supplies, i.e. medium-sized mortars, pestles, forceps, were washed in Liquinox (Alconox Inc., New York, NY) and bleached (10-20% Chlorox solution)(Chlorox Company, Oakland, CA) for ten minutes, oven dried for 30 minutes, and autoclaved on dry cycle for 20 minutes at 15 pounds pressure per square inch (psi). The centrifugations were performed in the microcentrifuge (Fisher Scientific, Model 235C, Springfield, NJ) at maximum speed of 10,000 revolutions per minute (rpm) (9680 Xg). Stock reagents were aliquoted into smaller quantities.

METHODS

IMMUNOHISTOCHEMICAL ANALYSES

The fundamental question addressed by this dissertation was whether, or not, mononuclear cells detected in the brain of cerebral malaria (CM) patients were activated macrophages and monocytes. To characterize the mononuclear phagocytes, the cells were immunophenotyped to assess their differentiation lineage and state of activation. Immunohistochemical (IHC) studies were performed on formalin-fixed, paraffin embedded tissue (PET) using monoclonal

antibodies with the specificity to study monocyte/macrophage differentiation, function, and structure.

The IHC methodology involves the specific recognition of an antigen fixed in a tissue section by an antibody labeled with specific indicator molecules for visualization (Elias 1990, Mackenzie 1992). There are two essential requirements of the IHC technique, (1) the use of antibodies with high sensitivity and specificity to the selected antigen; and (2) proper fixation and treatment of the tissue. This promotes optimal interaction between target molecules, antibodies and indicator reagents (Mackenzie 1992). To achieve the requisite level of sensitivity and specificity, monoclonal antibodies were employed for all IHC analyses.

Monoclonal antibodies, generated from a given clone, are immunochemically identical and react with a specific epitope on the antigen against which they are raised. Monoclonal antibodies were selected for their specificity and ability to characterize the leukocytes identified in the cerebral tissue, determine their state of activation, and detect the expression of tumor necrosis factor-alpha (TNF-α) in the tissue. The target tissue presented a challenge since formalin fixation and paraffin embedding often alters epitope recognition. The buffered formalin used to preserve the tissue crosslinks proteins and can disturb antigenicity. It can cause a conformational change of the epitope, chemical modification of the reactive amino acids in the antigen, cause steric hindrances. Moreover, it can extract the antigen itself, during the washing and embedding process (Mackenzie 1992).

In order for an antibody to detect a tissue antigen, the antigen must be

assessable to the antibody, remain *in situ* in the tissue (not washed way or dissolved out with reagents), maintain stability throughout the stringent conditions of the reaction, and be morphologically observable at the end of the analysis. The detection of an antigen in processed tissue can be highly affected by, (1) the quality of the target antigen in the tissue, (2) the type of fixative used to immobilize the antigen and preserve the tissue, (3) preparation of the tissue sections, (4) the permeability of reagents to enter the tissue and find the antigen and (5) the visualization of the antigen-antibody reaction (Mackenzie 1992).

Characterization of Mononuclear Phagocytes in Cerebral Tissue

In order to identify monocyte/macrophage, detect their state of activation, and cytokine secretion, monoclonal antibodies were employed that could react with formalin-fixed, paraffin embedded tissue (PET). Macrophage immunophenotyping was performed using MAC387 (Novocastra, Newcastle upon Tyne, UK), a broad spectrum macrophage marker and NCL-MACRO-3A5 (Novocastra) a macrophage specific marker. CD63 (PharMingen International, San Diego, CA) was used as a marker for cellular activation. Anti-TNF-α was employed for the detection of local protein production (Santa Cruz Biotechnology, Santa Cruz, CA). The Leica automated immunoanalyzer in the Histology department at MSU was used for MAC387, NCL-MACRO, 3A5 and CD63 immunophenotyping, following the manufacturers recommendations (Appendix B). The TNF-α was analyzed manually.

MAC387 - A Broad Spectrum Marker

MAC387 (Novocastra) is an IgG1, mouse, anti-human, IgG1 monoclonal antibody that phenotypically identifies neutrophils, monocytes, certain reactive macrophages, squamous mucosal epithelial cells, but is absent from lymphocytes. Granulocyte-Macrophage (GM-CSF) progenitor cells do not react with MAC387 suggesting that the cells become positive after divergence from their common committed stem cell (Goebeler et al 1994). MAC387 recognizes a calcium binding, myeloid-associated protein, known as calgranulin, leucocyte antigen L1, p6, calprotectin, cystic fibrosis antigen or calgranulin-related protein (CGRP). Calgranulin is a component of the 12 member S100 protein family located abundantly in the cytoplasm of granulocytes and macrophages (Goebeler et al. 1994, Wicki et al. 1996). The calgranulin gene is located on chromosome 1q21 and encoded from exons two and three (Wicki et al. 1996). Calgranulin undergoes a gross conformational change upon calcium binding, thus supporting the idea that this protein may be involved in calcium dependent signal transduction.

The MAC387 monoclonal antibody studies the antigenic markers of cells from the myeloid-macrophage lineage in formalin-fixed PET. However, it is not specific for macrophages, and will not detect their state of activation. MAC387 has been used to identify macrophage in ulcerative colitis (Waraich et al. 1997), medulloblastoma (Rossi et al.1991), non-neoplastic central nervous system disease (Esiri and Morris 1991), soft tissue sarcomas (Loftus 1991) and as antigenic markers of macrophages in atherosclerotic human arteries (Poston and Hussain 1993).

NCL-MACRO- 3A5, Macrophage Specific Marker

NCL-MACRO (Novocastra) is an mouse, antihuman, IgG2b, monoclonal antibody expressed in clone 3A5 developed to be specific for human macrophages in formalin-fixed PET. This monoclonal antibody was derived from a human spleen cell homogenate depleted of lymphocytes and erythrocytes immunized in the mouse. The cells were fused with the mouse myeloma cell line, Sp2/0-Ag14, to form a hybridoma (Jaspars et al. 1994, Novoscastra data sheet). NCL-MACRO-3A5 was selected because of its high specificity for detection of the monocyte/macrophage lineage. Unlike the other markers, NCL-MACRO did not react with myeloid cells, dendritic cells, lymphocytes or epithelial cells.

CD63 for Cellular Activation

CD is the abbreviation for "cluster of differentiation" which is an international nomenclature designed to clarify and organize monoclonal antibodies. The CD63 antisera (Pharmingen) is a mouse, anti-human, IgG1, kappa, monoclonal antibody expressed in clone H5C6 and purified in tissue culture. The expression of CD63 in cells is presumptive evidence of a state of cellular activity by the cells. The antibody reacts with a 53 kDa, type III lysosomal glycoprotein, expressed on activated platelets, monocytes and macrophages. This protein induces morphological changes in monocytes including cellular adhesion, extensive spreading on tissue culture dishes, proliferation, activation, and may play a role in antigen presentation (Koyama et al.1998, Woodhead et al. 1998). The lysosomal protein (CD63) activates neutrophils by altering their

ability to bind to integrin, CD11/CD18 (Skubitz et al. 1996). CD63 has also been referred to as lysosome integral membrane protein (LIMP), glycoprotein 55, melanoma-associated antigen ME491, pltgp40, LAMP-3 and is a member of the tetraspan transmembrane four superfamily (Israels et al. 2001).

Tumor Necrosis Factor-alpha (TNF-α) Immunohistochemical Staining

PET sections were cut into 5 micrometer (μM) sections by the Histology Department at Hurley Medical Center and incubated overnight at 37 degrees Centigrade (°C). The slides were de-paraffinized for two minutes each in three changes of xylene (J.T. Baker, Mallinckrodt Baker, Inc, Phillipsburg, NJ), hydrated twice in 100 percent (%) ethanol (J.T. Baker), once in 95% ethanol and rinsed in distilled water (dH_20), for two minutes each (Appendix C). For antigen retrieval, the slides were placed in plastic Coplin jars containing 50 milliliter (ml) Dako ® Target Retrieval Solution (Dako Corp., Carpinteria, CA) and steamed at 95°C for 25 minutes in a rice steamer (Black and Decker, Flavor Center Handy Steamer, Shelton, CA). The cooled slides were washed three times in dH_20 for five minutes each. Using the ImmunoCruz™ Staining System (Santa Cruz Biotechnology, Santa Cruz, CA), three drops (one drop equal to 40 μl) of peroxidase block was added to coat each slide to quench endogenous peroxidase. Slides were rinsed and then washed twice in phosphate buffered saline (PBS)(137 millimolar (mM) NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4) (Ambion Incorporated [Inc.], Austin, TX) for two minutes. To block nonspecific binding, they were incubated for 20 minutes with three drops of the Serum Block. Anti-TNF-α, the primary antibody, was an affinity-purified, mouse,

anti-human, IgG1, monoclonal antibody that corresponded to an amino acid sequence mapped at the amino terminus of the human TNF-α gene (Santa Cruz Biotechnology). Anti-TNF-α was diluted 1:50 in the serum block reagent. Three drops of antibody was added to the slides and incubated overnight at 4°C. The slides were rinsed, then washed twice for two minutes with PBS. The secondary antibody was a goat-anti-mouse biotinylated monoclonal antibody. Three drops were added to each slide, incubated for 30 minutes, and rinsed, then washed twice in PBS for two minutes. For detection, the slides were incubated for 30 minutes after adding three drops of the horseradish peroxidase (HRP)-streptavidin complex and washed twice in PBS for two minutes. The HRP substrate was prepared by adding five drops of 10X Buffer, one drop 50X hydrogen peroxide substrate, one drop DAB Chromogen, and 1.6 ml dH_20. Three drops of the HRP substrate was added to each slide and incubated for 10 minutes. The substrate was rinsed off with PBS and washed in dH_20 for two minutes. Gills Hematoxylin (Sigma Chemical, St. Louis, MO) was used for a 10 second counterstain and washed. The slides were dehydrated with 95% ethanol (J.T Baker) twice for 10 seconds, 100% ethanol (200 proof, dehydrate) (J.T. Baker) twice for 10 seconds, cleared three times through xylene (J.T. Baker) at 10 seconds each passage, mounted and coverslipped. Positive control tissue stained in parallel included a reactive lymph node (Hodgkin's lymphoma), a brain tissue (Alzheimer) control, and an internal patient control tissue (MP-5) used for each IHC run. The slides were scanned initially under 400X magnification and analyzed under 1000X. Between 800 -1000 cells were counted from which the

percent positive was calculated. When a slide contained few than 1000 cells, two or more slides were counted.

Detection of TNF-α mRNA in PET by *in situ* Hybridization - Overview

The *In situ* hybridization procedure was used to detect the TNF-α messenger RNA (mRNA) sequences in PET tissue sections. A labeled nucleic acid probe was prepared that hybridizes to complimentary nucleic acid sequences in the tissue. This procedure allowed for the visualization of the cellular source and location of the nucleic acid. A nonradioactive digoxigenin (DIG) labeled probe was generated from a 120 nucleotide sequence in exon one of the leader sequence of the TNF-α gene by the polymerase chain reaction (PCR) (Appendix D). PET was deparaffinized, hydrated and pre-treated to denature the tissue and remove some of the proteins making the TNF-α mRNA more accessible for hybridization. Slides were treated with proteinase K (Ambion Inc.) and hybridized with nonradioactive DIG labeled probes. The DIG labeled probes were detected after hybridization to target nucleic acids, by an anti-DIG-alkaline phosphatase antibody-conjugate (Roche Molecular Biochemicals). An enzyme-catalyzed color reaction with nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche Molecular Biochemicals) was used to visualize the hybrid molecules. The initial NBT/BCIP oxidation reaction was relatively slow and required incubation times up to three days. The procedure was modified with the addition of 10% polyvinyl alcohol to the NBT/BCIP (DeBlock and Debrouwer 1993). This detection modification enhanced the alkaline phosphatase reaction and prevented diffusion of reaction

intermediates, resulting in a twenty-fold increase in sensitivity, shorter incubation time, and decreased background.

Primer Preparation

The polymerase chain reaction (PCR) was used to prepare the DIG labeled probes of the leader sequence of TNF-α gene (Lion and Haas 1990). The TNF-α forward and reverse primers were synthesized at the Macromolecular Facility in the Biochemistry building at MSU. The nucleic acid sequence from the molecular cloning of TNF-α complementary DNA (cDNA) was published and downloaded from the GeneBank at the National Institute of Health (website: www.ncbi.nlm.nih.gov/entrez.cgi?val=339737, Wang et al. 1985, Nedwin et al. 1985). The sequences for the TNF-α primers are listed in Table 4. The primers were vacuum dried (Savant Instruments Inc. Speed Vac SC-100, Refrigerated Condensation Trap RT 100, Vacuum Gauge Model VG-5, and High Vacuum Pump VP 100, Farmingdale, NY) and reconstituted with 250 μl double distilled water (ddH_20).

Table 4. Sequence of TNF-α Forward and Reverse Primers

Forward primer sequence:	5' -cac acc ctg aca agc tgc cag gca g -3'
Reverse primer sequence:	5' -ctc ctc ggc cag ctc cac gtc ccg g -3'

TNF-α gene leader sequence, positions -100 to +10 bases relative to the start site of transcription in exon one. GeneBank at the National Institute of Health, www.ncbi.nlm.nih.gov/entrez.cgi?val=339737, Wang et al. 1985

An 260 nanometers (nm) optical density (OD) measurement was taken of primers diluted 1:200 (1 microliter (μl) primer in 199 μl ddH_20) using the GeneQuant RNA/DNA Calculator (Pharmacia, LKB Biochrom Limited, Science Park, Cambridge, England) (Appendix E). The primers were diluted to a stock concentration of 20 mM in ddH_20 using the calculated molecular weight (Table 5) from the GeneQuant reading. A 20 microMolar (μM) working solution was prepared from the stock solution. The diluted primers were stored at -20°C until amplification.

Table 5. Calculation of the Molecular Weight of the Primer

Nucleic acid	Formula
DNA oligonucletide	[(#dATP x 312.2) +(#dCTP x 288.2) +dGTP x 328.2) + (#dTTP x 303.2)] -61

To calculate the molecular weight of the primer, multiply the number of nucleotides in the primer by the molecular weight (the number in parenthesis) of each deoxynucleotide triphosphate (dNTP). Add all dNTPs and subtract -61 from the total (61 is the molecular weight of the terminal 5' phosphate and terminal 3' hydroxyl absent from the primer). # = the number of oligonucleotides in the primer.

Template DNA Extraction for Probe Generation

The target for probe preparation was human genomic DNA. Five ml of whole blood anticoagulated with acid citrate dextrose (Becton and Dickinson) was drawn for template deoxyribonucleic acid (DNA) extraction with the QIAamp® Blood Kit (QIAGEN LTD Incorporated, Chatsworth, CA). Four 200 μl aliquots were pipetted into 1.5 ml microfuge tubes to which 200 μl of cell lysis buffer, Buffer AL and 20 μl QIAGEN protease (17.86 mg/ml stock concentration)

were added. The tubes were vortexed (Deluxe Mixer, Scientific Product, Division of American Hospital Supply Corporation, McGraw Park, IL) for 15 seconds and incubated at 56°C for 10 minutes. After incubation, 200 μl of ethanol (200 proof, dehydrate) (Pharmco Products, Inc.) was added to each tube and vortexed (Scientific Products) for 15 seconds. The lysates were pipetted into QIAamp spin columns and centrifuged at maximum speed for one minute. As stated previously, all centrifugations were performed in the microcentrifuge (Fisher Scientific, Model 235C) at maximum speed of 10,000 rpm (9680 Xg). The collection tubes were discarded and the spin columns transferred to new, two ml collection tubes to which 500 μl Buffer AW1 was added. The tube/spin columns were centrifuged for one minute and collection tubes discarded. The spin columns were inserted into fresh two ml collection tubes and 500 μl of the final wash Buffer AW2 added, followed by a three minute centrifugation. The spin columns were inserted into 1.5 μl microfuge tubes and 100 μl ddH_20 added, which incubated for one minute and centrifuged for the same amount of time. The DNA was stored at -20° until analysis.

DNA Quantitation

DNA quantitation was based upon optical density (OD) measurements. An OD value of one at wavelength 260 nm corresponds to approximately 50 μg/μl of DNA (Sambrook and Russell 2001, Killeen 1997, Manchester 1995, 1996) A 1:200 dilution of the template DNA was prepared for nucleic acid quantitation (1.0 μl template DNA specimen added to 199 μl double distilled H_20). Using the GeneQuant RNA/DNA Calculator (Pharmacia) the following

readings were taken: OD readings at wavelengths, 260 and 280 nanometers (nm), protein determination, percent purity, and 260/280 absorbance ratios. The DNA concentration was calculated using the formula for double stranded DNA listed in Table 6 and the DNA stored at -20°C until amplification.

Table 6. Calculation of Nucleic Acid Concentration

Nucleic acid	Formula	Concentration
Double Stranded DNA (ds DNA)	$\frac{OD260 \times 50\ \mu g/ml \times dilution\ factor}{1000\ \mu l}$	X $\mu g/\mu l$ DNA

An optical density (OD) measurement of one at a wavelength of 260 nanometers (nm) corresponds to approximately 50 μg/ml of double stranded DNA. The X equals the value of DNA calculated from the formula in $\mu g/\mu l$.

Amplification of DIG Labeled TNF-α Probe by Polymerase Chain Reaction

The polymerase chain reaction (PCR) reaction volume was 100 μl. A master mix was prepared containing all PCR reagents except the template DNA (Lion and Haas 1990). The master mix included: 10X PCR Buffer (100 mM (hydroxymethyl)aminomethane hydrochloric acid (Tris-HCl), pH 8.3 [at 25°C], 500 mM potassium chloride (KCl), 15 mM magnesium chloride ($MgCl_2$), 0.1% weight/volume gelatin) (Perkin Elmer, Foster City, CA), 200 μM each of deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), 130 μM deoxythymine triphosphate (dTTP), (Perkin Elmer), 70 μM Digoxigenin-11-2'-deoxyuridine-5'-triphosphate, alkali stable (Roche Molecular Biochemicals, Mannheim Germany), 1.5 Units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin Elmer). The template DNA

was diluted to a concentration of 100 ng/μl and added to the master mixture. A negative control included all the above with the substitution of 200 μM dTTP and omission of Digoxigenin-11-2'-deoxyuridine-5'-triphosphate. The PCR reaction was carried out in the 9600 ThermalCycler (Perkin Elmer) using the cycling parameters shown in Table 7. The probe was labeled with the steroid hapten, digoxigenin which was amplified by PCR and electrophoresed.

Table 7. Cycling Parameters - TNF-α Digoxigenin Labeled Probe

# of Cycles	Step	Time (seconds)	Temp. (°C)
1	denature	120	93
33	denature	30	93
	anneal	40	55
	extension	40	72
1	denature	30	93
	anneal	40	55
	extension	420	72
	Hold	forever	4

Three repetitive incubations were performed for 35 cycles. Denaturation of dsDNA at 93°C. Primer annealing at the lower temperature of 55°C and extension of the ssDNA at 72°C.

Electrophoresis of Digoxigenin Labeled Nucleic Acids

DNA is a negatively charged molecule at neutral pH. When it is subjected to an electric current in an agarose gel, its migration rate is inversely proportional to the size of the molecule. Thus, the larger the DNA fragment, the slower its

rate of migration in the gel. The conformation is also a factor, in which linear molecules migrate faster, than folded molecules (Sambrook and Russell 2001). A 9 μl aliquot of the DIG labeled amplicon was mixed with 3 μl loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose [Sigma Chemical]) and pipetted into a 80 x 58 mm 2.5% agarose gel (DNA grade agarose), (Pel-Freez Clinical Systems LLC, Brown Deer, WI) diluted in 0.5X Tris:Borate:EDTA (TBE) buffer (0.9 M Tris [Sigma Chemical], 0.9 M Boric acid [J.T. Baker], 20 mM EDTA, pH 8.0 [Sigma Chemical]). An 5 μl aliquot of a molecular weight standard was pipetted to the top left well. The DNA Molecular Weight Marker VIII (Roche Diagnostics, Indianapolis, IN) was diluted 1:10 in loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose [Sigma Chemical]). Molecular Weight Marker VIII consisted of 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110, 67 base pair DNA fragments. The gel was immersed in the Hybaid Electro-4 (Life Technologies, Grand Island, NY) electrophoresis chamber containing approximately 200 ml 0.5X TBE buffer and oriented to allow the nucleic acids to migrate toward the positive electrode. A constant current of 100 volts (Model 200/2.0 power supply, Bio-Rad, Richmond, CA) was applied for 30 minutes until the bromophenol blue dye traveled two thirds the gel length.

Detection of Nucleic Acids

The gel was placed in a solution containing approximately 100-200 ml H_20 with a drop of 2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium bromide (ethidium bromide, 10 mg/ml) (Sigma Chemical) and mixed with gentle rotation

(Variable Rotator V, American Dade Division of American Hospital Supply Corporation, Miami, FL) for 5 -10 minutes. The gel was destained with tap water and visualized with placement onto the Chromato-Vue transilluminator Model 75-36 (UVP Inc, San Gabriel, CA). Nucleic acids were visualized at a wavelength of 254 nm. The gel was photographed with the Fotodyne FCR-10 camera (Fotodyne Inc. Hartland, WI) using type 667 black and white Polaroid ® film (Polaroid Corporation, Cambridge, MA) exposed for one second at *f*=8 and developed for 60 seconds. The DIG labeled probe was stored at -20°C until analysis. The entire PCR product was used as the DIG labeled probe for *in situ* hybridization without further purification.

In situ Hybridization Procedure

To eliminate contamination by exogenous RNases, preventive measures included using only RNase/DNase-free plastic, disposable supplies, treatment of glassware with 0.1% diethylpyrocarbonate (DEPC)(Sigma Chemical) and baking for 4 hours in a 300° oven (Sambrook and Russell 2001), preparation of reagents with DEPC treated H_20 (Ambion Inc.), decontamination of surfaces, supplies and equipment with 10% bleach (Chlorox, Chlorox Company) and RNaseZAP (Ambion Inc.). A new microtome blade was used by the Histology department for tissue sectioning. The blade was treated with RNaseZAP (Ambion Inc.) before and after each patient, and paraffin sections floated in DEPC treated water (Sigma Chemical).

To prevent detachment of the tissue sections from the glass slides, PET blocks were cut into 5 μM sections by the Histology Department at Hurley

Medical Center and placed on charged Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and incubated overnight at 37°C. The slides were deparaffinized by washing three times in xylene (J.T. Baker) for two minutes each and hydrated twice in 100% ethanol (J.T. Baker) for two minutes. Tissue sections were hydrated in descending grades of ethanol (100%, 95%, 70%, 50%) and 0.5X SSC (1X: 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) (Ambion Inc.) each for two minutes. The sections were was post fixed for 10 minutes in cold, 4% paraformaldehyde-phosphate buffered saline (PBS) (0.4 gm, paraformaldehyde, (Sigma Chemical), in 10 ml, PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4) (Ambion Inc.) and rinsed at room temperature in 0.5X SSC (Ambion Inc.) for 10 minutes. Each slide was permeablized with 100 μl Proteinase K (Ambion Inc.) solution (optimized concentration of 200 μg/ml) and incubated at 56°C for 30 minutes in a humid chamber containing approximately 100 ml Box Buffer (4xSSC, 50% formamide) (Ambion Inc.) in the bottom of the chamber. The slides were washed for five minutes in 0.5X SSC at room temperature and pre-hybridized to prevent background staining. The Pre-hybridization solution (50% formamide [Ambion Inc.], 5X SSC, [Ambion Inc.] 10% dextran sulfate [Sigma Chemical], 1X Denhardts [Sigma Chemical]) contained all the components of the hybridization solution except the probe. A 100 μl aliquot was added to each section and incubated at 56°C for one hour.

Sheared salmon sperm DNA (Ambion Inc.) was denatured by boiling for three minutes and cooled immediately in ice-water. The Hybridization solution (500 μl Pre-hybridization solution above, 5 μl Sheared Salmon Sperm DNA

[Ambion Inc.], 5 μl RNASE Inhibitor [Ambion Inc.], and 50 μl DIG labeled-Probe) was pipetted in 100 μl aliquots onto each section and mixed. The beta (β)-actin-DIG (Roche Molecular Biochemicals) control probe (100 μl Pre-hybridization solution, 5 μl denatured sheared salmon sperm DNA [Ambion Inc.], 5 μl RNASE Inhibitor [Ambion Inc.], 5 μl β-actin-DIG Probe [Roche]) was added in like manner. The slides were placed in a 80°C incubator for 10 minutes to enhance permeabilization and incubated overnight at 56°C.

Stringency Washes

Stringency washes were performed to remove nonspecific hybridization products that were partially but not entirely homologous to the probe. Such hybrids are less stable than perfectly matched hybridization and therefore, can be dissociated through washes of various stringencies.

The slides were washed with no agitation for one hour in 2X SSC (Ambion Inc), and one hour in 1X SSC (Ambion Inc) both at room temperature (RT), followed by a 30 minute wash in 0.5X SSC (Ambion Inc) at 37°C and 30 minutes 0.5X SSC wash at RT. The Washing Buffer (100 mM Tris-HCl, 150 mM NaCl, 0.3% polyoxyethylene sorbitan monolaurate [Tween 20][Sigma Chemical], pH 7.5) was added for five minutes. The sections were blocked in a Blocking Solution consisting of a 1:10 dilution of 10X Blocking Blocking Reagent (Roche Molecular Biochemicals) in Tris Buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5 [Ambion]) for 30 minutes.

Using the DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals), the Anti-Digoxigenin-Alkaline Phosphatase (AP) antibody (Roche Molecular

Biochemicals) was diluted 1:100 in Blocking Solution (50 μl Anti-DIG-AP in five ml Blocking Solution) and added to each tissue section and which were incubated overnight at 4°C. The slides were washed twice in Washing Buffer for 15 minutes each wash and equilibrated in Detection Buffer, (0.1M Tris-HCl, 0.1M NaCl, pH 9.5 [20°C]) for 20 minutes at RT.

Initially, 100 μl Color Substrate Solution (Detection Buffer, pH 9.5, and [18.75 mg/ml nitroblue tetrazolium and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 67% dimethyl formamide][NBT/BCIP](Roche Molecular Biochemicals) was added to each slide which reacted in the dark at RT up to three days. This Color Detection solution was modified with the addition of 10% polyvinyl alcohol (Sigma Chemical) and 1M magnesium chloride ($MgCl_2$) (Sigma Chemical) (DeBlock and Debrouwer 1993) to enhance detection. The sections reacted from four hours to overnight at RT. The color reaction was stopped by rinsing with sterile dH_20. Slides were counterstained for 10 seconds with Gills hematoxylin (Sigma Chemical), dehydrated twice in 95% ethanol (J.T. Baker) for 10 seconds, twice for 10 seconds in 100% ethanol (J.T. Baker) and three times for 10 seconds in xylene (J.T. Baker), dried and coverslipped.

Controls

Both positive and negative probe controls were used to verify the *in situ* hybridization methodology. The negative probe control also, served as an internal patient control. Each tissue was sectioned in quadruplicate, mounting two sections per slide depending of the architecture of the tissue, e.g. large tissue required four separate slides. One section received the DIG labeled

probe, while the other received the Pre-Hybridization solution with no probe (negative probe control). The positive control was a probe for the human β-actin mRNA, a molecule common to most cells. The human β-Actin-digoxigenin-(DIG)-labeled probe (Roche Molecular Biochemicals) was analyzed on control tissue and patient control tissue (MP-5). The patient control tissue was a cerebral tissue analyzed with each run serving as both a reagent and methodology internal control. There were three "tissue" controls run with each analysis including a reactive lymph node (Hodgkin lymphoma), a brain control (Alzheimer patient) and the internal MP-5 brain control mentioned above.

This method was chosen over Northern blot or *in situ* PCR because it preserved the morphology of the cerebral tissue and allowed the visualization of TNF-α mRNA *in situ*. *In situ* hybridization is similar to Northern blots and *in situ* PCR, however, the techniques differ in that the starting material for a Northern blot is a tissue digest, while the primary material for *in situ* hybridization and *in situ* PCR are the histological tissue sections (Wilcox 1993). With Northern blots, cellular relationships are lost and the mRNA concentration is averaged from all of the cells contained in the original sample. A few of the disadvantages of *in situ* hybridization are similar to those for *in situ* PCR. The technique is affected by the type of tissue fixative used, tissue adherence to slides, the stability of mRNA in paraffin embedded tissue, the length of fixation, permeabilization of the probe into the tissue, the type of probe (RNA, DNA or oligonucleotide) used, the type of labeling for the probe (radioactive vs non-isotopic), and the method of detection. *In situ* PCR methodology is problematic, the reasons includes: the target DNA

must be exposed destroying tissue morphology, the optimal concentrations of essential reagents such as primers, magnesium and DNA polymerase must be empirically determined, reactions carried out directly on glass slides are subject to the amplified nucleic acid floating away during stringency washes, as well as, the lack of tissue adherence to slides and tissue drying out (Nuovo 1997).

CYTOKINE POLYMORPHISM GENOTYPING

To detect allelic polymorphisms in the genes of pro and anti-inflammatory cytokines, genotyping was performed using sequence specific oligonucleotide primers (SSP) amplified by the polymerase chain reaction (PCR). DNA was extracted from frozen and PET from the parietal lobe of the cerebral cortex of fatal malaria patients and controls.

DNA Extraction of Frozen Cerebral Tissue

Cerebral tissue sections greater than (>) 10 square millimeters (mm^2) were placed into a mortar containing 100-200 milliliters (ml) liquid nitrogen (Aga Gas, Flint, MI). Using a cold pestle, the tissue was ground to a fine powder (Sambrook and Russell 2001) (Appendix F). Tissue specimens less than (<) 10 mm^2 were allowed to thaw and placed into a 100 mm petri dish and minced into small pieces. Both powdery tissue or minced fragments were scooped into 1.5 ml microfuge tubes containing 600 μl freshly prepared Lysis Buffer, pH 8.0: 50 mM Tris (hydroxymethyl)aminomethane hydrochloric acid (Tris-HCl), pH 7.2 (Sigma Chemical), 50 mM ethylenediamine tetraacetate, disodium salt (EDTA), pH 8.0 (Sigma Chemical), 3% sodium dodecyl sulfate (SDS) (Ambion Inc.), 1%

2-mercaptoethanol (Sigma Chemical), 12 μl Proteinase K (200 μg /ml) (Ambion Inc.) and filtered, nuclease free, dH_20 (Ambion Inc.). The mixture was sheared using a 20 gauge, one and one forth (1¼) inch needle fitted to a three ml syringe (Becton, Dickinson and Company, Parsippany, NJ). The microfuge tubes were incubated overnight at 55°C. The DNA was extracted by adding 600 μl (i.e., an equal volume) Tris-saturated Phenol:Chloroform:Isoamyl alcohol (25:24:1) (Ambion Inc.) and mixing for five minutes on the rotator mixer (Adams and Hamilton Nutator, Clay Adams, Becton, Dickinson and Company). The tubes were centrifuged for 15 minutes and 300 μl of the aqueous phase (upper phase) was very carefully pipetted into a new microfuge tube. The organic/protein interface was "back-extracted" in order to achieve maximum DNA recovery. Back extraction involved adding an equal volume of Lysis Buffer to the organic/interface solution, mixing, and centrifuging for 15 minutes. The aqueous phases from the back extractions were pipetted into fresh microfuge tubes in 300 μl aliquots. To precipitate the nucleic acids, 10 μl of 3M sodium acetate (Sigma Chemical), 6 μl mussel glycogen (5 mg/ml)(Ambion Inc.) and 600 μl ice-cold (-20°) 100% ethanol, i.e., two volumes (200 proof, dehydrate)(Pharmco Products, Inc., Brookfield, CT) were pipetted into the aqueous layer, mixed and incubated overnight at -20°C. Nucleic acids were pelleted by centrifugation for two minutes, and the supernatant decanted. The DNA was washed with 600 μl ice cold 70% ethanol (Pharmco Products, Inc.) and centrifuged for two minutes. The supernatant was decanted and the tubes air dried in a desiccator for about one hour. Nucleic acids were resuspended in 50 μl sterile, dH_20 (Ambion Inc.) and stored at -20°C until analysis (Sambrook and Russell 2001).

DNA Extraction from Formalin Fixed Paraffin Embedded Tissue (PET)

Frozen tissue was not available for six of the malaria project patients and PET was employed for DNA extraction. The formalin fixed PET was cut into ten micrometer (μm) PET sections and placed into 1.5 ml microfuge tubes by the Histology department at MSU. Each tissue was cut in triplicate. To minimize cross contamination, the microtome blade was cleaned with 10% bleach (Chlorox, Chlorox Company) between specimens. The tubes were stored at room temperature until use. The arduous, formidable task of extracting nucleic acids from PET resulted in the evaluation of five different PET protocols (Table 8). As a result, the number of triplicate tissue sections multiplied accordingly. The methodologies differed by deparaffinization reagents, extraction chemicals and chelation substances.

TABLE 8. Protocols for DNA Extraction of Paraffin Embedded Tissue

Protocols	Deparaffinization	Extraction Techniques	References
I	Xylene	Phenol:Chloroform:Isoamyl alcohol.	Frank et al. 1996 Wright and Manos, 1990
II	Heat	Salt extraction	Puregene Gentra Systems, Minneapolis, MN
III	Xylene	Chelex treatment Phenol:Chloroform:Isoamyl alcohol.	Baisse et al. 2000
IV	Heat	Salt extraction - QIAamp	QIAGEN Inc. Chatsworth, CA
V	Heat	Proteinase K for 5-7 days, Chelex treatment Phenol:Chloroform:Isoamyl alcohol.	Diaz-Cano and Brady 1997

Protocols for the extraction of DNA from PET differed by methods for deparaffinization and nucleic acid extraction techniques, i.e. organic vs salt

Protocol I: PET were dewaxed with 500 μl xylene (J.T. Baker) for five minutes, centrifuged for one minute and decanted (Wright and Manos 1990, Frank et al. 1996) (Appendix G). Xylene was removed with 500 μl 100% ethanol (Pharmco Products, Inc.), mixed for five minutes and centrifuged for two minutes. The tissue was allowed to air dry at room temperature. The protocol as described above for frozen tissue was followed, i.e. a Proteinase K (Ambion, Inc.) overnight digestion, extraction with Phenol:Chloroform:Isoamyl alcohol (Ambion Inc.) and precipitation with ethanol (Pharmco Products, Inc.). This procedure deviated from referenced procedures with the volume of ethanol used (500 μl instead of 1.0 ml for Wright and Manos 1990, or 100 μl for Frank et al. 1996) and the method of drying the nucleic acids (Wright and Manos dried under vacuum, while Frank et al. 1996 dried at 55°C).

Protocol II: The Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) was used omitting the xylene deparaffinization step (per telephone conversation with company). The manufacturer's protocol is outlined in Appendix H.

Protocol III: Seven μM PET sections were placed on glass slides which were dewaxed with a few drops of xylene (Sigma Chemical) until the paraffin dissolved (Baisse et al. 2000) (Appendix I). The slides were rinsed with methanol (Sigma Chemical) and rehydrated with a few drops of Tris-EDTA (TE) Buffer (1M Tris-HCl, pH 7.6, 0.5 M EDTA, pH 8.0, [Sigma Chemical]). The tissue was scraped off the slides into 1.5 microfuge tubes containing 27 μl TE Buffer, 3 μl Digestion Buffer (250 mM KCl, 150 mM Tris-HCl, pH 8.0, 1.5 mM

$MgCl_2$ (Sigma Chemical), 1.5% Tween-20 (Bio Rad, Richmond, CA), and 0.5 mg/ml Proteinase K (Ambion Inc.). The tubes were incubated overnight at 55°C. The lysates were boiled in an equal volume of 10% Chelex-100® (100-200 mesh, sodium free)(BioRad, Hercules, CA) followed by Phenol:Chloroform:Isoamyl alcohol extraction (Ambion Inc.). The extract was co-precipitated with ice cold (-20°) ethanol (200 proof, dehydrate) (Pharmco Products, Inc.) and 10 μg mussel glycogen (5 mg/ml)(Ambion Inc.) overnight at -20°C and re-suspended in sterile distilled water.

Protocol IV: This protocol used the QIAamp ® DNA Mini Kit (QIAGEN). The PET was deparaffinized in the ATL buffer during the 55°C incubation and DNA purified with QIAamp spin columns according to the manufacturer's protocol as outlined in Appendix J.

Protocol V: This protocol was a modification of a procedure described by Diaz-Cano and Brady (1997) (Appendix K). Ten μM PET sections were placed into 1.5 ml microcentrifuge tubes containing 600 μl Proteinase K Buffer (100 mM Tris-HCl, pH 7.6 (Sigma Chemical), 0.5% SDS (Ambion Inc.), 1 mM calcium chloride ($CaCl_2$) (Sigma Chemical), 100 μg/ml mussel glycogen (5 mg/ml, Ambion Inc.), and 12 μl Proteinase K (20 mg/ml)(Ambion Inc.). The PET were not deparaffinized prior to buffer digestion, since the paraffin melted (52 - 60°C) during the 55°C incubation releasing the tissue. This differed from the reference that deparaffinized with xylene. The tubes were incubated at 55°C and digested for 5-7 days with daily replacement of 12 μl Proteinase K (Ambion Inc.). After digestion, the lysate was boiled in 10% Tris-buffered Chelex-100 (BioRad), 100 mM Tris-HCl, pH 8.0 (Sigma Chemical).

DNA was extracted twice with the addition of 600 μl Tris buffered Phenol:Chloroform:Isoamyl alcohol (25:24:1)(Ambion Inc.), vortexed vigorously for 15 seconds, mixed for 10 minutes, and centrifuged for five minutes. Care was taken not to aspirate the Chelex beads when removing the aqueous phase from the digest. A "back-extraction" was performed after adjusting the volume to 600 μl with 3M sodium acetate. Diaz-Cano and Brady (1997) used 2.5 M ammonium acetate. The back extraction tubes were vortexed vigorously for 15 seconds, mixed for 10 minutes, and centrifuged for five minutes. The aqueous phases were pooled for each patient and 600 μl aliquots pipetted into fresh microfuge tubes. A second Phenol:Chloroform:Isoamyl alcohol extraction was performed on the aqueous phase as described above. The reference procedure performed an additional chloroform extraction which was omitted in this investigation. The nucleic acids were precipitated with two volumes of ice-cold 100% ethanol (200 proof, dehydrate)(Pharmco Products, Inc.) and incubated at -20°C overnight. The tubes were centrifuged for 15 minutes and washed with ice-cold, 70% ethanol (200 proof, dehydrate)(Pharmco Products, Inc.) and centrifuged for five minutes. The ethanol was decanted and tubes dried in a desiccator for one to two hours. The DNA was resuspended in TE buffer (100 mM Tris-HCl, pH 7.2, 1 mM EDTA, pH 8, [Sigma Chemical]) and stored at -20°C until analysis.

DNA Quantitation

A 1:200 dilution was prepared for nucleic acid quantitation, i.e., 1.0 μl nucleic acid specimen added to 199 μl double distilled H_2O. Using the

GeneQuant RNA/DNA Calculator (Pharmacia), OD readings at wavelengths 260 and 280, protein (mg/ml) determination, percent (%) purity, and 260/280 absorbance ratios were taken. The DNA concentration was calculated using the formula for dsDNA listed in Table 6. DNA extracted from PET could not be accurately analyzed spectrophotometrically due to interference by Chelex-100.

Cytokine Genotype Analysis

Using the DNA concentration calculated from GeneQuant measurements, the DNA was diluted in dd H_20 to achieve a standard nucleic acid concentration of 100 nanogram (ng)/μl. The appropriate number of Cytokine Genotyping Primer Set trays (pre-optimized oligonucleotide primers dried to a 96-well microtiter tray), (One Lambda Inc. Canoga Park, CA) and D-mix tubes (dATP, dCTP, dGTP, dTTP, cresol red, sodium salt, sucrose, gelatin, potassium chloride, magnesium chloride, hexahydrate, and Tris-HCl, pH 8.0 (One Lambda Inc.) were removed from the freezer and allowed to thaw at room temperature. The actual primer sequences for each cytokine were not published by the manufacturer, however, the primer specificity, nucleotide substitution, product size, and recognition sites are listed on Table 9 (One Lambda Inc.).

Table 9. Cytokine PCR Primers

CYTOKINE	PRIMER SPECIFICITY	SUBSTITUTION	SIZE (bp)	5' Recognition	3' Recognition
TNF-α	promoter	-G308A	125 125	-308	-200
IFN-γ	Intron 1 Intron 1	+A874T	250	+874 +874	+1100 +1100
IL-6	promoter	-G174C	175	-174 -174	-10 -10
TGF-β	codon 10 codon 10 codon 25 codon 25	+869T/T +869 C/C +915C/C +915 G/G	200	-150 -250 +74 +74	+29 +29 +250 +250
IL-10	promoter promoter promoter promoter promoter	-1082A, -819G -1082G,-819C -1082A, -819C - 819T, -592A -819C, -592C	300 300 300 250 250	-1082 -1082 -1082 - 819 - 819	-819 -819 -819 -592 -592

Cytokine primers for the PCR were dried to the wells of the 96 well microtiter tray. The specificity of each primer and the punitive size of the amplified product on gel electrophoresis are represented. One negative control well along with positive internal controls are incorporated in each microtiter tube. The primer sequences are the proprietary property of One Lambda Inc. (Canoga Park, CA). bp: base pair.

Each microtiter tray contains one negative control well consisting of the D-mix and Taq polymerase. Internal control primer pairs are included in every PCR reaction which amplifies a conserved region of the human β-globin gene (a gene present in all DNA samples) and used to verify the integrity of the PCR reaction (One Lambda, Inc.).

To the negative control tube at the top left microtiter well, 1 μl of sterile filtered ddH_20 was pipetted onto the side of the well. An 1 μl aliquot of *Taq* DNA polymerase (5 units/μl, Perkin Elmer) was pipetted into the D-mix tube, mixed

and pulse centrifuged. A 9 μl aliquot of D-mix:*Taq* mixture was added to the dH_20 in the negative control well. The diluted DNA specimen, 19 μl, was pipetted into the D-mix:*Taq* tube, mixed and 10 μl dispensed into each well of the microtiter tray. The tray was covered with the tray seal provided and taken for PCR amplification.

Theory and Application of PCR-SSP

The polymerase chain reaction (PCR) is an enzyme mediated, temperature sensitive procedure for the amplification of nucleic acids. Initially described in 1985, PCR amplification of nucleic acids is based on the reiteration of three incubation steps (denaturation, annealing and extension) performed at different temperatures. Nucleic acid amplification is mediated by a DNA polymerase enzyme generated from the thermostable bacterium *Thermus aquaticus* (Taq) which can withstand repeated heating without becoming inactivated (Saiki et al. 1988, Sambrook and Russell 2001). The cytokine genotyping PCR uses sequence-specific oligonucleotide primers (SSP). In this reaction, nucleic acid synthesis is crucially dependent upon correct base-pairing at the three prime (3') end. The PCR-SSP cytokine genotyping primers are designed to differ at the nucleotide that occurs at the extreme 3' terminus. Therefore, to detect allelic polymorphisms, the primers are prepared so that the nucleotide substitution is at the 3' terminus. Amplification of the target sequence is hindered if the 3' terminus of the primer does not match the target completely (Stachan and Read 1999).

The PCR was performed using the GeneAmp PCR System 9600

ThermalCycler (Perkin-Elmer, Norwalk, CT). The volume used in the PCR was 10 μl. Cytokine gene amplifications consisted of 30 cycles under the conditions of the One Lambda manufacturer (Table 10). The first incubation involved the denaturation of double stranded DNA (dsDNA) at 96°C for 130 seconds for one cycle. The SSP annealed to complementary regions of the single stranded DNA (ssDNA) at a lower temperature, 63°C, for 60 seconds. The ssDNA was extended by Taq polymerase in a 5' to 3' direction using the primers as the initiation site. There were nine cycles at 96°C for 10 seconds and 63°C for one minute. Twenty cycles followed at 96°C for 10 seconds, 59°C for 50 seconds, 72°C for 30 seconds. The tray was ramped to 4°C and held.

Table 10
PCR Amplification Conditions

# of Cycles	Step	Time (seconds)	Temp. (°C)
1	denature anneal/extend	130 60	96 63
9	denature anneal/extension	10 60	96 63
20	denature anneal extension	10 50 30	96 59 72
	Hold	forever	4

Repetitive PCR incubations were performed for 30 cycles. Denaturation of dsDNA at 96°C. Primer annealing at the lower temperature of 63°C for nine cycles and at 59°C for 20 cycles. The extension of the ssDNA was performed at 63°C for nine cycles and at 72°C for 20 cycles.

For PET, the amplification cycles were increased to 40 cycles (Table 11). The initial cycle was at 96°C for 130 seconds and 63°C for 60 seconds, followed by nine cycles at 96°C for 10 seconds and 63°C for one minute. Thirty cycles followed at 96°C for 10 seconds, 59°C for 50 seconds, 72°C for 30 seconds, which ramped to 4°C and held.

Table 11
PCR Amplification Conditions for Paraffin Embedded Tissue

# of Cycles	Step	Time (seconds)	Temp. (°C)
1	denature anneal/extend	130 60	96 63
9	denature anneal/extension	10 60	96 63
30	denature anneal extension	10 50 30	96 59 72
	Hold	forever	4

PET was amplified for 40 cycles. Denaturation of dsDNA at 96°C. Primer annealing at the lower temperature of 63°C for ten cycles and at 59°C for 30 cycles. The extension of the ssDNA was performed at 63°C for ten cycles and at 72°C for 30 cycles.

Electrophoresis

The PCR amplicons were electrophoresed using the Hybaid Electro-4 horizontal electrophoresis chamber (Life Technologies) and the Model 200/2.0 power supply (Bio-Rad). A 2.5% agarose gel (DNA grade agarose), (Pel-Freez

Clinical Systems LLC) diluted in 0.5X TBE buffer (0.9 M Tris, [Sigma Chemical], 0.9 M Boric acid [J.T. Baker], 20 mM EDTA pH 8.0, [Sigma Chemical]) was prepared using a 80 x 120 mm gel casting tray for three primer trays, or 80 x 58 mm tray for a single patient primer tray.

The entire PCR amplification product, approximately 10 μl from each microtiter well, was applied to the gel in succession. The loading (creosol red) dye was incorporated in the D-mix tube. A 5 μl aliquot of a molecular weight standard was pipetted to the top left well. The DNA Molecular Weight Marker VIII (Roche Diagnostics) was diluted 1:10 in loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose [Sigma Chemical]). The Molecular Weight Marker was comprised of DNA fragments: 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110, 67 base pairs. The gels were oriented to allow the nucleic acids to migrate toward the positive electrode. A constant current of 100 volts was applied for 25 minutes until the bromophenol blue dye traveled two thirds of the length of the gel.

Detection of Nucleic Acids

The gel was placed in a solution containing approximately 100-200 ml H_20 with a drop of ethidium bromide (10 mg/ml) (Sigma Chemical) and mixed with gentle agitation (Variable Rotator V) for 5 -10 minutes. The gel was destained with tap water and visualized with placement onto the Chromato-Vue transilluminator Model 75-36 (UVP, San Gabriel, CA). Nucleic acids were visualized at a wavelength of 254 nm. Each gel was photographed with the Fotodyne FCR-10 camera (Fotodyne Inc.) using type 667 black and white

Polaroid ® film (Polaroid Corp.) exposed for one second at *f*=8 and developed for 60 seconds. The photos were labeled with the specimen number, date, percent agarose, voltage and length of electrophoresis, i.e. 2.5% agarose, 100 volts/25 minutes.

Interpretation of Results

The amplification of sequence specific oligonucleotide primers by the polymerase chain reaction resulted in the presence or absence of a specific amplified DNA fragment. Unincorporated primer bands, the smallest molecules, migrated the fastest in the agarose gel. If the sequence specific primer was able to anneal to the target DNA, there would have been amplification of the target sequence. The size of the amplified DNA was larger than the primers, but smaller than the control, human globin product. A band was visible in the agar in between the unincorporated primers and the internal control. Thus, a positive reaction for a specific cytokine allele was visualized on the gel as an amplified DNA fragment between the internal control product band and the unincorporated primer band.

The first well in the microtiter tray was the negative control, containing D-mix, Taq polymerase but devoid of DNA and there were no amplification products observed in this well. The presence of a band would indicate DNA contamination, e.g. from a neighboring tube, or contaminated reagents. Each reaction tube also, contains a primer pair for a conserved region of the human beta globin gene, which serves as a positive internal control. Since the beta

globin gene is present in all DNA samples, its amplification verifies the integrity of the PCR reaction. A failure to amplify the internal control could result from pipetting errors, poor DNA quality, an insufficient quality of DNA, presence of inhibitors, etc. The appearance of a weak or absent internal control band when a positive cytokine band is present may be due to the difference in concentration and melting temperatures between the specific primer pairs and the internal control primer pair (One Lambda).

Statistical Analysis

Data compilation was accomplished on Excel spreadsheet computer program. Statistical analyses were achieved by the GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com computer program, in consultation with Dr. Gregory Fink, Department of Pharmacology Toxicology, MSU.

Immunohistochemical staining results generated data values for each stain. Individual data points were entered into the statistical program and analyzed by Kruskal-Wallis, non parametric, analysis of variance (ANOVA) using GraphPad InStat software. Kruskal-Wallis is a nonparametric test that compares three or more unpaired groups. The p value answers this question: "if the null hypothesis is true then what is the chance of obtaining a Kruskal-Wallis statistic as high as observed in this experiment (www.graphpad.com). ANOVA measures differences within and between groups based on the number of factors evaluated. When only two groups of non parametric data was analyzed (e.g. the

level of parasitemia versus degree of activation), linear regression was used to correlate the two independent variables.

The cytokine polymorphisms between TNF-α and anti-inflammatory cytokines were analyzed with Friedman's repeated measures ANOVA. With repeated measures ANOVA, there are three sources of variability: between columns (treatments), between rows (individuals) and random (residual). The repeated measures ANOVA controls for factors that cause variability between subjects and is more powerful because it separates between-subject variability from within-subject variability (GraphPad InStat).

Categorical data from cytokine polymorphism studies were also analyzed by Kruskal-Wallis, ANOVA. Chi-square analysis was performed on a subgroups of categorical data, which used a two by two contingency table to compare the means of two variables. This differed from the ANOVA which compares medians of multiple (three or more) factors and variables. Statistical significance was defined with a "p" value of ≤ 0.05.

Chapter 4

RESULTS

Chapter 4

IMMUNOHISTOCHEMICAL STAINING RESULTS

A critical study objective of this dissertation was to characterize the mononuclear phagocytes detected in cerebral tissue and determine their state of activation. This objective was accomplished by immunophenotyping cerebral tissue with macrophage specific markers and an activation marker. Two monoclonal antibodies were used to characterize the mononuclear phagocytes in paraffin embedded cerebral tissue, MAC387 (Novocastra, Newcastle upon Tyne, UK), a broad spectrum macrophage marker and NCL-MACRO-3A5(Novocastra), a macrophage specific marker. To detect cellular activation, the monoclonal antibody CD63 was used (Pharmingen International, San Diego, CA).

Differential Cell Counting and Enumeration of Immunohistochemical Stains

Immunohistochemical stains were performed for a total of 35 patients and controls. Initial focusing and scanning were performed at 400X magnification by brightfield light microscopy (Microstar IV, American Optical, Scientific Instrument Division, Buffalo, NY) to identify vessels and observe cellularity. The microscopic differential examination was performed within large to medium-sized cerebral blood vessels (small vessels occasionally) in several successive fields under 1000X magnification. The erythrocytes appeared as faint pinkish or clear refractory enucleate cells. The nuclei of the leukocytes appeared blue to blue-black depending of the class-specific coarseness of the nuclear chromatin, with light gray-blue to faint gray cytoplasm. Polymorphonuclear and band neutrophils

were identified by their segmented or sausage shaped nuclei and light gray cytoplasm. Lymphocytes were classified by their increased nuclear to cytoplasmic ratio, coarse nuclear chromatin and scant cytoplasm. Monocytes and macrophages appeared as large cells with irregularly shaped nuclei with "spongy" chromatin - parachromatin and abundant cytoplasm. The macrophages is the larger of the two cell types. Endothelial cells lined the cerebral blood vessels and were identified by flatten nuclei with hypopigmented chromatin and abundant cytoplasm that formed the tight vascular junctions.

The vessels were analyzed for the presence or absence of positive staining cells at 1000X oil immersion. Every leukocyte was enumerated and categorized as either a positive or negative staining cell using a five key Laboratory Counter (Clay Adams, Division of Becton, Dickinson and company, Parisippany, NJ). Between 800 to 1000 total leukocytes were counted and the percentage of positive cells calculated (Table 12).

Table 12

Calculation of Percent Positive Cells for Immunohistochemical Stains
$\frac{\text{\# Positive cells counted}}{\text{total number of cells counted}}$ X 100 = percent (%) positive

The percent positive cells was calculated by counting the all cells and designating them as either positive or negative cell based on the presence of red-reddish brown granulation. The number of positive staining cells was divided by the total number of cells counted and multiplied by 100.
#: the number of positive cells counted in 1000 cells

A positive staining result was reported when the cell showed various intensities of red to red-brown granulation in the cytoplasm. A negative result was reported for cells that stained blue with no red pigmentation (or very faint granulation) in the cytoplasm. Endothelial cells were identified as large cells with flattened nuclei that lined the cerebral vessels. Endothelial cells (or erythrocytes) were not counted during the assessment of positive and negative staining cells. When a slide contained fewer than 1000 cells, two or more slides were counted and the percent positive calculated. Microscopic photography was performed on the Nikon Optiphot Biological microscope with Nikon camera (Nippon Kogaka K.K, Chiyoda-Ku, Tokyo, Japan) using Kodak Porta 160 color negative 135/36 film (Eastman Kodak Company, Rochester, NY). The data from macrophage immunophenotyping is shown in Table 13.

Table 13. Macrophage Immunophenotyping Results

	MAC387	MACRO-3A5	CD63
CM	**35%** (2 - 66)	14.5% (1.1 - 43.0)	56.6% (28.5 - 82.0)
NCM	**23.8%** (8.5 - 42.5)	15.5% (2.5 - 30.2)	43.1% (23 - 67)
COC	**20.9%** (0 - 46.5)	16.7% (0 - 42.0)	46.8% (32.7 -67.5)
	p= 0.06	p = 0.93	p= 0.076

The table shows the median percent positive cells that reacted with macrophage monoclonal antibodies. The range from lowest to highest percent positive detected is shown in parenthesis. The groups were not statistically significant by Kruskal-Wallis, nonparametric analysis of variance (ANOVA) using the GraphPad InStat software.

Characterization of Mononuclear Phagocytes in Paraffin Embedded Cerebral Tissue using MAC387

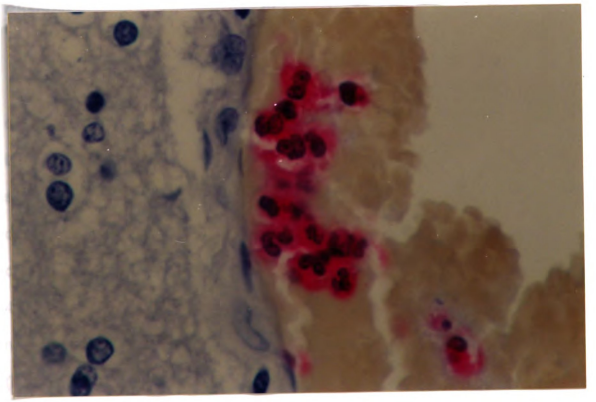
The MAC387 monoclonal antibody was used in an immunohistochemical staining reaction and the antigen-antibody bond was visualized by a labeled secondary antibody (biotinylated horse radish peroxidase). MAC387 positive cells included neutrophils, monocytes and certain reactive macrophages. The cells demonstrated brilliant brick-red (Vector Nova Red, Vector Laboratories, Burlingame, CA) intensely staining granulation uniformly dispersed throughout the cell. The endothelial cells and cerebral cells failed to demonstrate MAC387 positive granulation. Erythrocytes also gave a negative staining reaction (1000X) (Figures 1 and 2).

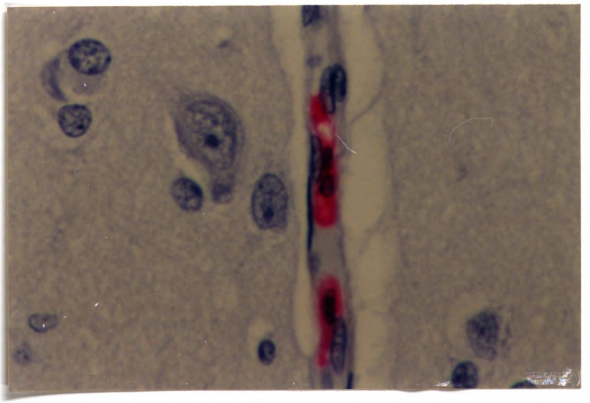
The average number of MAC387 positive cells was 35.0% in CM patients and the values ranged from 2 - 66%. In NCM patients, the mean was 23.8% positive cells, ranging from 8.5 to 42.5%, while the mean value for COC patients was 21.1% MAC387 positive cells, ranging from zero to 46.5%. Although the number of positive cells was greater in the CM patient group, this was not quite statistically significant, $p=0.0641$, by ANOVA. An outlier in both the COC and CM patient groups may have skewed the results of these groups. One patient (00-41) in the COC group had a much higher number of positive cells (46.5%) compared with other COC patients whose values were much lower, while a CM patient (MP15) showed a much lower number (2%) of positive cells compared with other CM patients. The COC patient was diagnosed with *Salmonella typhimurium* meningitis and bacteremia with 1200 white blood cells per cubic millimeter in the cerebral spinal fluid. The Outlier test (GraphPad InStat,

www.graphpad.com/calculators/Grubbs.cfm) failed to alter the statistical data. The Outlier test, also called the ESD method (extreme studentized deviate), was performed to determine whether one of the values was a signficant outlier from the rest. Although the aforementioned data points were furthest from the rest, they were not considered significant outliers when the Grubbs' test was performed.

Figure 1. MAC387 staining of PET. This photo shows MAC387 positive neutrophils, monocytes and reactive macrophages in a large cerebral vessel containing many red blood cells. MAC387 positive cells show brillant red granulation. The colored granules are not present in the RBC (1000X)

Figure 2. MAC387 stained cells in small vessel. A MAC387 postive macrophage in a small cerebral blood vessel. Positive cells show intense red granulation in cytoplasm and nuclei (1000X).





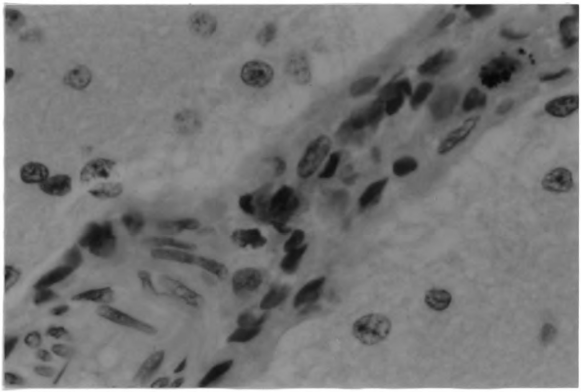
Characterization of Mononuclear Phagocytes in Paraffin Embedded Cerebral Tissue using NCL-MACRO-3A5

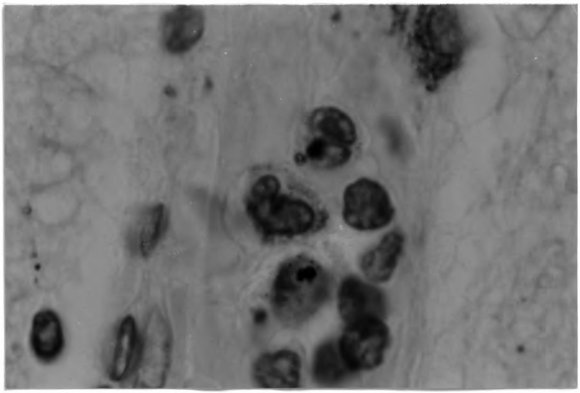
NCL-MACRO-3A5 positive cells showed reddish-brown granulation in the cytoplasm of macrophages within cerebral blood vessels (Figures 3 and 4) . Lymphocytes, neutrophils, and endothelial cells failed to produce positive staining reactions with the antibody. Their nuclei appear blue with grayish-blue cytoplasms. Golden-yellow to brown-black malaria pigmentation, hemozoin, was observed in phagocytes that had engulfed parasite infected RBCs (Figures 3 and 4). Erythrocytes appeared as clear opalescent bodies in the cerebral microvessel and failed to show a positive staining reaction with this antibody (Figure 4)(1000X).

The macrophage specific marker, NCL-MACRO-3A5, showed that there were slightly more macrophages detected in the cerebral tissue of the COC (16.7%) group compared with CM (14.5%) and NCM (15.5%) (Table 12). However, the variation between the groups was not statistically significant, p=0.93, ANOVA. There was a wide range of values observed within each study group. The positive staining values in the CM group ranged from 1.1% to 43% positive cells, while NCM values ranged from 2.5 to 30.2%. The NCL-MACRO-3A5 positive cells in COC control group were between zero and 29% with one outlier value at 42% (00-41). Although this value was furthest from the rest, it was not considered a significant outlier when the Grubbs' test was performed (GraphPad InStat, www.graphpad.com).

Figure 3. MACRO-3A5 positive macrophages in a cerebral blood vessel. MACRO-3A5 positive macrophages shows red-brown granules in the cytoplasm (400X). Yellow-black malaria pigment hemozoin is observed in the cytoplasm of a macrophage.

Figure 4. MACRO-3A5 immunostained macrophages in a small cerebral blood vessel. The macrophage cytoplasm demonstrated red-brown granulation with MACRO-3A5. A few macrophages are shown with yellow-black malaria pigment from phagocytosized malaria parasites (1000X).





Characterization of Macrophage Activation Using CD63

The monoclonal antibody, CD63, was used to detect a state of activation by the macrophages. CD63 positive cells expressed abundant reddish-brown granulation throughout the cell (Figures 5 and 6). A low power (100X) magnification of the cerebral tissue reveals several microvessels with a moderate number of CD63 positive staining cells in a CM patient (Figure 5). The microvessels are engorged with malaria parasites. Lining the blood vessels are some CD63 positive endothelial cells that display a slight amount of reddish-brown granulation in the cytoplasm (Figure 6). The CD63 negative cells fail to show any reddish granulation.

The data showed that 56.6% of the macrophages in CM patients were activated compared with 43.1% of NCM and 46.8% of COC patients. The variation among the groups was not quite statistically significant, p=0.076 ANOVA. The range of CD63 positive cells detected in CM patients was from 28.5 to 82%, 23 to 67% in the NCM group, and 32.7 to 67.5% for the COC control group.

Degree of Parasitemia and Macrophage Activation

Parasitemia was ranked empirically, from 0 to 4+ based on the degree of parasitization. Figure 7 displays a microvessel classified as having 4+ parasitization, engorged with a massive amount of sequestered pRBCs. Sequestered pRBC were detected in all CM patients, and in 50% of children with NCM. On average, CM patients had an average parasitemia score of 2.7, while parasitemia in NCM averaged 1.4 and COC patients were, by definition,

aparasitemic. There was a direct correlation between number of cells activated and degree of parasitization from CM patients compared with the NCM group, p= 0.006, by linear regression with correlation coefficient r=0.6155, r squared = 0.3788 (GraphPad InStat).

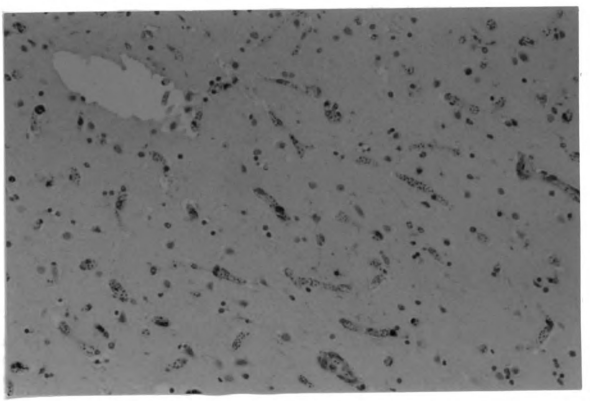
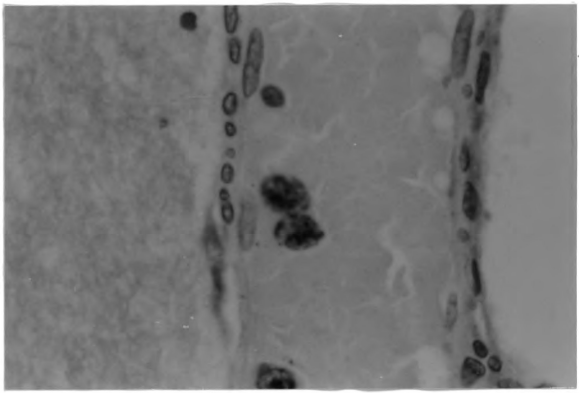
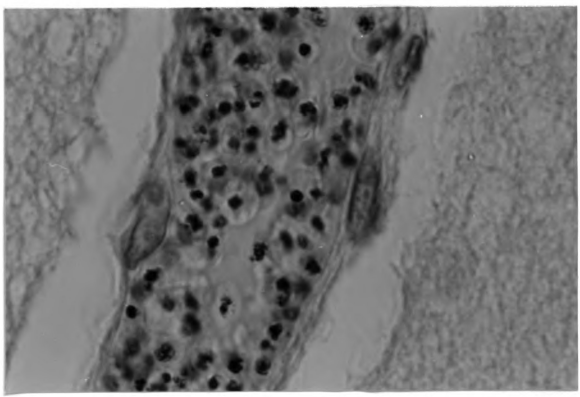


Figure 5. CD63 positive microvessels.
Low power magnification displays hyperparasitized microvessels that also show CD63 positive red-brown granules (100X).

Figure 6. CD63 reactive cells.
CD63 Immunophenotyping demonstrates mononuclear phagocytes with red-brown granulation and malaria pigment. Endothelial cells also display faint CD63 positive granules (1000X)

Figure 7. Malaria sequestration of pRBCs.
Cerebral blood vessel engorged with malaria infected RBCs (1000X). Yellow-black malaria pigment displayed inside red blood cells. No leukocytes observed.





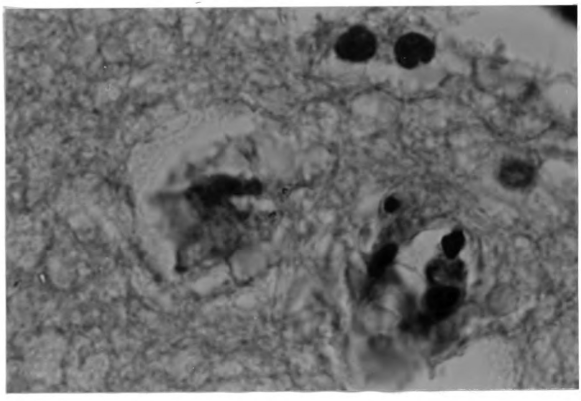
Local TNF-α Cytokine Expression in Cerebral Tissue

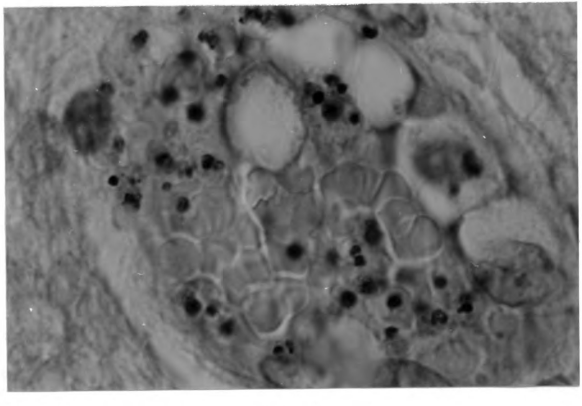
The secretion of TNF-α protein was demonstrated in the parietal lobe of the cerebral tissue CM patients and controls. A cerebral vessel packed with pRBC shows the local production of TNF-α at the site of infection in a CM patient (MP6)(Figures 8 and 9). TNF-α is shown as orange-brown pigmentation in the cytoplasm of cerebral macrophages. Erythrocytes appeared as colorless, refractory cells, many containing parasites as evidenced by the brown-black cytoplasmic aggregates.

The TNF-α protein was detected in 11.8%, 19.8% and 15.4% of CM, NCM and COC patients, respectively. The NCM patients had a slightly higher number of cells and vessels staining positive for TNF-α. There was no significant difference between the groups, $p = 0.3621$ by ANOVA (GraphPad InStat).

Figure 8. TNF-α vessel packed with pRBC (1000X)
Microvessel engorged with pRBC. Unstained macrophages surrounded with TNF-α granulation. Malaria parasites appear as brown-black aggregates in RBCs.

Figure 9. TNF-α positive macrophage
A small cerebral blood vessel contains malaria pRBC and macrophage with TNF-α positive granulation. (1000X)





IN SITU HYBRIDIZATION RESULTS

In situ hybridization was performed to detect the presence of TNF-α messenger ribonucleic acid (mRNA) in the cerebral tissue. The slides were scanned initially under 400X magnification and analyzed under 1000X oil immersion. A cell was identifed as positive if it contained dark-blue to black granules of various sizes. Generally, the number of granules per cell was greater than three granules. A cell was deemed negative if no granules or, a hazy, indistinct granule was observed.

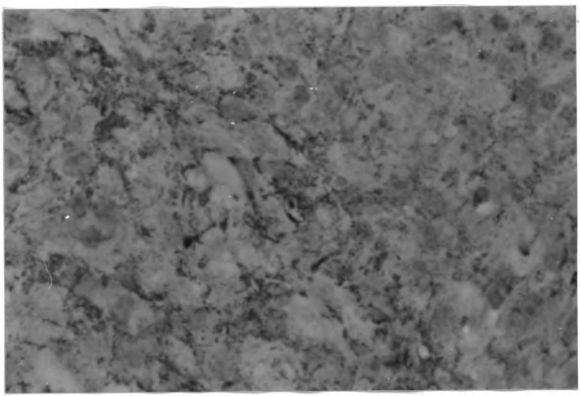
Twenty 1000X oil immersion fields were evaluated for the presence of the dark-blue to black formazan granules (Silamut et al. 1999, Wilcox 1993). A negative result was pronounced if 0 - 20 fields contained no positive cells. The slide was considered positive if least one cell in all twenty fields contained positive granules. On average, the number of positive cells per 1000X field was generally greater than three.

Each patient was analyzed in duplicate, with and without (negative internal control) the DIG-labeled probe. DIG-labeled-β-actin served as a positive probe control (Figure 10 and 11). Since β-actin is a component of most nucleated cells, this molecule was highly observable. Blue-black punctate granules were observed in the cytoplasm of various cells demonstrating the presence and distribution of β-actin mRNA within the cells. Cells with no granulation were deemed a negative result. TNF-α mRNA was observed as fine to coarse black-blue granules in cytoplasm of leukocytes shown in Figures 12 and 13. Malaria parasites were differentiated by a ten-fold greater size and denser staining quality. The TNF-α mRNA was detected in 47% (9 of 19) CM patients. In NCM

patients, TNF-α mRNA was present in 38% (3 of 8 patients), and 29% (2 of 8) in COC patients. There was no significant statistical difference between the groups (p=0.0939, by ANOVA (GraphPad InStat).

Figure 10. ISH DIG-labeled, beta actin control probe (1000X magnification). Numerous blue black granules in the cytoplasm. Blue-black punctate granules in the cytoplasm of various cerebral cells, demonstrate the presence and distribution of β-actin mRNA within the cells.

Figure 11. ISH DIG-labeled, beta actin control probe (1000X magnification). Positive cells contain blue-black cytoplasmic granules



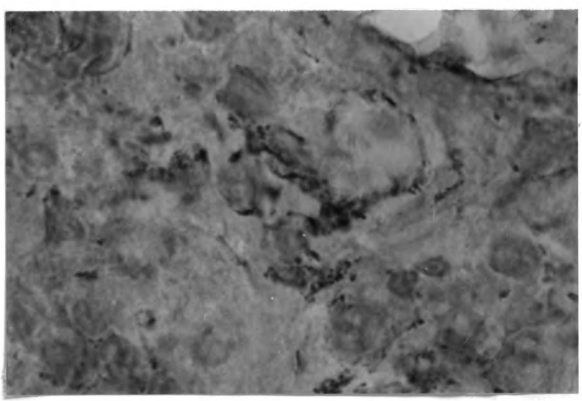
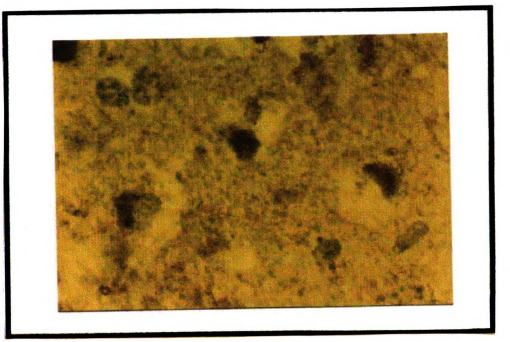
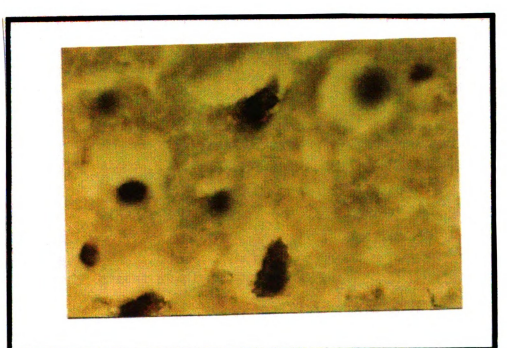


Figure 12. *In situ* hybridization of TNF- α mRNA in a CM patient (1000X). TNF-α mRNA observed as fine to coarse black-blue granules in cytoplasm of leukocytes. The large, black granules of malaria parasites differentiates them from the small in situ hybridization granules.

Figure 13. *In situ* hybridization of TNF- α mRNA in a CM patient (1000X). TNF-α mRNA was observed as fine to coarse black-blue granules in cytoplasm of leukocytes. Malaria parasites were ten-fold greater size with dense black granules.





CYTOKINE POLYMORPHISM RESULTS

Cytokine genotyping was performed to detect polymorphisms in the genes of pro- and anti-inflammatory cytokines. Genetic substitutions in the promoter regions, introns and leader sequences of the cytokines were analyzed using SSP-PCR. The cytokine polymorphisms are categorized by their putative production phenotypes as shown in Table 14.

TABLE 14. CYTOKINE POLYMORPHISM GENOTYPE AND PRODUCTION PHENOTYPES

CYTOKINE	LOW	INTERMEDIATE	HIGH
TNF-α	-308 G/G	N/A	-308 G/A -308 A/A
IFN-γ	+874 A/A	+874 T/A	+874 T/T
IL-6	-174 C/C	-174 C/G	-174 G/G
IL-10	**(-1082/-819/-592)** ACC/ACC ATA/ATA ACC/ATA	**(-1082/-819/-592)** GCC/ACC GCC/ATA	**(-1082/-819/-592)** GCC/GCC
TGF-β	10 T/T 25 C/C	10 T/C 25 C/G	10 C/C 25 G/G

Cytokine polymorphisms associated with putative production phenotypes.
TNF-α= tumor necrosis factor-alpha, IFN-γ=interferon-gamma, IL-6=interleukin-six, IL-10 interleukin-ten and TGF-β transforming growth factor-beta
One Lambda Corporation

Amplified nucleic acids were detected on ethidium bromide stained 2.5% agarose gels and photographed after UV illumination (Figures 14 and 15). The DNA Molecular Weight Marker VIII, marked "M," is observed in the small well, at the top upper left corner of the gel. The locations of the primers in each

amplification well are listed successively, along with representative gels (Figures 14 and 15.

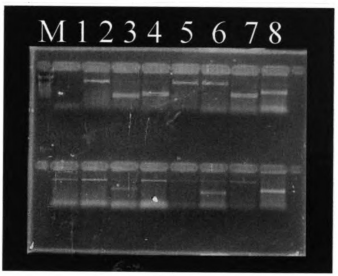
Figure 14 shows a gel of CM patient with following genotype:

Cytokine	Genotype	Putative Production Phenotype
TNF-α	-308 G/G	low production phenotype
TGF-β	10T/T, 25 G/G	high production
IL-10	-1082 A/A, -819 T/C, -592 A/A (ATA/ ACA)	low production phenotype
IL-6	-174 (G/G)	high production phenotype
IFN-γ	+874 (A/A)	low production phenotype

Figure 15 shows a gel of CM patient with following genotype:

Cytokine	Genotype	Putative Production Phenotype
TNF-α	-308 G/A	high production phenotype
TGF-β	10T/T, 25 G/G	high production
IL-10	-1082 G/G, -819 T/C, -592 A/A (GTA/GCA)	high production phenotype
IL-6	-174 (G/G)	high production phenotype
IFN-γ	+874 (A/A)	low production phenotype

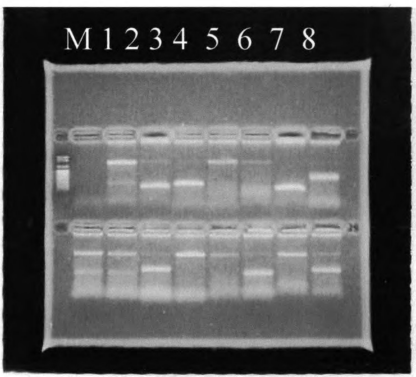
Figure 14 - Cytokine Genotyping -308 (G/G)
(TNF-α low production phenotype)



Top Row			**Bottom Row**	
Marker	Molecular weight marker			
Lane 1	Negative control		IL-10	-1082G, -819C
Lane 2	TNF-α	-308A	IL-10	-1082A, -819C
Lane 3	TNF-α	-308G	IL-10	-819T, - 592A
Lane 4	TGF- β	Codon 10T	IL-10	-819C, -592C
Lane 5	TGF- β	Codon 10C	IL-6	-174C
Lane 6	TGF- β	Codon 25C	IL-6	-174G
Lane 7	TGF- β	Codon 25G	IFN-γ	+874T
Lane 8	IL-10	-1082A, -819T	IFN-γ	+874A

GENOTYPE: TNF-α: -308 G/G (low production phenotype), TGF-β: 10T/T, 25 G/G (high production), IL-10: -1082 A/A, -819 T/C, -592A/A, (ATA, ACA) (low production phenotype), IL-6: -174 (G/G) (high production phenotype, IFN-γ: +874 (A/A) (low production phenotype).

Figure 15 - Cytokine Genotyping -308(G/A)
(TNF-α High Production Phenotype)



Top Row			**Bottom Row**	
Marker	Molecular weight marker			
Lane 1	Negative control		IL-10	-1082G, -819C
Lane 2	TNF-α	-308A	IL-10	-1082A, -819C
Lane 3	TNF-α	-308G	IL-10	-819T, - 592A
Lane 4	TGF- β	Codon 10T	IL-10	-819C, -592C
Lane 5	TGF- β	Codon 10C	IL-6	-174C
Lane 6	TGF- β	Codon 25C	IL-6	-174G
Lane 7	TGF- β	Codon 25G	IFN-γ	+874T
Lane 8	IL-10	-1082A, -819T	IFN-γ	+874A

GENOTYPE: TNF-α: -308 G/A (high production phenotype), TGF-β: 10T/T, 25 G/G (high production), IL-10: -1082 G/G, -819 T/C, -592A/A, (GTA, GCA) (high production phenotype), IL-6: -174 (G/G) (high production phenotype, IFN-γ: +874 (A/A) (low production phenotype).

Frequency of TNF-α Gene Polymorphism

The polymorphism investigated in the TNF-α gene was located at position -308. Guanine at position -308 (G/G) has been associated with low TNF-α production, while the substitution of adenine, whether homozygous or heterozygous at position -308 (A/A) or (G/A), has been identified with high production.

The frequency of polymorphisms in the TNF-α gene is presented by clinical study groups and categorized by the putative production phenotypes. In this study, the homozygous -308 (A/A) allele was not detected. The study population had a gene frequency of 71% with -308(G/G), the putative low production phenotype, while 29% displayed the -308 (G/A) putative high producer phenotype.

Table 15. Frequency of TNF-α Cytokine Polymorphisms

GROUPS	Low production -308 (G/G)	High Production -308 (G/A)
CM	70% (14/20)	30% (6/20)
NCM	88% (7/8)	12% (1/8)
COC	50% (3/6)	50% (3/6)
OVERALL FREQUENCY	71% (24/34)	29% (10/34)

Substitution of guanine to adenine at position -308 of the TNF-α promoter. The difference between the groups was not statistically significant, p=0.22 (ANOVA) (GraphPad InStat). The value in parenthesis is the number of patients per study group. CM: cerebral malaria, NCM: non cerebral malaria, COC: coma of other causes. G: guanine, A: adenine

The three clinical study groups were statistically evaluated within and between groups (Table 15). TNF-α cytokine polymorphisms were detected in CM patients at a frequency of 70% for the putative low production phenotype and 30% displayed the heterozygous substitution, -308(G/A), associated with high production. The NCM control group had an 88% frequency for the low production phenotype and 13% were classified as high producers. COC control patients were equally divided with 50% demonstrating the putative low and high production phenotypes. Although there were numeric differences between the medians of the low and high production phenotypes, the variation between the groups was not statistically significant (p=0.22, by ANOVA).

Of the ten patients with the -G308A substitution associated with high TNF-α production, 60% (6 of 10) died from CM, 10% (1 of 10) died from NCM, and 30% (3 or 10) from COC. Chi-square analyses were performed to compare TNF-α CM to NCM (p=0.434) and CM to COC (p=0.326). The variation was considered not significant, with the note that with such small values, the Chi-square p value was not accurate. Chi-square used a two by two contingency table to compare two variables. This differed from the ANOVA which compares multiple factors and variables.

Frequency of IFN-γ Gene Polymorphism

The polymorphisms investigated for IFN-γ were located in the first intron of the gene at position +874 downstream of the transcriptional start site. Genotypes that contained thymine (T) at position +874 were associated with high

cytokine production, whereas genotypes containing adenine (A) were reported to be low producers. The genotypes and putative production phenotypes are listed in Table 16.

Table 16. Frequency of IFN-γ Cytokine Polymorphism

GROUPS	Low +874 (A/A)	Intermediate +874 (T/A)	High +874 (T/T)
CM	75% (15/20)	25% (5/20)	0
NCM	50% (4/8)	50% (4/8)	0
COC	67% (4/6)	33% (2/6)	0
OVERALL FREQUENCY	68% (23/34)	32% (11/34)	0

Substitution of thymine (T) at position +874 associated with high cytokine production, while, adenine (A) is associated with low production. The variation between the groups was not statistically significant, $p = 0.15$, by ANOVA (GraphPad InStat). The value in parenthesis is the number of patients per study group. CM: cerebral malaria, NCM: non cerebral malaria, COC: coma of other causes.

Of the three possible IFN-γ genotypes, only two were detected in this study population. None of the patients demonstrated the +874 (T/T) genotype associated with high IFN-γ production. The overall polymorphism frequency for the total study population was 68% (23 of 34) for the low production phenotype (+874A/A), while, 32% (11 of 34) were categorized as intermediate producers.

Comparing each group separately, it was noted that, 75% of the CM patients had the low producer phenotype. There was an equal distribution of the low and intermediate production phenotypes in the NCM control group. The

COC controls were categorized as 67% low producers and 33% intermediate. The variation between the groups was not statistically significant different, p=0.15, by ANOVA.

Frequency of Interleukin-6 (IL-6) Gene Polymorphism

A biallelic polymorphism in the IL-6 gene was associated with a substitution of cytosine [C] by guanine (G) at position -174 of the promoter region. Neither the homozygous allele, C/C, at position -174 associated with low cytokine production, nor the heterozygous C/G that represents intermediate production were detected in the study population. The homozygous -174 (G/G), associated with high cytokine production was detected in 100% of the patients in all three study groups.

Frequency of Interleukin-10 (IL-10) Gene Polymorphism

Three base pair substitutions in the IL-10 promoter region at positions -1082, -819 and -592, relative to the transcription start site, have been linked to the cytokine's production level. Alleles containing G at position -1082 encode for high IL-10 production, while alleles containing A at position -1082 correlate with low IL-10 production. The three positions -1082, -819 and -592 comprise nine inherited haplotypes which can be expressed in an abbreviated fashion, i.e. ACC/ACC. The order of the nucleotides correlate to the three loci at positions -1082, -819 and -592 of the haplotype . The haplotype comprised of -1082(A/A), -819(C/C), -592(C/C) would be abbreviated ACC/ACC for this homozygote.

The patients in this study were categorized based on the proposed low, intermediate and high production phenotype frequencies. The haplotype GCC/GCC, associated with high production, was detected in 6% (2 of 34) of the study population, while 41% (14 of 34) presented the haplotypes, GCC/ACC and GCC/ATA consistent with intermediate production and 53% (18 of 34) possessed the low producer haplotypes, ACC/ACC, ATA/ACC, ATA/ATA (Table 17).

Table 17. Frequency of Interleukin-10 Cytokine Polymorphisms

GROUPS	LOW PRODUCTION ACC/ACC, ATA/ATA, ACC/ATA	INTERMEDIATE GCC/ACC, GCC/ATA	HIGH PRODUCTION GCC/GCC
CM	45% (9/20)	50% (10/20)	5% (1/20)
NCM	75% (6/8)	25% (2/8)	0
COC	50% (3/6)	33% (2/6)	17% (1/6)

The haplotypes of -G1082A, -T819C, -A592C can be expressed in as an acronym based on the nucleotides. Using the haplotypes at positions -1082, -819 and -592, the nucleotides at each position form an acronym, i.e. ACC/ACC for genotype -1082(A/A), -819(C/C), -592(C/C). The table categorizes the low, intermediate and high production phenotypes by genotypes. The variation between the groups was statistically significant by ANOVA, p=0.0291. The value in parenthesis is the number of patients per study group. CM: cerebral malaria, NCM: non cerebral malaria, COC: coma of other causes.

In the CM patients, 45% of the patients presented haplotypes associated with the low producer phenotype, 50% with intermediate production and 5% representing a high production phenotype. The NCM control group was equal

divided into low and intermediate production phenotypes, with no high producers. COC controls had 50%, 33% and 17% frequencies representing low, intermediate and high production phenotypes, respectively. There was a statistically significant difference observed between the high producer, GCC/GCC haplotype compared with the ACC/ACC, ATA/ATA, ACC/ATA low producer haplotypes, p=0.0291 using ANOVA (GraphPad InStat). A two by two statistical comparison of the clinical groups CM to NCM (p=0.2) and CM to COC (p=0.21) revealed no significant difference between the groups by Chi-square (GraphPad InStat).

Frequency of TGF-β Gene Polymorphism

Polymorphisms in the leader sequence of the *TGFB* gene at positions +869 and +915 correlate with TGF-β production ability. A substitution of thymine (T) by cytosine [C] at position + 869 changes the amino acid at codon 10 from leucine to proline, and a substitution of cytosine by guanine (G), results in a change from arginine to proline at codon 25. For both polymorphisms, the allele encoding proline is associated with lower TGF-β synthesis. Patients homozygous for leucine at codon 10, and arginine at codon 25 are associated with high production of TGF-β. There were three possible inheritable haplotypes for each codon, and nine haplotype combinations (Table 18). Codon 10 (+869), combinations 10 T/T, T/C, and C/C were associated with low, intermediate and high cytokine production, while at codon 25, C/C, C/G and G/G, are similarly characterized.

The patients and controls in this study were categorized as either low, intermediate or high producers of TGF-β based on their haplotype inheritance. There were 79% (27 of 34) in the study population categorized as high producers, 18% (6 of 34) as intermediate producers and 3% (1 of 34) possessing the low producer phenotype.

Table 18. Frequency of Transforming Growth Factor-β Cytokine Polymorphisms

GROUPS	LOW PRODUCTION C/C-G/G* C/C-C/C T/C-C/C T/T-C/C	INTERMEDIATE T/C-G/C* C/C-G/G T/T- G/C	HIGH PRODUCTION T/T-G/G* T/C-G/G
CM	5% (1/20)	20% (4/20)	75% (15/20)
NCM	0	13% (1/8)	88% (7/8)
COC	0	17% (1/6)	83% (5/6)
Overall	6% (1/34)	18% (6/34)	79% (27/34)

The table represents the low, intermediate and high production phenotypes and the associated haplotype at two loci, codons 10 and 25. The variation between the groups was statistically significant, p=0.0339 by ANOVA (GraphPad InStat). The value in parenthesis is the number of patients per study group.
* The codon 10 genotype is shown first followed by the genotype at codon 25 (One Lambda, Canoga Park, CA). T= thymine, C= cytosine, G= guanine.

In the CM group, 5% of the patients presented haplotypes associated with the low producer phenotype, 20% with intermediate production and 75% representing a high production phenotype. There were no NCM patients

associated with low production, but 13% displayed the intermediate production phenotype, while 88% demonstrated high production capacity. The COC controls had a 17% frequency of intermediate production and 83% showed the putative high production phenotype. The variation between the groups was statistically significant, p=0.0339 by ANOVA (GraphPad InStat).

Correlation of Combinations of Cytokine Genotype Polymorphisms

The -G308A polymorphisms in the TNF-α gene has been associated with high production of the cytokine and fatal outcomes in cerebral malaria. With this fact is mind, an investigation was performed to correlate TNF-α polymorphisms with polymorphism in other cytokines. The patients and controls were sub-categorized based on the putative TNF-α production phenotypes and re-analyzed. There were 24 patients and controls with the putative TNF-α low production phenotype comprised of 13 CM, seven NCM and three COC. Ten patients and controls demonstrated the high TNF-α production phenotype including seven in the CM group, one with NCM and three from the COC group. When the three pro-inflammatory cytokines were compared for high TNF-α production, high IL-6 production, and low, intermediate and high IFN-γ production. There was significant variation among the groups, p=0.0202, by repeated measures ANOVA (GraphPad InStat). In like manner low TNF-α, with high IL-6 production, and low, intermediate and high IFN-γ groups had significant variation between the groups, p=0.0174, by repeated measures ANOVA.

Correlation Between TNF-α Low Production Phenotypes and IL-10

The 24 patients and controls with the TNF-α low production phenotype were analyzed against IL-10 low, intermediate and high producer phenotypes (Table 19). When the 14 TNF-α low producer CM patients were separated and analyzed for their IL-10 production phenotypes, there were 57% with low, 36% intermediate and 7% with the high production phenotype. Of the seven individuals in the NCM group, 86% had the low production phenotype, 14% showed intermediate production capability and none were high producers. The COC group presented twice as many low producers (67%), compared with high (33%) and no intermediate producers. There was a statistically significant difference between the groups, p=0.038 by ANOVA.

Table 19. A Correlation of 24 Patients with TNF-α Low Production Phenotypes with IL-10 Polymorphism

GROUPS	LOW IL-10 LOW TNF-α	INTERMEDIATE IL-10 LOW TNF-α	HIGH IL-10 LOW TNF-α
CM	57% (8/14)	36% (5/14)	7% (1/14)
NCM	86% (6/7)	14% (1/7)	0
COC	67% (2/3)	33% (1/3)	0%

The value in parenthesis is the number of patients per study group.
The patients were separated into two groups based on their putative TNF-α production phenotype. The patients demonstrating low, intermediate and high IL-10 putative phenotypes and TNF-α low producers are listed above. p = 0.038 ANOVA. CM - cerebral malaria, NCM- non cerebral malaria, COC - coma of other causes

Correlation Between TNF-α High Production Phenotypes and IL-10

The six TNF-α high producer CM patients were analyzed in like manner, for IL-10 frequency and showed 17% (low), 83% (intermediate) with no high producers (Table 20). The one NCM control expressed the intermediate production phenotype, only. The COC group was equally divided among the IL-10 production levels (Figure 20). There was no statistical difference between the high TNF-α producers evaluated against the IL-10 producer phenotypes, p=0.1787 by ANOVA.

Table 20. A Correlation of 10 Patients with TNF-α High Production Phenotypes with IL-10 Polymorphisms

GROUPS	LOW IL-10 HIGH TNF-α	INTERMEDIATE IL-10 HIGH TNF-α	HIGH IL-10 HIGH TNF-α
CM	17% (1/6)	83% (5/6)	0
NCM	0	100% (1/1)	0
COC	33% (1/3)	33% (1/3)	33% (1/3)

The patients were separated into two groups based on their putative TNF-α production phenotype. The patients demonstrating low, intermediate and high IL-10 putative phenotypes and also TNF-α high producers are listed above. p=0.1787 by ANOVA (GraphPad InStat).
CM: cerebral malaria, NCM: non cerebral malaria, COC: coma of other causes

Correlation Between TNF-α Low Production Phenotypes and TGF- β

After controlling for the low TNF-α producer phenotype, the incidence of the low, intermediate and high TGF-β production phenotypes were analyzed in

the 14 CM patients (Table 21). The frequencies observed in these patients was 7% low, 21% intermediate and 71% high. In the seven NCM group, there were 14% classified as intermediate producers, compared with high 86% high producers. Only the high production phenotype was detected in the COC group. The variation between the groups was statistically significant, p=0.0171 by ANOVA.

Table 21. A Correlation of 24 Patients with TNF-α Low Production Phenotypes with TGF-β Polymorphism

GROUPS	Low TGF-β Low TNF-α	Intermediate TGF-β Low TNF-α	High TGF-β Low TNF-α
CM	7% (1/14	21% (3/14)	71% (10/14)
NCM	0	14% (1/7)	86% (6/7)
COC	0	0	100% (3/3)

The patients were separated into two groups based on their putative TNF-α low production phenotype and low, intermediate and high TGF-β putative phenotypes (p=0.0171)(ANOVA, GraphPad InStat).
CM: cerebral malaria, NCM: non cerebral malaria, COC: coma of other causes

Table 22. A Correlation of 10 Patients with TNF-α High Production Phenotypes with TGF-β Polymorphism

GROUPS	Low TGF-β High TNF-α	Intermediate TGF-β High TNF-α	High TGF-β High TNF-α
CM	0%	17% (1/6)	83% (5/6)
NCM	0	0	100% (1/1)
COC	0	33% (1/3)	67% (2/3)

The patients were separated into two groups based on their putative TNF-α high production phenotype and low, intermediate and high TGF-β putative phenotypes, p= 0.2000 by ANOVA (GraphPad InStat).
CM: cerebral malaria, NCM: non cerebral malaria, COC: coma of other causes

Correlation Between TNF-α High Production Phenotypes and TGF-β

The six TNF-α high producer CM patients showed 17% with intermediate production, 83% high and no low producers (Table 22). The one NCM control expressed the putative high production phenotype, only. The COC group exhibited 33% intermediate producers and 67% high producers. There was no statistical difference between the high TNF-α producers evaluated against the TGF-β producer phenotypes, p=0.2000 by ANOVA (GraphPad InStat).

Correlation Between TNF-α Low Production Phenotypes and Anti-inflammatory Cytokines IL-10 and TGF- β

Given the above results, low TNF-α producers were compared with both IL-10 and TGF-β low, intermediate and high production phenotypes. The 14 CM patients, seven NCM and three COC controls with the TNF-α low production

phenotype were analyzed against anti-inflammatory cytokines IL-10 and TGF-β. There was a statistically significant difference between the groups, p=0.0078 by Friedman, nonparametric repeated measures ANOVA (GraphPad InStat).

Table 23. Correlation Between TNF-α Low Production Phenotypes and Anti-inflammatory Cytokines IL-10 and TGF- β

GROUPS	IL-10	TGF-β
CM		
Low	57%	7%
Intermediate	36%	21%
High	7%	71%
NCM		
Low	86%	0%
Intermediate	14%	14%
High	0%	86%
COC		
Low	66%	0
Intermediate	33%	0
High	0%	100%

The patients were separated into two groups based on their putative low TNF-α production phenotype and correlated with anti-inflammatory cytokines IL-10 and TGF-β, p= 0.008 by ANOVA (GraphPad InStat).
CM: cerebral malaria, NCM: non cerebral malaria, COC: coma of other causes

Correlation Between TNF-α High Production Phenotypes and Anti-inflammatory Cytokines IL-10 and TGF- β

In like manner the high TNF-α producers were compared with the anti-inflammatory cytokines. The six CM patients, one NCM and three COC controls were analyzed against the anti-inflammatory cytokines. The data was considered significant, p= 0.0152 by Friedman, nonparametric repeated measures ANOVA (GraphPad InStat).

Table 24. Correlation Between TNF-α High Production Phenotypes and Anti-inflammatory Cytokines IL-10 and TGF- β

GROUPS	IL-10	TGF-β
CM		
Low	17%	0%
Intermediate	83%	17%
High	0%	83%
NCM		
Low	0%	0%
Intermediate	100%	0%
High	0%	100%
COC		
Low	33%	0
Intermediate	33%	33%
High	33%	67%

The patients were separated into two groups based on their putative high TNF-α production phenotype and correlated with anti-inflammatory cytokines IL-10 and TGF-β, p= 0.0152 by ANOVA (GraphPad InStat).
CM: cerebral malaria, NCM: non cerebral malaria, COC: coma of other causes

Chapter 5

DISCUSSION

IMMUNOHISTOCHEMICAL STAINS

IN SITU HYBRIDIZATION

Chapter 5

DISCUSSION

CHARACTERIZATION OF MONONUCLEAR PHAGOCYTES BY IHC

The fundamental question addressed by this dissertation was whether or not mononuclear cells detected in the brain of cerebral malaria (CM) patients were activated macrophages and monocytes. This inquiry was important because the detection of macrophages in cerebral tissue was previously associated only with murine CM, and not human CM. Unexpectedly, mononuclear phagocytes were detected in the brain of Malawian children dying from CM (Mackenzie et al. 1999). This observation prompted an investigation for the characterization and functionality of those cells using immunohistochemical stains (IHC).

Detection of Macrophages in Cerebral Tissue

The association of monocyte/macrophage function with the ages of the children in the study population was pondered. Although immunodeficiency has been associated with various cellular lineages of the newborn, previous literature reviews supports the fact that based on the ages of the children in this study, the monocytes/macrophages had a functional capacity equal to adult cells. Post mortem tissue from children dying of CM and controls was immunophenotyped with macrophage specific markers that demonstrated the location, staining intensity and morphology of monoclonal antibody positive cells. Since cells of the monocyte/macrophage lineage are heterogeneous in

morphology and function, two cell-specific monoclonal antibodies were directed against cerebral mononuclear cells for identification Table 13). The staining pattern of the MAC387, broad spectrum, monoclonal antibody demonstrated the presence of many myeloid, monocytic and macrophage cells in cerebral tissue of CM patients and controls with NCM and COC (figures 1 and 2). The lymphocytes did not react with this marker and were identified by their nuclear and cytoplasmic morphology. Although there was a greater number of MAC387 positive cells in CM patients compared with controls this was not statistically significant by ANOVA. The NCL-MACRO-3A5, differentiated the positive staining macrophages from other intravascular cells (figures 3 and 4). Macrophages were randomly distributed within small and large cerebral vessels. However, there was no significant difference between the study groups for the detection of macrophages in cerebral microvasculature. This may mean that the presence of macrophages in cerebral vessel was not associated with any particular pathology.

CD63 data showed that the majority of the macrophages in CM patients were activated compared to controls with half as many activated cells (figures 6 and 7). Although, less than 20% of the total invading cells in cerebral tissue at the time of death were macrophages, the data showed that these cells were more likely to be activated than resting in CM patients compared with controls (Table 13). This finding was important for two reasons. First, it provided evidence for the detection of activated macrophages and monocytes in cerebral tissue in fatal malaria. Secondly, the data suggested that the activated

macrophages may play a role in the pathogenesis of CM. The IHC data supports the hypothesis that activated monocytes and macrophages were present in cerebral tissue of fatal malaria patients, as well as control patients

TNF-α Protein Detection

The IHC stains revealed the local production of TNF-α protein in macrophages and some neuronal cells in cerebral microvessels. Children with severe, NCM, demonstrated more TNF-α expression (19.8%) than COC (15.4%) and CM patients (11.4%). This is consistent with the literature which associates elevated TNF-α levels with the severity of malaria, but does not relate it specifically with the development of CM. The data suggested that macrophages and neuronal cells secrete TNF-α in the local cerebral environment. However, a quantitative analysis was not able to be performed on neuronal cells. The identification of sites of TNF-α production might permit a more detailed analysis of the relationship between TNF-α and clinical illness. Moderate cytokine secretions can provide beneficial effects to suppress parasitic growth, but excessive amounts can be damaging. The major sources for TNF-α production are activated macrophages and monocytes.

In Situ HYBRIDIZATION

In situ hybridization (ISH) was used to detect TNF-α mRNA expression in cerebral tissue at the cellular level. ISH allows for the inspection of cells in their proper morphological context. Using ISH, the mRNA of TNF-α was detected in

the cerebral tissue of more CM patients (47%) than controls (NCM 38%, COC 29%). A frustration of the procedure was the presentation of indistinct, unevenly distributed blue-black formazan granules of fine to coarse consistency. The ISH reaction used a nonradioactive, digoxigenin-labeled probe and employed an empirical staining method that cannot be accurately quantitated (Fenhalls et al, 2000, Wilcox 1999, Nuovo 1997). While positive granules were detected in tissue hybridized with probe and absent from tissue with no probe, the interpretation was subjective and did not allow for quantitation. The positive cells were identified by the nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) positive staining reaction within a minimum of 20 successive 1000X oil immersion fields. A negative result does not necessarily imply an absence of TNF-α mRNA, it merely means that, using the current methodology, the TNF-α mRNA was not detected. The differential process undertaken to problem solve the negative test results include: assessing the quality of the tissue, the specificity of the probe, the concentration of Proteinase K used for permeabilization, antibody concentration used for detection and chromogen stability. The reasons for the lack of detection include those stated above under the limitations of the procedure, but also could have resulted from RNASE enzyme inactivation of *in situ* RNA occurring post mortem. Measures to prevent RNASE contamination included the addition of an RNASE inhibitor to the hybridization solution, treatment of supplies and surfaces with RNASE ZAP, baking of glassware and use of disposable RNASE/DNase free plasticware. Since the quantity of probe used may not have been sufficient to penetrate into

the tissue, a repeat analysis with a greater amount of probe may prove helpful. Other modification to enhance sensitivity include the extension of hybridization times, and prolonging incubation with anti-Digoxigenin (DIG) to increase detection. Also, the anti-DIG antibody may have been unstable (It has been reported that the supplier previously had experienced problems with the specificity of the antibody in the past (personal communication). A future goal is to repeat the *in situ* hybridization with another DIG-labeled probe, decrease the concentration of proteinase K used for permeablization for better tissue morphology, re-evaluate the hybridization period and antibody incubation times (e.g. increase to 48 hours instead of overnight).

The patients with negative results were randomly dispersed and not associated with the age of the PET or clinical condition. Regardless of the subjectivity of the results, there was an unexpected association between the detection of TNF-α mRNA and the amount of parasitemia observed. Patients with detectable TNF-α mRNA exhibited elevated parasitemia. TNF-α mRNA was also detected in two COC patients, hence no parasitemia, diagnosed with Reye's syndrome and viral encephalopathy. TNF-α mRNA was not detected in ten CM, six of whom had low parasitemia and three with increased parasitemia. Thus, the negative results are cautiously interpreted and may merely mean lack of detection.

One of the research goals was to detect the expression of TNF-α in cerebral tissue at the message and protein levels. Both IHC stains (cited above) and *in situ* hybridization were employed to ascertain the presence of TNF-α

protein and mRNA production in cerebral tissue. Every CM and NCM case demonstrated some degree of TNF-α protein in the cerebral tissue. Although many in the COC group showed some TNF-α protein, it was at a lower level and two patients failed to show any protein. These studies examined the expression of both TNF-α mRNA and protein in cerebral tissue. A conclusion drawn from these observation is that studies of mRNA expression alone do not indicate whether translation into the functional cytokine will occur. The detection of the TNF-α protein does not indicate when the protein was secreted. The secretion of TNF-α by neuronal cells was also observed in a few CM patients and warrants further study to identify these neurological cells.

In summary, there was no correlation between the detection of TNF-α mRNA and the quantity of protein production. Patients with negative TNF-α mRNA tended to have the lowest percentage of TNF-α protein positive cells. Some patients with high TNF-α protein did not express the presence of its message. The IHC data indicate that activated macrophages were detected in the cerebral tissue CM patients and controls. The IHC and ISH studies provided evidence that there was a local production of TNF-α mRNA and protein in the cerebral tissue. However, the expression of TNF-α was not confined to CM patients alone. This fact is consistent with the literature, since severe malaria (NCM) is associated with a higher TNF-α serum level than CM.

Chapter 6

DISCUSSION

DETECTION OF POLYMORPHISM IN THE GENES OF PRO- AND ANTI-INFLAMMATORY CYTOKINES

Chapter 6

DISCUSSION

DETECTION OF POLYMORPHISM IN THE GENES OF PRO- AND ANTI-INFLAMMATORY CYTOKINES

Malaria infection prompts the host to initiate a massive inflammatory response to eradicate the intracellular parasitic invasion of RBCs. The severe manifestations of malaria are caused not by the *P. falciparum* parasite itself, but by the excessive production of inflammatory cytokines. The activation of phagocytes to kill the parasites requires the production of powerful pro-inflammatory cytokines whose overproduction can have major systemic effects. Activated macrophages are a significant source for the production of pivotal pro-inflammatory cytokines associated with malaria pathology. The intravascular accumulations of large numbers of malaria infected RBCs can generate a local concentration of cytokine secreting, inflammatory mediators. On the other hand, anti-inflammatory cytokines function to downregulate pro-inflammatory cytokines which minimizes the toxicity of prolonged exposure. The outcome of infection teeters unsteadily between the appropriate expression of both pro- and anti-inflammatory cytokines.

Polymorphisms in the genes of cytokines have been associated with their rate of transcription and production. The pathogenesis of CM may involve both the excessive production of pro-inflammatory cytokines and a defective negative feedback mechanism. With the exception of TNF-α, no data exists about an association between polymorphism in the genes of cytokines and the outcome of

malaria. Genetic polymorphism is defined as the existence of multiple alleles at a specific genetic locus. Allelic polymorphisms in the genes of cytokines result in accelerated *in vitro* gene transcription and increased protein.

This dissertation postulates that CM might be related to a genetic propensity for an inappropriate production of pro- or anti-inflammatory cytokines. It investigates polymorphisms in the genes of pro and anti-inflammatory cytokines of children dying from CM, and controls with NCM and COC. The results presented here support this hypothesis.

Quantitative cytokine analyses were not performed for several reasons: because of the instability of the cytokines in serum samples, the intermittent expression of cytokines in peripheral circulation, the unavailability of serum samples from this study population and the fact that serum concentrations do not adequately express the level of cytokine production in the local tissue. This study utilized the established relationships between cytokine gene polymorphism and cytokine production levels presented in the literature (Hutchinson et al. 1999, Wilson et al. 1997, Turner et al. 1997).

Tumor Necrosis Factor-alpha (TNF-α) Gene Polymorphism

TNF-α is a central mediator of inflammation and plays a pivotal role in the outcome of malaria. TNF-α functions to suppress parasitic growth, induces increased adhesiveness of endothelial cells for polymorphonuclear leukocytes, monocytes and macrophages, and induces the synthesis of other pro-inflammatory cytokines such as interleukin-1 (IL-1) and IL-6 (McGuire et al. 1994, Stuber et al. 1996, McGuire et al. 1999). The polymorphism investigated for the

TNF-α gene is located in the promoter region at position -308 relative to the start site of transcription. The substitution of guanine (G) by adenine (A) affects the regulation of the *TNFA* gene and is associated with increased cytokine production (Wilson et al.1997). The homozygous G/G at the position -308 is associated with low TNF-α production, while the homozygous -308 (A/A) and heterozygous -308 (G/A) genotypes are associated with high TNF-α production and the fatal outcome from severe malaria.

The patients in this study were categorized into the putative low or high TNF-α production phenotypes based on their allelic polymorphisms (Table 15). The observed frequencies between CM patients (70%) and controls (NCM [88%] and COC [50%]) for the TNF-α low production phenotype were not statistically significantly different. Nonetheless, the majority of children (70%) that died from CM had the genotype for low TNF-α production. These observation indicate that other factors were associated with their fatal outcome.

Interferon-gamma (IFN-γ) Gene Polymorphism

IFN-γ is the principal factor associated with the activation of macrophages which stimulates the secretion of various cytokines, especially TNF-α. None of the patients in this study demonstrated the homozygous genotype (+874 T/T) associated with high IFN-γ production, instead, the vast majority of patients in all three clinical groups demonstrated the putative low producer phenotype. There was no statistical difference between the groups (Table 16). These observations suggest that the genetic predisposition to produce low amounts of IFN-γ may

indicate an impairment in one arm of the immune system, e.g NK or Th1 cells.

Interleukin-6 Gene Polymorphism

Elevated IL-6 levels have been described in both human and murine CM, however, it has not been implicated in the pathogenesis of the cerebral complications. The bi-allelic G to C polymorphism in the promoter region at position -174 was reported to have functional importance in modulating the expression of the IL-6 gene. The -174 (C/C) genotype has been associated with low serum levels, while the C/G and G/G genotypes represent intermediate and high production phenotypes, respectively. This study did not detect any difference in IL-6 genotypes between patients and controls, all displayed the high producer -174 (G/G) genotype.

The unanticipated finding that every patient in the study population presented the same genotype, regardless to clinical group status is an enigma. It could mean that the IL-6 -174 gene locus is not associated with disease, just not associated with malaria. IL-6 downregulates the production of TNF-α and IFN-γ. These observations suggest that an elevated production of IL-6 may serve as a natural feedback or negative regulator for the secretion of IFN-γ.

Interleukin-10 (IL-10) Gene Polymorphism

The IL-10 gene is highly polymorphic and various haplotypes result in differential cytokine expression. This study analyzed three dimorphic polymorphisms within the IL-10 promoter region at positions -1082, -819 and -592 relative to the transcriptional start site, which have been linked to the

cytokine's production level (Pelletier et al. 2000). The patients in this study were categorized as either low, intermediate or high producers of IL-10 based on haplotype polymorphisms. The presence of guanine instead of adenine at position -1082 in the IL-10 promotor was associated with a higher IL-10 serum level (Maurer et al. 2000).

All three study groups, demonstrated the putative low or intermediate production phenotypes, predominantly (Table 17). There were no patients with the high production phenotype in the NCM group, while only one child in the CM and COC, respectively, displayed the haplotype associated with high production. There was a statistically significant difference between the low phenotype compared to the intermediate and high production phenotypes.

IL-10 is an anti-inflammatory cytokine, produced by Th2 lymphocytes. It suppresses cellular immunity, inhibiting pro-inflammatory cytokines while stimulating B cells and promoting humoral immunity. The significance of this observation is that a high IL-10 could hamper the secretion of pro-inflammatory cytokines, such as IFN-γ, by Th1 cells, causing a Th1/Th2 imbalance. This fact will be explored further in the conclusion chapter.

Transforming Growth Factor-beta Gene Polymorphism

Transforming growth factor-beta (TGF-β) is a pleiotropic, multifunctional cytokine considered to be a physiologic antagonist of TNF-α. Polymorphisms in the leader sequence of the *TGFB* gene at positions +869 and +915 are associated with the cytokine's expression. A substitution of thymine by cytosine

at position +869 relative to the start site of transcription changes the amino acid coding sequence. Position +869 makes up codon 10 of the amino acid sequence for leucine, and this substitution changes the amino acid from leucine to proline. In like manner, a substitution of cytosine by guanine at position +915 results in a change from arginine to proline at codon 25. For both polymorphisms, the allele encoding proline is associated with lower TGF-β synthesis. The combination of codons 10 and 25 result in naturally occurring haplotypes. There are three possible inheritable haplotypes for each codon, and nine haplotype combinations. These nine inheritable haplotypes can be grouped into three phenotypic categories based on in vitro cytokine production (Wilson et al. 1997, Pelletier et al. 2000).

The study groups were categorized as either low, intermediate or high producers of TGF-β based on their individual genotypes at positions 10 and 25. The data showed that the high production phenotypes greatly out numbered the frequency of low and intermediate production haplotypes in all three study groups (Table 18). Only one patient, a CM patient, displayed a polymorphism associated with low TGF-β production. All but one NCM patient, presented the putative high production phenotype, with the exception showing the intermediate production phenotype. Only one COC demonstrated the polymorphism associated with the intermediate production phenotype, while all others were deemed high producers.

TGF-β mediates the control and clearance of malaria parasites with direct effects on parasite sequestration by downregulating the expression of cell

adhesion molecules, ICAM-1 and VCAM-1 (Omer and Riley 1998). A crucial role for TGF-β is controlling the transition between pro- and anti-inflammatory cytokine responses during malaria infection.

In this study, the vast majority of the patients in all three study groups demonstrated the haplotype associated with the putative high TGF-β production phenotype. One supposition is that at the onset of infection, the elevated expression of TGF-β would be anti-inflammatory, inhibiting the actions of pro-inflammatory cytokines. Whether the TGF-β polymorphisms observed in this study are associated with the pathogenesis of CM cannot be concluded based on the results of this limited investigation. Based on these observations, this data indicates that there may be a propensity of an inappropriate cytokine expression. This will be explored further in the conclusion chapter.

Correlation of TNF-α with Anti-inflammatory Cytokine Polymorphisms

Synergism exists between certain cytokines, such as TNF-α and IFN-γ resulting in a much greater biologic effect than would be expected from either cytokine alone (Day et al. 1999). Arguably, one can hypothesize that synergistic cytokine effects may result from polymorphisms in the genes of specific cytokines. This study investigated the assumption that the interactive effects of combinations of genetic polymorphisms may affect clinical outcome. It analyzed the relationships between combinations of putative cytokine production phenotypes within and between the study groups.

Since the expression of TNF-α is documented to be associated with the

clinical outcome of CM, the data was re-categorized based upon the polymorphisms associated with low (-308 G/G) or high (-308 G/A) TNF-α production phenotypes. The three study groups were divided into six groups based upon TNF-α production and correlated with polymorphisms of anti-inflammatory cytokines.

Correlation of TNF-α Production Phenotypes with IL-10

The study population was re-categorized based on the (-308 G/G) allelic polymorphism associated with low TNF-α production, and analyzed by the low, intermediate and high IL-10 production phenotypes. A majority of cases in all study groups (CM 57%, NCM 86% and COC 67%) showed the low IL-10 production phenotype (Table 19). While most of the CM group presented the IL-10 low production phenotype, about one-third had a capacity for intermediate production, and one patient, demonstrated the IL-10 putative high producer phenotype. The NCM group had the highest percentage with the low IL-10 production phenotype.

The study groups were also divided into the TNF-α high producer phenotype and correlated with IL-10 polymorphisms (Table 20). Patients with a genetic predisposition to secrete increased amounts of TNF-α also, demonstrated a higher capacity to produce IL-10. In the CM and NCM groups, the vast majority (83% and 100%, respectively) demonstrated intermediate IL-10 production capacity, while, the COC was equally divided. A possible explanation is that TNF-α has the unique ability to upregulate IL-10 expression in human monocytes. It induces IL-10 mRNA translation and subsequent protein secretion

(Wanidworanun and Strober 1993).

The significance of these observations is that the two can function reciprocally. Elevated TNF-α can stimulate the production of IL-10, while, elevated IL-10 can downregulate the production of TNF-α (May et al. 2000). The inheritance of the genetic propensity to produce increased amounts of TNF-α may also be counterbalanced by a gene for enhanced IL-10 expression. However, when the gene for low TNF-α is inherited along with the gene for intermediate/high IL-10 production, this may represent an impaired Th1 arm of the immune system.

Correlation of TNF-α Production Phenotypes with TGF- β

The TGF-β putative low, intermediate and high production phenotypes were separated into two groups based on TNF-α production phenotypes. An unexpected finding of the study was that the majority of the patients had the genotype for high TGF-β production. There was a statistically significant difference observed with the inheritance of both the low TNF-α producer phenotype and the high TGF-β, while the high TNF-α control group failed show any significant difference (Table 21).

TGF-β is a pleiotropic cytokine that is pro-inflammatory at low concentrations and anti-inflammatory at high concentrations. At the onset of an infection at low levels, the inflammatory mediators function to eradicate the pathogenic organisms. Later in the infection at high concentrations, TGF-β downregulates the pro-inflammatory cytokines and the toxic effect that could

occur with their prolonged expression. TGF-β is the main negative regulator of macrophage activation. It deactivates macrophages and reduces their capacity to release peroxides (de Waal Malefyt et al. 1991). The correlation between low TNF-α and high TGF-β indicates a negative regulation on TNF-α production. It is speculative to suggest that this haplotype may protect against the pathologic effects of TNF-α overproduction.

Correlation of TNF-α Production Phenotypes with Anti-inflammatory Cytokines IL-10 and TGF- β

Both anti-inflammatory cytokines, IL-10 and TGF-β, were evaluated after grouping the study population into either TNF-α low or high production groups which generated highly significant (p=0.001) correlations (Tables 23 and 24). The anti-inflammatory function of IL-10 may not have been dominant. The patients had the genotype associated with low (45%), intermediate (50%) or high (5%) production. TGF-β appears to be the pivotal cytokine in these analyses. At high concentration it functions as an anti-inflammatory cytokines with the ability to inactivate macrophages and lymphocytes. Although high TNF-α producers can up regulate macrophage expression, the counter-effect by TGF-β is unknown. A NCM patient with the TNF-α high production phenotype had the low IFN-γ phenotype counterbalanced with high TGF-β and intermediate IL-10 phenotypes from anti-inflammatory cytokines. The macrophages accounted for 18% of the total cerebral leukocyte population, and only 24% were activated. This could have the effect of an anti-inflammatory cytokine predominance, resulting in dysfunctional macrophages and an immunologic imbalance.

Chapter 7

CONCLUSION

Chapter 7

CONCLUSION

The data from this dissertation indicated that the mononuclear cells detected in cerebral tissue were activated macrophages, and that there was a local production of TNF-α protein and mRNA. This would lead one to conclude that macrophages are the source of local TNF-α production. Patients with CM may be genetically programmed to produce low amounts of pro-inflammatory cytokines, TNF-α and IFN-γ, high IL-6 and elevated amounts of anti-inflammatory cytokines IL-10 and TGF-β. Following is a model that would fit these data.

Early in malaria infection, non specific mediators including, monocytes, macrophages, neutrophils, gamma:delta T cells (γδ) and natural killer cells (NK) are activated to control malarial parasitemia. The central mediator in CM appears to be the activated macrophage. Malarial antigens stimulate the macrophages to produce TNF-α and IL-12 which activates NK cells. The NK cells, along with γδ and Th1 cells, secrete IFN-γ which functions to transform the macrophage to full activation. The activated macrophages in turn secrete TNF-α, and other pro-inflammatory to suppress parasitic growth.

Macrophages play an essential role in both specific and nonspecific immunity against malarial parasites. The macrophage is able to undergo phagocytosis and some killing before full activation. However, after activated, macrophages acquire an enhanced capacity to perform complex functions attributable to their defense against malarial parasites, including enhanced

phagocytosis and killing, antigen processing and presentation to lymphocytes, and secretion of cytokines. After the phagocytosis and degradation of malarial parasites, macrophages process antigens which are then presented T helper cells by MHC class II molecules.

Malarial protozoa have evolved to either evade or resist the innate immune surveillance of mononuclear phagocytes. The data indicated that over 50% of the macrophages of CM patients were activated, yet the patients ultimately, died. Therefore, macrophage activation was apparently not the fundamental feature essential for protection. The macrophages may have become dysfunctional after phagocytosizing toxic malarial products, e.g. GPI and hemozoin, or downregulated by the deactivating effects of anti-inflammatory cytokines.

If cytokines play an important pathogenetic role, it is likely to be at the local tissue level, where *P. falciparum* schizogony occurs and parasitic products are released. The data showed the local production of TNF-α in cerebral vessels. Macrophages serve as a major source of TNF-α and other pro-inflammatory cytokines which function to inhibit parasitic proliferation. TNF-α augments killing of *P. falciparum* by activating neutrophils, monocytes and macrophages, enhancing phagocytosis, stimulating the production of other pro-inflammatory cytokines, and upregulating cell adhesion molecules.

The impact of the allelic polymorphism on the pathogenesis of severe malaria, is speculative. The genetic predisposition to produce low amounts of IFN-γ may indicate a defect in one arm of the immune system, such as Th1 cells

or NK cells. Allelic polymorphism associated with low IFN-γ production may cause in an underproduction of IFN-γ resulting in decreased amounts of activated macrophages. Fewer activated cells would lead to decreased parasite killing.

A Th1/Th2 lymphocyte imbalance may have been genetically selected for through cytokine polymorphisms. This would have the effect of downregulating the inflammatory mediators. IL-6 and IL-10, both produced by Th2 cells, can downregulate the expression of IFN-γ. The genetic propensity to produce elevated levels of IL-6 may inappropriately downregulate the production of IFN-γ by Th1 lymphocytes. An overproduction of IL-6 might ultimately inhibit Th1 cell expression resulting in a Th1/Th2 imbalance. An elevated expression of IL-6 may serve as a natural feedback or negative regulator for the secretion of IFN-γ. The polymorphism for elevated IL-6 production coupled with the genetic propensity for low IFN-γ production, may have resulted in the downregulation of pro-inflammatory cytokines during the inflammatory challenge. Thus, during the early immunologic response to malaria parasites, the genetic propensity to produce elevated levels of IL-6 may have inappropriately, downregulated the production of IFN-γ by NK cells and Th1 lymphocytes.

Anti-inflammatory cytokines function to downregulate the toxic effects of pro-inflammatory cytokines. IL-10 (produced by Th2 cells) suppresses Th1 cells (cellular immunity) and promotes humoral immunity. IL-10 normally functions to inhibit TNF-α overproduction induced by malarial parasites. Allelic polymorphism in the IL-10 genes of CM patients demonstrated, the genotype associated with

intermediate and high production. IL-10 may have functioned to downregulate pro-inflammatory cytokines that were also genetically predisposed for low cytokine production. Thus, IL-10 may have downregulated the secretion of IFN-γ, also crippling the activation of macrophages.

Early in infection low concentrations of TGF-β recruits monocytes and macrophages to the site of injury and activates phagocytosis. At low concentrations, TGF-β stimulates pro-inflammatory cytokines that control parasite growth. Later in infection, TGF-β downregulates pro-inflammatory cytokine responses to limit the development of inflammation-associated pathology (Omer et al. 2000).

TGF-β downregulates the expression of pro-inflammatory cytokines by inhibiting the production of IFN-γ and TNF-α while simultaneously upregulating IL-10. TGF-β also, functions as the main negative regulator of macrophage activation. The patients and controls in this study all demonstrated the genotype associated with the high TGF-β production phenotype. This would imply that TGF-β could have deactivated the macrophages and suppressed the T helper cells. In the midst of a massive parasitic challenge, it is tempting to speculate that the effects of TGF-β may have prevented the attainment of 100% macrophage activation in CM patients and controls.

The pathogenesis of CM is still incompletely understood, however it is known to be caused by a combination of factors. It maybe too simplistic to designate a single allelic polymorphism with all the pathologic manifestations of CM. Instead, a possible explanation is that the interactive effects of

combinations of genetic polymorphisms working in combination may be a major pathologic determinant. The synergistic effects of combinations of cytokine polymorphisms may have affected the clinical outcome of these children. The majority of the patients hd the genotype associated with the production of low amounts of pro-inflammatory cytokines, TNF-α and IFN-γ (which may have result in a weakened inflammatory response), elevated IL-6 (which may have functioned to downregulate pro-inflammatory and stimulate B cells), and elevated amounts of anti-inflammatory cytokines, IL-10 and TGF-β (which can inactivate macrophages, suppress T helper cells, and neutralize pro-inflammatory cytokines).

Results of this study demonstrated the local production of TNF-α in cerebral tissue along with activated monocytes and macrophages. Cytokine genotyping characterized CM patients with the genotype to produce low amounts of pro-inflammatory cytokines, TNF-α and IFN-γ, high IL-6 and elevated amounts of anti-inflammatory cytokines IL-10 and TGF-β. Whether these allelic combinations are contributory to the pathogenesis of CM warrants further study in a larger population.

APPENDICES

Appendix - A

Post Mortem Tissue Site and Labeling Codes

SITE	LABEL
Pituitary	A1
Frontal lobe	B1
Parietal lobe	B2
Temporal lobe	B3
Occiptal calcarine fissure	B4
Hippocampus	B5
Basal ganglia (caudate)	B6
Thalamus	B7
Midbrain	B8
Pons	B9
Medulla	B10
Cerebellum (peripheral)	B11
Cerebellum (dentatenucleus)	B12
Spinal cord	B13
Lung: RLL	D2
Lung: LUL	D3
Liver	F4
Right kidney	G1
Spleen	J2

Appendix - B

IMMUNOHISTOCHEMISTRY
MAC387, NCL-MACRO-3A5, CD63

Procedure for CD63

1. Block for endogenous peroxide in 3% Hydrogen Peroxide for 10 minutes at RT.
2. Rinse in running tap water to remove hydrogen peroxide for 5 minutes.
3. Place in Tris Buffered Saline for 5 minutes.
4. Perform Heat Induced Antigen Retrieval using 10 mM Citrate Buffer at pH 6.0 for 30 minutes in the steamer. Upon completion of the 30-minute incubation in the steamer allow the sections to stand m the jar for 10 minutes.
5. Rinse in three changes of distilled water.
6. Place in Tris Buffered Saline + Tween 20 for 5 minutes.
7. Incubate sections with Super Block for 5 minutes.
8. Rinse sections in two changes of TBS+TW20.
9. Incubate sections with Mouse anti-Human CD 63 diluted in normal antibody diluent@ 1:75 for 60 minutes.
10. Rinse sections in two changes of TBS+TW20.
11. Incubate sections with Biotinylated horse anti-Mouse for 30 minutes.
12. Rinse sections in two changes of TBS + TW20.
13. Incubate sections with ready to use Avidin-Biotin Horseradish Peroxidase labeling reagent for 30 minutes.
14. Rinse sections in two changes of TBS + TW20.
15. Incubate sections with (prepared just prior to use) NOVA RED substrate for 15 minutes.
16. Rinse sections in running tap water for 5 minutes.
17. Counterstain m Lerner 2 hematoxylin for 1- ½ minutes.
18. Differentiate in 1% Glacial Acetic water for 2 - 3 dips.
19. Blue in running tap water for 2 minutes.
20. Dehydrate through ascending grades of ethyl alcohol.
21. Clear in several changes of Xylene.
22. Coverslip with a synthetic mounting media.

Appendix - C

IMMUNOHISTOCHEMISTRY - TUMOR NECROSIS FACTOR-α

	Deparaffinize	3X in Xylene - 2 minutes each
	Hydrate	2X in 100% ETOH - 2 minutes each
		95% ETOH - 2 minutes
		Wash in dH_20 - 2 minutes
	ANTIGEN RETRIEVAL	Citrate buffer: Heat at 95°C for 25 minutes
	Cool	Cool slides for approximately - 20 minutes.
	Wash	3X - dH_20 - 2 minutes each.
	Endogenous Peroxidase	3 drop Peroxidase Block - 5 minutes
	Wash	Rinse PBS - Wash in PBS 2X - 2 minutes
	Serum block	3 drops Serum Block for 20 minutes. Drain
	PRIMARY antibody	3 drops PRIMARY Antibody - overnight at 4°C
	DAY TWO	
	Wash	Rinse PBS - Wash in PBS 2X - 2 minutes
	SECONDARY Antibody	3 drops SECONDARY Antibody for 30 minutes.
	Wash	Rinse PBS - Wash in PBS 2X - 2 minutes
	HRP-STREP COMPLEX	3 drops HRP-streptavidin complex - 30 min
	Wash	Rinse PBS - Wash in PBS 2X - 2 minutes
		<u>Prepare HRP Substrate</u> dH_20 1.6 ml 800 μl 10X Buffer 5 drops 100 μl 50X H_20_2 1 drop 40 μl DAB Chromogen 1 drop 40 μl (1 drop = 40 μl)
	HRP SUBSTRATE	3 drops HRP substrate mixture 10 minutes
	Wash	Rinse PBS - Wash in PBS 2X - 2 minutes
	Counterstain	Gills hematoxylin for 10 seconds. Wash
	Dehydrate	95% ETOH - twice for 10 seconds, each

Appendix - D

Human tumor necrosis factor alpha (TNFA) gene, allele TNFAp4, promoter region and partial CDS

LOCUS: HSU42625 597 bp DNA PRI 05-JUN-1996
DEFINITION: Human tumor necrosis factor alpha (TNFA) gene, allele TNFAp4, promoter region and partial CDS.
ACCESSION U42625
VERSION: U42625.1 GI:1353717
SOURCE: human.
ORGANISM: Homo sapiens (Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.)

Human tumor necrosis factor alpha (TNFA) gene, allele TNFAp4, promoter region and partial CDS
LOCUS: HSU42625 597 bp DNA PRI 05-JUN-1996
DEFINITION: Human tumor necrosis factor alpha (TNFA) gene, allele TNFAp4, promoter region and partial CDS.
ACCESSION U42625

BASE COUNT 154 a 185 c 151 g 107 t
ORIGIN

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1    ttcctgcatc ctgtctggaa gttagaagga aacagaccac agacctggtc
cccaaaagaa
61   atggaggcaa taggttttga ggggcatggg gacggggttc agcctccagg
gtcctacaca
121  caaatcagtc agtggcccag aagacccccc tcagaatcgg agcagggagg
atggggagtg
181  tgaggggtat ccttgatgct tgtgtgtccc caactttcca aatccccgcc
cccgcgatgg
241  agaagaaacc gagacagaag gtgcagggcc cactaccgct tcctccagat
gagctcatgg
301  gtttctccac caaggaagtt ttccgctggt tgaatgattc tttccccgcc
ctcctctcgc
361  cccagggaca tataaaggca gttgttggca cacccagcca gcagacgctc
cctcagcaag
421  gacagcagag gaccagctaa gagggagaga agcaactaca gacccccccct
gaaaacaacc
481  ctcagacgcc acatcccctg acaagctgcc aggcaggttc tcttcctctc
acatactgac
541  ccacggctcc accctctctc ccctggaaag gacaccatga gcactgaaag
catgatc
```

Appendix E

Protocol for the GeneQuant RNA/DNA Calculator/Spectrophotometer

1. Turn on machine from the rear, will read Instrument "Initializing."
2. DILUTION OF SPECIMENS (1:200)
   a. Pipette 199 μl ddH_2O in a microtube. Add 1.0 μl of specimen using the PA2 Pipetteman. Care should be taken to avoid any external drops of fluid on the pipette tip. Specimen dilution should be done in the biological hood.
3. INSTRUMENT SET UP
   a. Set parameters by pressing SET UP, then ENTER to accept the values. Parameters should read as follows:

Path length:	10
Use 320nm	Yes
Factor	dsDNA 50

   b. Press SET REF to default the rest of the unnecessary parameters.
4. CLEAN CUVETTE
   a. Before taking a reading, acid rinse the cuvette. Squirt dilute 0.5N HCl into the cuvette and discard. Rinse three times with ddH_2O.

PROCEDURE

1. To correct for background:
   a. Fill the cuvette with ddH_2O used to resuspend samples and cap.
   b. Press SET REF, screen will read "Please Wait."
   c. Then Insert Reference (i.e. ddH_2O blank).
   d. After reading is taken, screen reads Remove Reference.
   e. To confirm background correction, press ABS then SELECT to cycle through the wavelengths. All should read 000 Au.
   f. Remove ddH_2O with Pasteur pipette.
2. To read samples:
   a. Place the entire 200 μl sample into cuvette using Pasteur pipette
   b. Press SAMPLE, screen will read "Please Wait."
   c. Insert the Sample, when prompted.
3. The GeneQuant will read the sample. The screen will read "Remove Sample."
4. Data may now be collected by pressing these respective buttons:
   a. Press ABS, then press SELECT to cycle through wavelengths

b. PROTEIN (mg/ml)

c. PURITY (%)

d. RATIO (260/280)

e. Remove sample and rinse out cuvette.

5. When all reading have been taken, rinse out the cuvette first, with 0.5N HCl or 0.5N NaOH. followed by three rinses with ddH_2O, (in squirt bottles)

6. When finished, turn off the machine and return the cuvette to drawer.

Reference: Instruction Manual Gene Quant Calculator

Appendix F

DNA Extraction of Frozen Cerebral Tissue

Phenol:Chloroform:Isoamyl Alcohol Procedure

SPECIMEN NUMBER_____Date_____

Day 1		Prepare lysis buffer:	500 μl Tris HCl, 500 μl EDTA, 1.5 ml 3% SDS, 50 μl 2-mercaptoethanol, 2.45 ml dH_20
		Grind/macerate tissue:	Liquid nitrogen or sterile forceps
		Lysis Buffer:	Add 600 μl lysis buffer
		Proteinase K:	Add 12 μl proteinase K
		Vortex & Incubate: **Don't vortex after this step**	Vortex and incubate at 55°C for 3 - 24 hrs Time: Describe:
Day 2		Phenol:chloroform: isoamyl alcohol:	Add 600 μl Phenol:Chloroform:Isoamyl alcohol:
		Mix & Centrifuge	Mix gently 5 minutes. Centrifuge for 15-20 minutes at maximum speed
		Aspirate & transfer	Aspirate aqueous layer, transfer 300 μl to new microfuge tube
		Glycogen	Add 6 μl Glycogen
		Sodium Acetate	Add 10 μl NaOAc to tube
		100% ETOH	Add 600 μl, ice cold, 100% ETOH
		Incubate	Incubate in freezer overnight. Time:
Day 3		Warm	Bring tubes to room temperature, 15 minutes
		Centrifuge	Centrifuge for 2 minutes, at RT Describe:
		70% ETOH	Add 600 μl 70% ETOH - ice cold
		Centrifuge	Centrifuge for 2 minutes, at RT
		Aspirate	Aspirate supernatant, dry tube by inverting 5 minutes
		Desiccate	Dry tubes in desiccator for 45 minutes. Time:
		Reconstitute	Add 100 μl sterile, H_20 to resuspend the nucleic acids

Appendix G

DNA Extraction from Paraffin Embedded Cerebral Tissue with Xylene

Specimen Number:_____Date_____

Day 1		Tissue	Place 10 mm PET in microfuge tube.
		Xylene	Pipet 500 μl xylene into tube and rotate for 5 minutes.
		Centrifuge	Centrifuge for 2 minute at maximum speed. Dry
		Prepare lysis buffer:	500 μl Tris HCl 500 μl EDTA, 1.5 ml 3% SDS, 50 μl 2-mercaptoethanol, 2.45 ml dH_20
		Lysis Buffer:	Add 600 μl lysis buffer
		Proteinase K	Add 12 μl proteinase K
		Vortex & Incubate: Don't vortex after this step	Vortex and incubate at 55°C for 3 - 24 hrs Time: Describe:
Day 2		Phenol:chloroform: isoamyl alcohol:	Add 600 μl Phenol:Chloroform:Isoamyl alcohol:
		Mix & Centrifuge	Mix gently 5 minutes. Centrifuge for 15-20 minutes at maximum speed
		Aspirate & transfer	Aspirate aqueous layer, transfer 300 μl to new microfuge tube
		Glycogen	Add 6 μl Glycogen
		Sodium Acetate	Add 10 μl NaOAc to tube
		100% ETOH	Add 600 μl, ice cold, 100% ETOH
		Incubate	Incubate in freezer overnight. Time:
Day 3		Warm	Bring tubes to room temperature, 15 minutes
		Centrifuge	Centrifuge for 2 minutes, at RT Describe:
		70% ETOH	Add 600 μl 70% ETOH - ice cold
		Centrifuge	Centrifuge for 2 minutes, at RT
		Aspirate	Aspirate supernatant, dry tube by inverting 5 minutes
		Desiccate	Dry tubes in desiccator for 45 minutes. Time:
		Reconstitute	Add 100 μl sterile, H_20 to resuspend the nucleic acids

Appendix - H

DNA Extraction from Paraffin Embedded Cerebral Tissue Purgene Salt Extraction

Specimen Number:_____Date_____

Day 1		Tissue	Place 30 μm paraffin-embedded tissues in microfuge tube.
		Cell Lysis	Pipet 600 μl Cell Lysis Solution into tube and heat at 65°C for 15 minutes.
		Homogenize	Using sterile forceps, remove tissue and place in fresh tube of 600 μl Cell Lysis Solution. Homogenize tissue.
		Proteinase K	Add 12 μl Proteinase K Solution (20 mg/ml)
		Incubate	Vortex and Incubate overnight at 55°C. Time:
Day 2		RNASE A	Add 3 μl RNASE A Solution to the cell lysate.
		Incubate	Mix the sample for 5 minutes. Incubate at 37°C for 1 hour.
		Centrifuge	Centrifuge for 1 minute.
		Protein Precipitation Solution	Add 200 μl Protein Precipitation Solution to the microfuge tube.
		Vortex vigorously	Vortex vigorously at high speed for 20 seconds (important!!) to mix the Protein Precipitation Solution.
		Centrifuge	Centrifuge for 3 minutes at maximum speed.
		Aspirate	Pipet off aqueous phase, 150-300 μl to new 1.5 ml tube.
		Glycogen	Add 6 μl mussel glycogen (30 μg)
		100% ETOH	Add 600 μL of ice cold 100% ETOH (2X volume). Mix for 5 minutes
		Freezer	Incubate in freezer overnight at -20°C. Time:
DAY 3		Centrifuge	Centrifuge the samples at max speed for 5 minutes.
		Aspirate	Carefully remove the supernatant. Discard
		70% ETOH	Add 600 μl 70% ETOH (ice cold). Mix for 5 minutes.
		Centrifuge	Centrifuge at max speed for 5 minutes
		Aspirate	Aspirate the supernatant. Discard
		Desiccate	Dry the pellet in desiccator for 2 hours.
		Resuspend	Resuspend the dried pellet in 40 μL of sterile water.

APPENDIX I

DNA Extraction from PET with Chelex - BioTechnique Procedure

Specimen Number:_____**Date**_____

D a y 1		Xylene	Add xylene to PET slides for 5 minutes.
		Methanol	Add methanol for 5 minutes.
		Scrape	Scrape tissue off slides place in microfuge tube.
		Buffer	Add to the tube: 27 mL of TE buffer, and 3 µL of digestion buffer.
		Incubate	Vortex and Incubate overnight at 55°C.
D A Y 2			Remove from incubator. Bring to room temperature.
		10% Chelex	Add 30 µL of 10% Chelex-100 resin in TE to the tube. Make sure Chelex is in suspension.
		Boil	Place into boiling water bath for 10 minutes (Wrap tops of tubes with parafilm to prevent leaking during boil.)
		Centrifuge	Centrifuge for 10 minutes.
		Phenol: chloroform: isoamyl	Add 60 µL Phenol:Chloroform:Isoamyl
		Rotate	Invert several times, allow to rotate at RT for one hour.
		Centrifuge	Centrifuge for 15-20 minutes at full speed.
		Aspirate	Remove the aqueous phase to a clean 1.5 mL tube.
		Glycogen	Add 30 µg of mussel glycogen (0.5 µl to 300 soln)
		100% ETOH	100 µL of ice cold 100% ETOH.
		Freezer	Incubate in freezer overnight at -20°C.
D A Y 3		Centrifuge	Centrifuge the samples at max speed for 20 minutes at 4°C
		Aspirate	Carefully remove the supernatant. Discard
		70% ETOH	Pipet 100 µl 70% ETOH. Mix
		Centrifuge	Centrifuge at max speed for 20 minutes
		Aspirate	Aspirate the supernatant. Discard
		Desiccate	Dry the pellet at in desiccator for 2 hours.
		Resuspend	Resuspend the dried pellet in 40 µL of sterile water.

USE 5 µl OF SAMPLE PER PCR REACTION!!!!!

Appendix J

QIAamp DNA MINI KIT

SPECIMEN NUMBER_____Date_____

Day 1	Mince Tissue	Cut up tissue. Place into a 1.5-ml microcentrifuge tube
	ATL BUFFER	Add 180 μl of Buffer ATL
	Proteinase K	Add 20 μl Proteinase K, mix by vortexing
	Incubate	Incubate at 56^{0}C, overnight. Pulse cfg
Day 2	BUFFER AL	Add 200 μl Buffer AL. Vortex for 15 sec
	Incubate	Incubate at 70^{0}C for 10 min. Pulse cfg
	100% Ethanol	Add 200 μl 100% Ethanol, vortexing for 15 sec.
	QIAamp column	Pipet into QIAamp spin column. Label 2 collection tubes.
	Centrifuge	Centrifuge for 1 min at 8000 rpm.
	Collection tube #1	Place QIAamp column in a clean 2-ml collection tube
	BUFFER AW1	Add 500 μl Buffer AW1 without wetting the rim.
	Centrifuge	Centrifuge for 1 min. at 8000 rpm . Place QIAamp spin column in clean collection tube
	Collection tube #2	Place QIAamp spin column in clean 2-ml collection tube
	BUFFER AW2	Add 500 μl Buffer AW2 without wetting the rim.
	Centrifuge	Centrifuge for 3 min. Discard tube
	1.5 microfuge tube	Place spin column in a 1.5 ml microfuge tube
	Water	Add 100 μl distilled water.
	Incubate	Incubate at room temperature for 1 min
	Centrifuge	Centrifuge for 1 min.
	2nd Wash	Add 50 μl distilled water. Incubate 5 minutes. Cfg 1 min.
	3rd Wash	Add 50 μl distilled water. Incubate, centrifuge for 1 minute

Appendix K

DNA Extraction from Paraffin Embedded Cerebral Tissue with Chelex

(Diaz -Cano and Brady 1997 Procedure)

Specimen Number:_____Date_____

Day 1		Prepare Buffer	**PROTEINASE K BUFFER** Tris-HCl, 1 M 500 µl SDS, 10% 250 µl CaCl2, 10 mM 500 µl dH_20 3.75 ml
		Buffer	Add 600 µl Buffer to tube
		Glycogen	Add 6 µl Glycogen
		Proteinase K	Add 12 µl Proteinase K
		Incubate	Vortex and Incubate overnight at 55°C for 5 days.
Day 2-7			Add 6 µl Proteinase K daily for 5 days
DAY 7			Remove from incubator. Bring to room temperature.
		10% Chelex	300 µL of 10% Chelex to two tubes. Chelex in suspension.
		Boil	Boil for 10 minutes (Wrap tops of tubes with parafilm)
		Phenol: chloroform: isoamyl	Add 600 µL Phenol:Chloroform:Isoamyl (equal volume) Vortex vigorously for 15 sec. Rotate at RT for 10 min.
		Centrifuge	Centrifuge for 5 minutes at full speed
		Transfer	Pipette aqueous phase into a clean 1.5 ml tubes.
		Back Extract	Add 300 µl Ammonium acetate, vortex and centrifuge
		Transfer	Pipette aqueous phase into a clean microfuge tube
		Phenol: chloroform: isoamyl	Add 600 µL Phenol:Chloroform:Isoamyl to aqueous phase for 2nd extraction Vortex vigorously for 15 sec. Rotate at RT for 10 min.
		Transfer	Transfer aqueous phase to new tube
		100% ETOH	Add 600 µL of ice cold 100% ETOH. (2 volumes)

D a y 8		Freezer	Incubate in freezer overnight at -20°C.
		Centrifuge	Centrifuge the for 15 minutes.
		Aspirate	Carefully remove the supernatant. Discard
		70% ETOH	Add 600 µl 70% ETOH (ice cold). Mix
		Centrifuge	Centrifuge at max speed for 5 minutes
		Aspirate	Aspirate the supernatant. Discard
		Desiccate	Dry the pellet in desiccator for 2 hours.
		Resuspend	Resuspend the dried pellet in 40 µL of sterile water.

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