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Cerebral Malaria

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Malaria infection of the Central Nervous System (CNS) can cause a severe neurological syndrome termed Cerebral Malaria (CM). The central neuropathological feature of CM is the preferential sequestration of parasitised red blood cells (PRBC) in the cerebral microvasculature. The level of sequestration is related to the incidence of cerebral symptoms in severe malaria. Other neuropathological features of CM include petechial hemorrhages in the brain parenchyma, ring hemorrhages and Dürck's granuloma's. Immunohisto-chemical and electron microscopy studies have shown widespread cerebral endothelial cell activation and morphological changes occur in CM, as well as focal endothelial cell damage and necrosis. The immune cell response to intravascular sequestration appears to be limited, although activation of pigment-phagocytosing monocytes is a late feature. The mechanisms by which PRBC cause coma in malaria remain unclear. *In vitro* parasitised erythrocytes bind to endothelial cells by specific, receptor mediated interactions with host adhesion molecules such as ICAM-1, whose expression on cerebral endothelial cells is increased during CM as part of a systemic endothelial activation. Induction of local neuro-active mediators such as nitric oxide and systemic cytokines like TNFα may be responsible for the rapidly reversible symptoms of the coma of CM. The recent cloning of the parasite ligand PfEMP-1, thought to mediate binding to host sequestration receptors, promises further insight into the relationship between patterns of sequestration and the incidence and pathogenesis of coma in cerebral malaria.

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Introduction

The Clinical Syndrome of Cerebral Malaria. Malaria remains a major health care problem in many parts of the world, exacting a high cost in health, economic and social terms on many developing countries. Human malaria infection is caused by four *Plasmodium* parasites, of which *P. falciparum* is the commonest and most serious pathogen. Infection can be asymptomatic or lead to a mild febrile illness, but a small minority of patients suffer severe complications affecting a number of organs. The best known of these potentially fatal sequelae of severe disease is Cerebral Malaria (CM). This review will limit itself to describing the neuropathological features of cerebral malaria as caused by *P. falciparum*, and investigating our current understanding of the pathophysiological mechanisms thought to underlie this syndrome.

Variation in the clinical presentation of severe malaria has caused problems in precisely defining the nature of the disease. Work in clinical centers around the world during the 1970's and 80's sought to define strict diagnostic and prognostic features of severe disease to enable accurate clinical diagnosis, treatment and research. This culminated with a set of guidelines published by the World Health Organization (106). The definition of severe malaria according to these guidelines is the appearance of one or more severe complications in addition to a positive blood smear diagnostic of malaria infection. The complications include severe anemia, acidosis, respiratory distress, jaundice, renal failure, disseminated intravascular coagulation, hyperparasitemia, shock or cerebral malaria.

The most important complication of severe disease is cerebral malaria (102, 103). This is a neurological syndrome consisting of a diffuse, potentially rapidly reversible encephalopathy associated with loss of consciousness and fitting. The level of consciousness can range from confusion or stupor to coma. The patient can become unconscious very quickly and is unresponsive to pain, visual or verbal stimuli. There are often fits which can be prolonged as status epilepticus, but few localizing neurological signs (such as weakness, hemiplegia or cranial nerve defects) to indicate pathology in a specific part of the brain, although isolated cases have been reported (45). There have also been reports of a specific cerebellar syndrome both during and after disease (18).

Estimation of the level of consciousness uses the Glasgow Coma Score (or modified Blantyre score for children) to measure responses to verbal and painful stimuli, with cerebral malaria defined by a score of less than 11/15. Localizing neurological signs are occasionally present but by no means common although the presence of global signs such as extensor or flexor posturing, opisthotonus and muscular rigidity are associated with a poor prognosis (60). One striking clinical finding is the apparently rapid reversibility of cerebral symptoms. A child in a deep coma may be fully conscious and alert a matter of hours later. Seizures in cerebral malaria are common and also associated with a poor prognosis. A large EEG study carried out on Kenyan children indicates a high rate of sub-clinical status epilepticus which can be mistaken for coma (Dr J. Crawley, personal communication).

Cerebral malaria generally has a poor prognosis, with mortality of 30-50% even with active treatment and support (13, 37). There has been debate as to the rate of permanent neurological complications following recovery from cerebral malaria, which was initially thought to be very low (82). Careful follow up studies have shown that it is higher than originally thought, with up to 10% of patients suffering some sort of neurological impairment ranging from weakness and hearing impairment to severe symptoms such as quadriplegia, epilepsy and cortical blindness (12, 13). In addition there are possible unmeasured longer term consequences such as subclinical learning difficulties in children who recovered from infection.

Severe malaria is uncommon compared to mild or asymptomatic malaria infection (56). However, its prevalence in Africa, Asia and South America and the high mortality rate from cerebral malaria ensure that it remains a major health problem. Estimates of the incidence of cerebral malaria are difficult to acquire but some figures suggest that it may be responsible for the death of up to 0.5 million children every year in Africa alone (57, 105). Thus research into the spectrum of disorders which make up severe malaria has focused on the syndrome of CM. In consequence we have a better idea of how malaria affects the brain than other organs such as the lung or kidney, although our understanding of the pathophysiology of cerebral malaria remains inadequate.

The Neuropathology of Malaria

The first modern studies of the pathological features of malaria were published in the late 19th century. As mentioned in the introduction to this Symposia the marshes of Italy provided a fertile breeding ground for the *Anophalene* mosquito, and the illness was prevalent around Rome at this time. Consequently the seminal works in this field were produced by Marchiafava and colleagues (11, 54, 55). Since then the pathological anatomy of malaria has been studied in different populations by a number of authors (1, 2, 19, 23-26, 28, 32, 47, 50, 52, 61, 68, 73-75, 86, 87, 89, 92, 94, 98, 100, 108). Several factors have made these studies difficult. The availability of tissues from post-mortems has been restricted because malaria mainly occurs in developing countries where religious and cultural objections to autopsy persist. In addition detailed clinical data have not always been available to compare with the pathological findings, and given the heterogeneity in clinical presentation this has hampered clinico-pathological correlations.

The common feature of these different studies has been the heterogeneity in neuropathological findings. This is in part due to variations in the time to death, degree of treatment and supervening pathology from severe malaria in other organ systems. There is also a wide geographical variation in the clinical presentation of severe malaria in terms of the prevalence and severity of the neurological manifestations compared to the other syndromes of severe disease such as renal failure and anemia (58). This may in part be due to geographical strain variations in parasite virulence, or host immunity. Where pathological changes occur they are often non-specific and even these changes, such as petechial parenchymal hemorrhages and edema, seem to vary both within the brain in a particular patient and between different cases. However, certain features do recur.

Sequestration. Erythrocytes infected with the late maturing stages of the *Plasmodium* parasite (the trophozoite / 'ring' stages and schizonts) disappear from the free circulation, causing a drop in the observed peripheral parasitemia. These parasitised red blood cells (PRBC) become preferentially localized in the deep vascular beds of vital organs, a process termed sequestration (10, 59, 71, 104). The sequestered parasites accumulate in the brain, lung, gut and heart, and have been recorded to a differing extent in many other organs of the body in different cases. Sequestration was initially thought to be limited to capillaries rather than arteries or veins, but several studies have shown adherence of PRBC to the larger calibre venules of the cerebral circulation. Sequestration of parasitised erythrocytes in a cerebral microvessels from fatal cases of CM is shown in figures 1 and 2.

There has been great debate as to the specificity of cerebral sequestration in cerebral malaria as opposed to non-cerebral cases. The first pathological studies of tissues from cases of fatal malaria demonstrated that this process occurred preferentially in the brain in cases of cerebral malaria, and offered this as a putative explanation for the development of coma in these cases (55). Quantitative studies of sequestration in different organs from fatal cases confirmed that sequestration of PRBC in the cerebral microvasculature was significantly associated with clinical

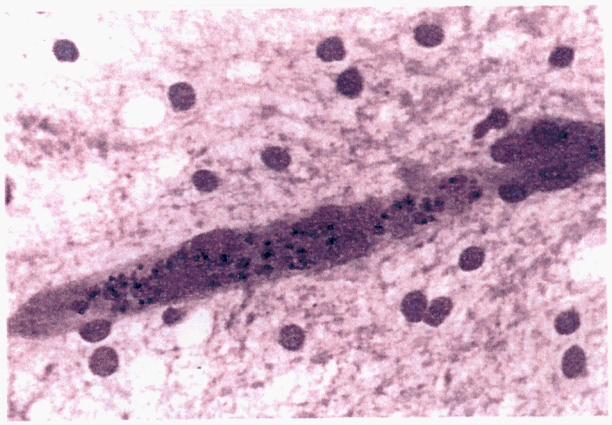

Figure 1. A cerebral microvessel showing sequestration of parasitised erythrocytes (Giemsa stain, x100)

cerebral malaria (52, 74). Sequestration in the brain was higher in these patients than in other organs, thus the clinical syndrome of cerebral malaria appeared to be associated with cerebral sequestration. However the specificity of sequestration in the brain to CM and the link between sequestration and the incidence of coma has been questioned, and some authors have felt that cerebral sequestration also occurs in non-cerebral malaria (28, 47, 73, 86, 89). This has led to an alternative proposal that coma is in fact unrelated to parasite sequestration, which is merely an epiphenomenon.

A recent study of 50 adult Vietnamese patients has helped clarify this issue (Turner et al in preparation). The histological observation of PRBC sequestration in cerebral microvessels is clearly related to time to death, both due to the duration of drug treatment before death and natural immune clearance of the parasite. In rigorously defined groups of cerebral and non-cerebral cases there was definite evidence for PRBC sequestration in some non-cerebral cases, and all cerebral cases, who died soon after admission to hospital (and thus after a shorter duration of illness and treatment). Late deaths in both groups showed no observed sequestration in either group, although there were signs of past PRBC localization such as malaria pigment deposition. Quantitatively there was significantly more sequestration in the brains of cerebral than non-cerebral cases. These results indicate that, in this adult Asian group, cerebral sequestration is not exclusively limited to cerebral malaria, but is also seen in the brains of some cases who did not develop the neurological complications. However, quantitatively there are more PRBC in the brains of CM, and no case of CM was seen without cerebral sequestration. Thus it seems that sequestration of PRBC is necessary but not solely sufficient to cause cerebral malaria. Similar studies have not been conducted in African patients with CM. They represent a younger age group due to endemic exposure to the disease from birth causing progressive development of protective immunity. However, a consensus of pathological studies from a

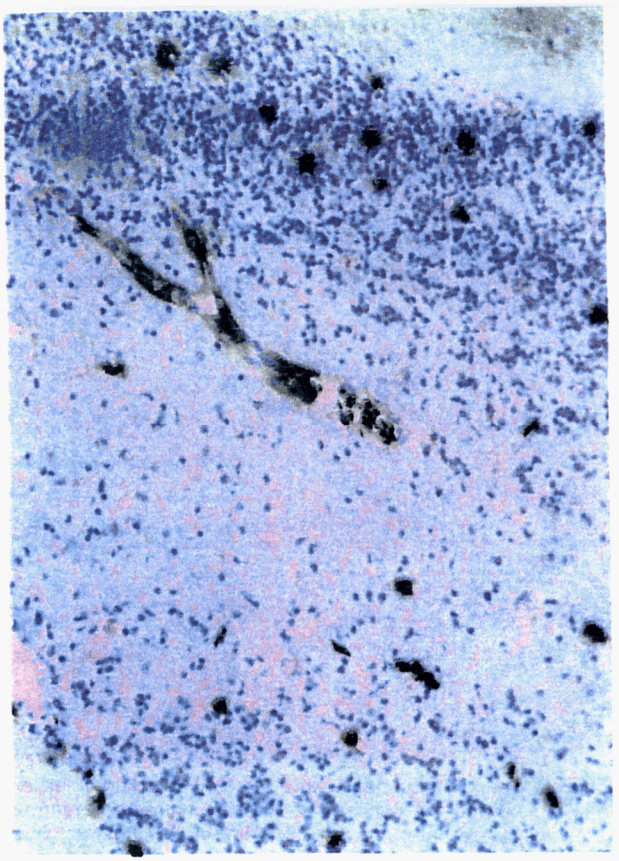

Figure 2. Immunohistochemical staining of brain from a case of cerebral malaria with labelling of sequestered PRBC by a monoclonal antibody against the *P. falciparum* MSP-1 antigen (Indirect peroxidase method, hematoxylin counterstain, x 63)

variety of populations favor the process of PRBC sequestration in the brain as a common feature of cerebral malaria.

Sequestration of PRBC does not seem to be uniform within the brain. Aikawa's group have proposed preferential sequestration in white versus grey matter (61), and differential sequestration in the cerebellum compared to the cerebral cortex in both simian models and man (84, 85). They have suggested that this difference may underlie the occurrence of cerebellar symptoms in disease. The cases of CM from Vietnam also show differential rates of sequestration within different areas of the brain, with the cerebral cortices and cerebellum being preferentially affected when compared to mid-brain structures or the brainstem (Turner et al in preparation). This implies that coma is not solely related to the effects of specific brainstem sequestration as opposed to cortical or cerebellar disease.

Hemorrhages. There are two types of hemorrhage in the brain in malaria; punctiform and ring hemorrhages. Punctiform or petechial hemorrhages of normal erythrocytes surrounding a ruptured cerebral vessel are common in cerebral malaria, and have been used as a diagnostic feature of the disease in macroscopic examination of the freshly cut post-

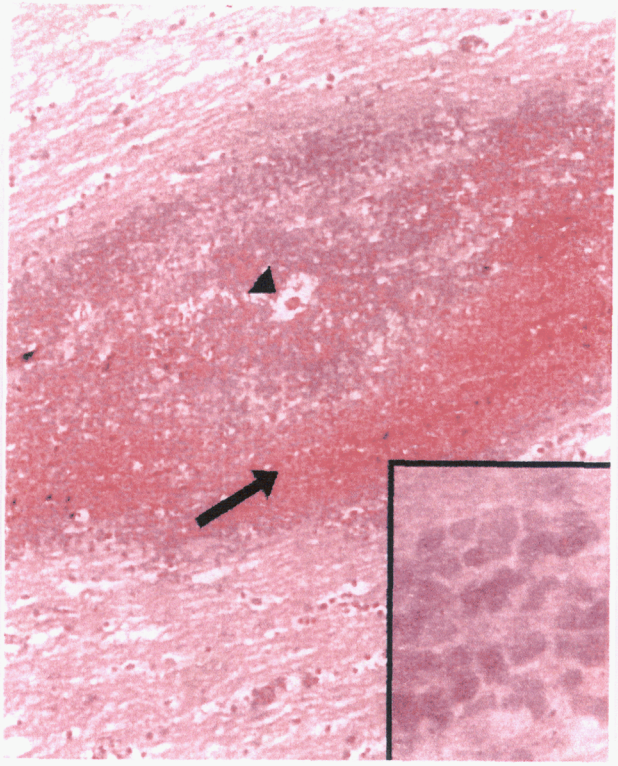

Figure 3. A ring hemorrhage. Note the central ruptured vessel (arrowhead) and the concentric rings of uninfected erythrocytes (inset) and outer ring of PRBC (arrow), free pigment and host monocytes (H&E, x40).

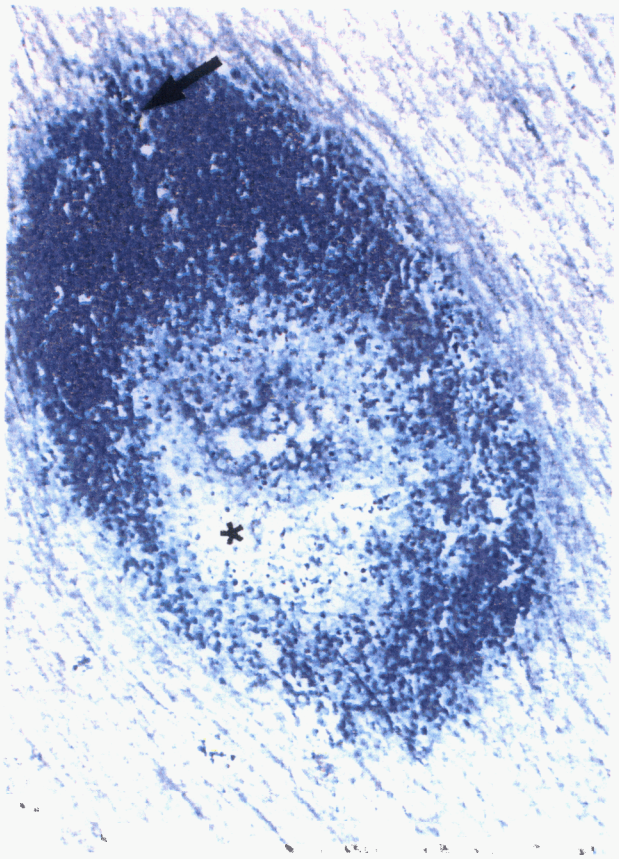

Figure 4. A ring hemorrhage, stained with the Luxol blue/cresyl violet method to demonstrate the focal gliosis (star) around the vessel, which is seen entering the lesion (arrow) (H&E, x40)

mortem brain. There is a reported predilection for the white matter, as opposed to the grey matter distribution of some encephalitides (which illustrates the non-specific nature of these hemorrhages to malaria). The presence of petechial hemorrhages in the brain appears to be linked to the prevalence and distribution of sequestration, e.g. they occur more frequently in areas of high sequestration and are more frequent in cerebral than non-cerebral malaria. However, these hemorrhages can be seen in other organs in malaria, such as the kidney and heart, and are occasionally seen in the brain in cases of non-cerebral malaria. They are also present in several other unrelated diseases, such as terminal asphyxia, barotrauma, and carbon monoxide poisoning. This would seem to argue that petechial hemorrhages are probably a reflection of a pathological process, presumably in part related to hypoxia, which may be accentuated by sequestration during malaria and cause their increased frequency in CM.

In contrast the ring hemorrhage is a neuropathological feature unique to malaria. The lesion consists of a series of concentric rings surrounding a central necrosed cerebral vessel. The outermost ring contains a mixture of parasitised erythrocytes, free pigment and host monocytes, with an inner layer of uninfected erythrocytes and gliosis surrounding the vessel. The features of the ring hemorrhage are shown in figures 3 and 4. This distinctive pattern may indicate the pathogenesis of this lesion to be a 're-perfusion' type injury, where renewed and perhaps unregulated restoration of flow to a cerebral microvessel previously filled with sequestered PRBC and host monocytes causes rupture of the damaged vessel, forcing the contents out into the brain parenchyma followed by a leakage of unparasitised erythrocytes. Again these lesions can occur in all parts of the brain, but have not been reported in other organs. They are not limited to CM, and have been observed less frequently in the brain of cases of NCM, which would be compatible with the low but definite rates of sequestration seen in some brains from patients who do not develop cerebral symptoms or coma before death.

The other lesion peculiar to the brain in malaria is the Dürck's granuloma. In his treatise on the origin of the cerebral granuloma which now bears his name (although it had been recognized by Marguelis in 1914), Dürck described multiple circumscribed diffusely scattered cellular reactions in the brains of fatal malaria cases. He attributed this reaction to glial cell proliferation associated with ruptured vessels and necrotic areas caused by hemorrhage, which represented an inflammatory defense process. An example of a Dürck's granuloma from a case of fatal cerebral malaria is shown in figure 5. Various theories

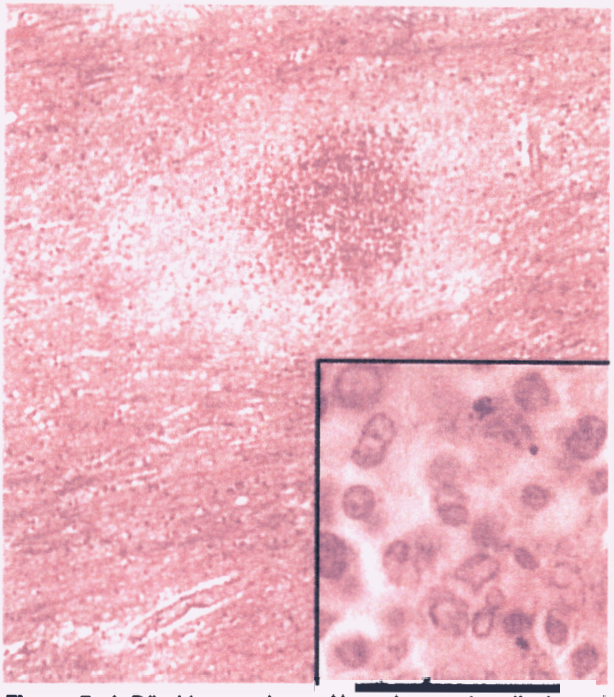

Figure 5. A Dürck's granuloma. Note the patchy gliosis and extravasated host monocytes phagocytosing pigment (inset) (H&E, x40)

have been proposed to explain the genesis of this lesion, such as cerebrovascular leakage of plasma proteins and platelets (69, 75). Rigdon clearly delineated simple 'petechial' hemorrhages and the so-called 'ring' hemorrhage from the cellular granuloma which he felt did not necessarily have to be associated with pre-existing hemorrhage (75). Once endothelial cell damage had allowed the leakage of exudate, cells and parasites into the perivascular space there would be a glial and immune reaction forming a granuloma. An alternative explanation is that ring hemorrhages and granuloma's represent a temporal spectrum of the same lesion. Granulomas occur in proximity to ring hemorrhages, which can themselves show differences in their size and maturation indicative of temporal development within an affected brain. Granuloma's have not been reported in the absence of ring hemorrhages and certain features are similar between the two, such as the patchy gliosis surrounding a necrotic ruptured vessel, the presence of parasite debris in the parenchyma and a host reaction to it (i.e. host monocytes and glial cells phagocytosing pigment). Granulomas may thus simply be what remains after the red cells, infected and uninfected, are cleared from a hemorrhage and this begins to be organized by the host response. Similarly, petechial hemorrhages may be the result of vessel rupture in areas of no sequestration, where parasites and their products cannot, or have not yet, elicited any host reaction. If the two share a common cause, then both may show extravascular iron deposition, parasite antigens and host leukocytes. However, such detailed studies on the genesis of these lesions have not yet been conducted.

Cerebral Endothelial Cell Changes. Malaria is principally a hematogenous infection, and sequestration of PRBC in the brain is an intravascular process with entry of parasites into the brain parenchyma being very rare. This means that the cerebral endothelial cell (EC) is a key interface between the blood space and the brain in CM. Focal areas of endothelial cell degeneration, hyperplasia and necrosis can be seen in CM, frequently in areas where hemorrhages and granulomas are also found. However such changes may be secondary to local ischemia and reperfusion, and it is clear that PRBC adhesion to cerebral endothelial cells does not inevitably cause destruction of the EC. Several different mechanisms have been proposed to account for both reversible and permanent damage to EC function in CM, including hypoxia due to sequestration, intravascular complement activation and vasculitis, metabolite competition or local parasite toxin release. Intra-luminal deposition of fibrin-platelet thrombi is a variable feature, although some authors feel that it plays a major role in EC damage in CM (73, 95).

There is immunohistochemical evidence for EC activation during CM in humans (67, 98) and both murine and simian models of the disease (1, 2, 107). Ultrastructural studies of EC in human CM (52) have shown morphological changes of activation with membrane ruffling, lipid droplet accumulation and formation of pseudopodia which are seen to interact with host leukocytes and PRBC. Simian models of CM (53, 76) have revealed the capacity of EC to phagocytose PRBC. Clearly the interaction of endothelial cells and parasitised erythrocytes and their role in transduction of stimuli to the brain microglia will be an important area of future research.

Focal Brain Ischemia And Neuronal Cell Loss. Patches of parenchymal necrosis and peri-vascular edema, along with neuronal cell loss, have been thought to represent the neuropathological correlates of ischemia secondary to sequestration. However most neuropathological studies have noted that the degree of ischemic change in the brain in CM is not great enough to account for coma as a purely 'hypoxic' event. The reversibility of coma in CM and the large numbers of patients who recover complete neurological function would argue against widespread permanent hypoxic damage, or reperfusion injury, as a pathological mediator in most cases of CM. How a vessel which is completely blocked by PRBC could continue to allow adequate oxygen perfusion to the surrounding brain is an intriguing question.

Neuronal cell loss has not been a notable feature in most neuropathological studies of malaria. Rigdon mentioned two types of pathological changes, the first being degeneration of brain cells and the second

focal degeneration of brain tissue. Marchiafava and Bignami had reported chromatolysis in brain cells and related this to several cases where bulbar (brain stem) symptoms were most severe or longer-lasting after recovery. Rigdon concentrated on depletion of the Purkinje cells of the cerebellum, relating this to chronic neurological sequelae suffered after the acute malaria attack, although his studies concentrated on animal models of the disease and did not attempt to allow for the superadded effect of terminal hypoxia on Purkinje cell loss. Little else has been reported on the subject of neuronal toxicity until recently, when circulating antibodies in sera from human cerebral malaria patients have been found to inhibit Purkinje cell growth *in vitro* (14).

Cerebral Edema and Raised Intracranial Pressure. Cerebral edema was thought to be a major factor in contributing to morbidity and mortality during cerebral malaria. However the most recent radiology and pathology studies imply that the majority of patients, even those who die, do not have a significant degree of edema. On the basis of radiography, intracranial pressure monitoring and some pathological data a subset of patients do clearly suffer severe cerebral edema which can be associated with brainstem displacement and death, or if survived, is associated with permanent neurological deficit after recovery (49). Although treatment with mannitol helped reduce intracranial pressure in some of these patients the effects were not long-lasting and the rises were difficult to control (64). A study of the efficacy of corticosteroid treatment in adult Thai groups of cerebral and non-cerebral malaria found them to be deleterious in cerebral malaria (101), in contrast to its protective role in childhood meningitis for instance. Thus although most patients seem to be able to survive cerebral malaria without developing cerebral edema, a subset do develop this complication in a very aggressive form, which is associated with a poor prognosis.

Pathological studies would seem to support this, as the incidence of widespread interstitial cerebral edema as judged by brain weights, macroscopic and histological criteria is low in both Adult Vietnamese patients (Turner et al in preparation) and African children (50). The occurrence of localized areas of perivascular edema is a more common, though still a focal feature. Some authors believe this to be a major neuropathological feature of CM which reflects a widespread disturbance in blood brain barrier permeability (73). However the role of post-mortem retraction artefact in creating this appearance has not been adequately addressed.

Deposition and Phagocytosis of Malaria Pigment. Even when a patient survives the course of CM long enough to receive treatment, they may still remain in coma and die with little histological evidence of PRBC sequestration in the brain. However both light and electron microscopy will confirm the presence of malaria pigment in the brain. This remains free in the circulation, or can be phagocytosed by circulating blood monocytes and neutrophils, cerebral endothelial cells and is most frequently seen in the meninges and choroid plexus. It also remains in the ghosted erythrocyte membranes which continue to adhere to EC's after schizogony and rupture of the infected erythrocyte. It has been shown that pigment load in the body (as measured by leukocyte phagocytosis of pigment granules) is a significant prognostic feature, and may reflect the previous parasite load (65). Whether the pigment which remains in the brain influences the course of disease by stimulating cytokine release from monocytes or glial cells is unknown, but phagocytosis of pigment by peripheral blood monocytes has both toxic and stimulatory effects. One animal study has suggested that some neurons can phagocytose malaria pigment (42), but most pigment seen in CM is in the vascular space or phagocytic cells associated with it.

Summary of Neuropathology of Malaria. Most of the neuropathological changes in CM have been recognized since the earliest studies of Marchiafava, and subsequent studies have differed only in their interpretations. One reason for this is the variation between the findings in different cases, even when clinical criteria are strict. The weight of evidence of these studies does allow us to draw some conclusions as to the pathogenesis of cerebral malaria. Firstly there is little evidence for embolic disease as a cause of cerebral symptoms, and widespread vascular thrombosis or demyelination have also been excluded. The lack of ischemic change also rules out hypoxia as a major mechanism of neuronal damage. The variability in pathological findings and causes of death within the clinical grouping of 'cerebral malaria' suggests that there are different groups within this category, including those patients who are susceptible to developing cerebral edema and have a poor prognosis.

The Role of Animal Models In Examining the Neuropathology of Cerebral Malaria

Several animal models of CM have been developed to study the neuropathology of disease in a more controlled setting. These are predominantly either the murine or simian models, which use a number of different combinations of host and parasite species. The main rodent models for malaria are the rat -*Plasmodium chabaudi* model and infection of inbred CLB, CBA or BALB/c mice with *P. yoelli* or *P. berghei*. *P. berghei* strain ANKA infection of CBA mice is reported to lead to 'cerebral malaria' in that the mice develop neurological symptoms and the great majority die (95%). There is histological evidence of cerebral edema, petechial hemorrhages and sequestration of host monocytes in cerebral vessels (63). There is evidence for hemiplegia, fitting and coma and this has been proposed to validate this combina-

tion as a model of human cerebral malaria. Hunt's group have examined the function of the blood brain barrier in a mouse model using direct measurement of dye extravasation and morphological changes in a retinal wholemount preparation (17). Evan's blue dye extravasation and focal petechial hemorrhages in the brain parenchyma were observed in mice who succumbed to a neurological syndrome with some similarities to human cerebral malaria. Hemorrhages and fluorescent dye extravasation were also observed in the retinal model, used as a way of examining the function of the retinal blood barrier. These findings imply that the blood brain barrier is compromised in the mouse model, although an important difference exists between this and the human disease, namely the sequestered cells, which are host leukocytes rather than infected erythrocytes.

The fact that it is host immune cells which sequester has led to some disagreement about the relevance of the rodent model to the human situation, and it seems clear from further studies that there is a degree of auto-immune disease occurring in the mouse. The release of systemic cytokines, especially TNFα, is important in this process (35), and seems to be linked to activation of host CD4+ T-lymphocytes (34). Impairment of host CD4+ cells due to murine AIDS also protects mice against the effects of cerebral malaria (27). Damage to the brain appears to be mediated in part by the sequestered neutrophils, because antibody treatment or neutrophil depletion decreases brain pathology (29). Therefore this model has been useful in examining the contribution of the host immune system and cytokines to the pathophysiology of disease, but there are dissimilarities between the pathological details which imply important differences between murine and human disease.

There have been notable advances in developing monkey models in the past 10 years, mainly due to the work of Aikawa and Guysin. They have reported infection of Aotus, Owl, Japanese and Rhesus monkeys with both *Plasmodium coatneyi* and *Plasmodium fragile* (30, 46, 53). This has led to re-examination of an older primate model developed in the 1970's, the squirrel monkey infected with the human malaria *Plasmodium falciparum* (38).

Some, but not all, of these combinations produce a very close match of human severe malaria with cerebral involvement. Clinically the monkeys develop hyperparasitemia, anemia and are severely ill, often dying from infection with a proportion of cases showing cerebral involvement. Splenectomy of the animal influences the course of infection and reduces cerebral sequestration. Histologically they show circulating parasitised erythrocytes expressing knob proteins and rosetting, and cerebral sequestration of PRBC. Parasites taken from the monkeys have been shown to cytoadhere to endothelial cell cultures *in vitro*, and immunohistochemistry has revealed the expression of antigens on cerebral microvessels in infected animals which cross react with antisera to human TSP, CD36, ICAM-1 and E-selectin, which are known to be host sequestration receptors in the human (53). However, the clinical picture is not identical to that in humans, in that it lacks notable pathology in other organs, despite appreciable sequestration in other tissues such as the lung and heart. Neurological symptoms and cerebral pathology also vary, with cerebral edema being common in the Saimiri but not Rhesus monkeys.

In conclusion animal models of malaria have provided evidence for the possible major factors in the pathophysiology of malaria, namely sequestration, cytokine release and the role of the host immune system. The monkey models mirror human infection closely but are impracticble for the generation of transgenic animals to examine the effect of specific gene 'knockouts.' The mouse model would be ideal for this purpose but has important differences in the role of receptor-mediated sequestration in disease. Most of the data from these models appear to support the possibility that host molecules could support adhesion of parasitised erythrocytes in organs which are affected during malaria.

The Pathophysiology of Cerebral Malaria

The main contribution of neuropathological data on CM to our understanding of the pathogenesis of the disease concerns the relationship between parasite sequestration and coma. It is clear that whereas sequestration is necessary to cause coma, coma does not always follow in the presence of sequestration. Several other factors have been proposed which may influence the course of an individual infection. Research on parasite virulence factors has centered on the ability of parasites to adhere to other cells (cyto-adherence) (9, 40, 71) and the induction of soluble circulating host mediators (cytokines and nitric oxide) by parasite products (20, 21, 44). Host factors such as genetic polymorphism may also underlie variation in disease. Well known genetic polymorphisms between populations (such as the incidence of red cell disorders like sickle cell and thallasemia) are known to be associated with resistance to malaria infection. The search for host genes (such as specific HLA types) which influence susceptibility to severe malaria is already underway (39) and a polymorphism has recently been discovered in the promoter region of the TNFα gene (57), which is associated with susceptibility to CM.

Two main theories are currently being tested to explain the clinical and pathological features seen in cerebral malaria. The two alternative hypotheses are that the coma of cerebral malaria is either due to local parasite sequestration, or a 'toxic' phenomenon due to the systemic release of parasite or host mediators. The truth probably lies in a combination of these processes.

The apparent reversibility of the coma, combined

with the lack of ischemic damage and low levels of permanent neurological complications, has led Clark and colleagues to propose that coma is mediated by short lived molecules which affect cerebral function, rather like the reversible encephalopathy of hepatic failure, or an anaesthetic. This 'cytokine' theory states that as coma is not always accompanied by sequestration of parasites; this is actually an epi-phenomenon. Indeed some authors feel that sequestration is not specific to falciparum malaria but also occurs in *P. vivax* infection (81). Parasites, either locally in the brain or systemically, could induce release of soluble host mediators such as TNFα or nitric oxide (NO), which are very short lived and could diffuse into the brain substance to interfere with neuronal function (20, 21). The role of these mediators in suppressing consciousness in other diseases and their relationship to sleep is currently being investigated. The measurement of TNFα in malaria showed a significant increase in circulating levels in African children which was related to disease severity (36, 48). However, not all conditions associated with systemic release of TNFα are associated with coma.

The stimulation of TNFα secretion by malaria infection could occur locally in the brain, due to microglial or endothelial secretion, or be induced systemically due to the effects of circulating, secreted parasite 'toxins'. The concept of a soluble parasite derived toxin is not new, but efforts to find the molecule have been unsuccessful until recently (7, 83). *In vitro* the exposure of macrophages to parasites causes them to secrete TNFα, which can occurs after phagocytosis of parasites or pigment or by exposure to soluble *P. falciparum* antigens (93). A soluble phospholipid moiety released from the erythrocyte surface during infection can also induce monocyte production of TNFα (44). Different strains of the parasite vary in their ability to elicit TNFα production (4).

In the 'sequestration' theory the main factor which has been proposed to contribute to coma is the sequestration of parasites in cerebral blood vessels (9, 10). This theory arose from pathological examination and went through various stages to account for the action of sequestration in causing cerebral impairment, such as 'stagnant hypoxia' or ischemia due to mechanical vascular obstruction, thrombo-embolic disease due to aggregates of parasites, leukocytes and fibrin, or direct neuronal and endothelial toxicity. Reversal of coma could be due to the clearance of parasites, but the lack of ischemic damage would suggest that even when vessels contain many infected cells the blood supply to that area is not compromised. The two theories are not incompatible, as local sequestration may cause greater release of toxic mediators, but evidence for true cerebral malaria occurring in the absence of cerebral sequestration has so far been circumstantial.

How could these two theories be tested? Post mortem tissues currently give the best available 'window' through which to examine pathological processes, and have helped to show that cerebral sequestration is necessary, although not sufficient, to cause cerebral coma. It seems therefore that systemic cytokine release in the absence of sequestration is unlikely to be the cause of coma in cerebral malaria. The key question of whether there is evidence for local production of cytokines or NO in the brain during coma, and conversely whether they occur in the absence of coma, is currently being addressed. No immunohistochemical data exists on the localization of cytokines or nitric oxide synthase in the brain in cerebral malaria. However, systemic measurements of soluble nitrite by-products of nitric oxide breakdown in humans so far support NO secretion as being protective in CM, rather than being associated with it (22).

Molecular Studies Related to the Pathophysiology of Cerebral Malaria

Parasitised erythrocytes show a propensity to bind to other cells *in vitro*. This is manifested as autoagglutination (binding to other infected erythrocytes), cytoadherence (binding to endothelial and immune cells) and rosetting (binding to uninfected erythrocytes). Cytoadherence to vascular endothelium is thought to represent the *in vitro* correlate of sequestration. The binding of parasitised erythrocytes to endothelial cells noted in most pathological studies has thus been proposed to be the mechanism underlying the sequestration of parasites in organs. The specific binding of PRBC to the lining of blood vessels was confirmed using electron microscopy, which also identified changes to the red cell membrane characterized by the appearance of electron dense 'knobs' (51, 96). There was an initial debate as to whether the knob positive (+) phenotype was necessary for cytoadherence, but it has become clear that individual knob negative (-) strains can sequester *in vivo* and cytoadhere *in vitro*. Variations between strains in their ability to cytoadhere in different organs may represent a parasite virulence factor.

Rosetting occurs spontaneously *in vitro* and has been hypothesized to occur via lectin-like molecules on the red cell surface (15, 99). It can be inhibited with sulphated glycoconjugates (80). The rate of spontaneous rosetting is a strain specific property and there have been several studies into the possible relationship between rosetting of field isolates and disease severity. Theoretically strains which rosette more might have an increased ability to cause vascular obstruction in concert with endothelial cytoadherence, due to clogging of cerebral vessels by rosettes as well as sequestered PRBC. All but one of these studies has shown a consistent correlation between rosetting frequency and severe disease (3, 97).

Host Receptors for Parasitised Erythrocytes

Molecule	Summary Information	Proof of Receptor Function *in Vitro*	Expression in the Brain *in Vivo*
Thrombospondin (TSP) (77)	Trimeric glycoprotein ~ 420 kDal. Cell matrix adhesion molecule. Secreted by endothelial cells, platelets and monocytes	PRBC bind to purified protein Binding inhibited by polyclonal antisera Receptor for PRBC in an *ex vivo* model of adhesion in rat mesenteric venules	Synthesised and secreted by cerebral endothelial cells. Only weakly expressed in human cerebral malaria and simian models of CM
CD36 (Platelet glycoprotein IV) (5, 66, 70)	Globular glycoprotein ~ 88 kDal. Cell surface receptor for matrix proteins including TSP. Expressed by mature monocytes, platelets and endothelial cells	PRBC bind to purified protein and recombinant protein expressed on cell lines. Monoclonal antibodies and peptide analogues of the binding site inhibit adhesion to nascent molecule. Binds nearly all field isolates of parasites	Widespread endothelial marker but very sparsely expressed on brain and glomerular endothelium. Recognised (and reported to be up-regulated) on EC's in animal models of CM, but no evidence for up-regulation on human cerebral EC's
ICAM-1 (CD54) (8)	80-110 kDal adhesion molecule, member of Ig superfamily, 5 tandem repeat domain structure. Co-stimulatory signal for MHC signalling, by binding to the ligand LFA-1 on leukocytes. Cytokine-inducible expression on endothelial cells	PRBC bind to purified and recombinant protein, and cell lines expressing or transfected with recombinant ICAM-1. High levels of ICAM-1 binding associated with cerebral malaria in case control study of cytoadherence from African field isolates	Expressed basally on human and simian cerebral endothelium. Expression up-regulated in human cerebral malaria, and co-localises with sites of parasite sequestration. Inducible expression noted in the brain of a murine model of cerebral malaria.
VCAM-1 **AND** **E-selectin** (67)	6 or 7 domain adhesion molecule of the Ig superfamily ~ 110 kDal. Expressed inducibly on endothelium. Cell surface adhesion molecule of the Selectin family; complex domain structure. Expression restricted to activated endothelial cells	Adherence of selected parasite lines to recombinant proteins. Can be inhibited by antisera. Very few field isolates show significant levels of binding to these molecules	Very little basal expression in human or simian brain. Increased expression in human and simian cerebral malaria. Distribution of E-selectin co-localises with parasite sequestration
Chondroitin-Sulphate A (79)	Poly-sulphated matrix protein, expressed on several molecules on endothelial and other cell surfaces	Parasites adhere to purified protein. Adhesion inhibited by competition and polyclonal antisera.	Unknown One study implies this molecule to be an important receptor for sequestration in the placenta

Table 1.

Parasites Adhere to Specific Receptors in the Cerebral Microvasculature. The process of sequestration has been modelled *in vitro* using cultured parasite lines which demonstrate cytoadherence to endothelial and other cell lines. This adhesion process has been shown to be mediated by specific host sequestration receptors which have been identified and characterized using a variety of techniques (9, 71). The first identified receptor was thrombospondin (TSP), a trimeric extracellular matrix glycoprotein secreted by endothelial cells and involved in both cell-matrix and matrix-matrix interactions (77). The next was CD36, a platelet, monocyte and endothelial expressed cell surface glycoprotein (5, 66, 70). The third was ICAM-1, a cell adhesion molecule of the Ig superfamily (8). Other cell adhesion molecules involved in leukocyte adhesion to endothelial cells in sites of inflammation have also been shown to support adhesion of selected strains of PRBC to cultured endothelial cells, including E-selectin and VCAM-1 (67). The most recent molecule identified as supporting adhesion of PRBC is the extracellular adhesion molecule Chondroitin sulphate A, which could be expressed on a variety of sulphated glycoproteins on endothelial cells (78). The characteristics of these receptors, and a summary of the evidence suggesting that they function as PRBC receptors, is shown in Table 1.

These molecules have all been shown to support the adhesion of cultured parasite lines *in vitro*. However to be of potential importance as sequestration receptors *in vivo* these molecules should be expressed in tissues in which PRBC sequestration occurs *in vivo*, Also, there should be a relationship between the incidence of sequestration *in vivo* and the dysfunction of the tissue during the clinical syndrome of severe disease during life. These criteria have been examined in the brain in CM. A quantitative immunohistochemical study of the distribution of the putative sequestration receptors on cerebral microvascular endothelial cells showed that ICAM-1, E-selectin and VCAM-1 expression was increased on cerebral vessels during cerebral malaria compared to controls, whereas CD36 and TSP expression was scattered and low in controls and did not increase during disease. A significant correlation was shown between the expression of all the sequestration receptors and the localization of PRBC in cerebral microvessels (98). Thus expression of sequestration receptors was increased during cerebral malaria, and where these receptors were expressed on cerebral vessels there was a significantly higher chance of observing sequestration in these vessels. Parasite sequestration in a cerebral microvessel expressing increased levels of VCAM-1 is shown in figure 6.

There is evidence to support the role of host sequestration receptors in the CNS in PRBC localization *in vivo* from a SCID mouse model (107). This showed increased expression of murine ICAM-1 on cerebral capillaries after systemic TNFα treatment of the mouse, and subsequent injection of human *P.*

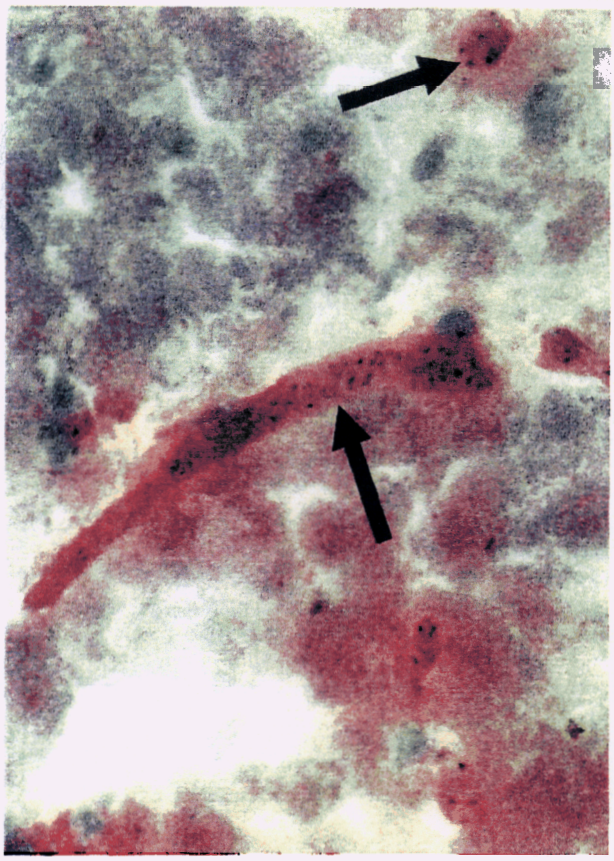

Figure 6. Immunohistochemical staining of cerebral microvessels for VCAM-1 showing sequestered parasitised erythrocytes (arrows) (APAAP method, hematoxylin counterstain, x63)

falciparum parasitised erythrocytes led to retention of these cells in the brain in preference to other organs. This could be partially prevented by treatment with an anti-murine ICAM-1 antibody, and was interpreted as showing ICAM-1-mediated sequestration of parasitised erythrocytes in the mouse brain. However parasitised human erythrocytes do not bind to recombinant murine ICAM-1 i*n vitro* so the role of other receptors in supporting human parasite adhesion to mouse molecules should also be considered.

Preferential sequestration of PRBC in the brain in CM does not result solely from selective expression of sequestration receptors in this site. Other tissues showed constitutive expression of some of the receptors in both controls and cerebral malaria. The upregulation of sequestration receptors on brain EC's was part of a systemic picture of endothelial activation, with increased expression also seen on the vascular beds of the lung, kidney and muscle. The sequestration receptors ICAM-1, VCAM-1 and E-selectin were first characterized as leukocyte adhesion molecules as well as being markers of endothelial activation, due to their upregulation on endothelial cells during inflammatory responses. Upregulation on cerebral EC's has been observed in multiple sclerosis, HIV encephalitis and other inflammatory conditions of the CNS. Indirect measurement of systemic endothelial activation using soluble circulating levels of sICAM-1, sVCAM-1 and sE-selectin in the sera of living malaria patients, and immunohistochemistry on dermal microvessels in skin biopsies, indicates that systemic endothelial activation occurs during life in both cerebral, non-cerebral and non-severe malaria, as well as cases of bacterial sepsis (Turner G. et al. submitted). A number of studies using the circulating levels of soluble adhesion molecules as surrogates for endothelial activation *in vivo* (33, 41, 43) have shown increases in soluble Cell Adhesion Molecules (sCAM) in both severe and mild malaria compared to controls, and levels of these molecules increase in many other inflammatory and neoplastic disorders. Measurement of sCAM levels in the CSF may allow examination of how specific a raised sCAM level seen in the serum is to endothelial activation in the CNS, as opposed to other vascular beds.

From these studies a number of conclusions can be drawn. Endothelial expression of sequestration receptors in the brain occurs at basal levels in the normal brain, but increases in malaria. However their expression is not limited to the brain, being constitutive in normal tissues such as lung, muscle and gut. Levels of sequestration receptor expression may also be raised in non-severe malaria and other conditions, and systemic endothelial activation as measured by indirect methods occurs in other diseases as a non-specific response. Whether differences in the regional distribution of receptor expression within the brain (either basally or during disease) can explain the differential patterns of sequestration mentioned earlier is unknown, but it would seem that other factors in addition to host sequestration receptor expression influence the pattern of preferential PRBC sequestration in the brain in CM.

There remains the possibility that other receptors, important in establishing sequestration *in vivo*, have not yet been identified *in vitro*. This is especially true of receptors which could mediate 'rolling interactions' between PRBC and cerebral endothelial cells. The paradigm of leukocyte adhesion has provided examples of an adhesion molecule family, the selectins, which mediate initial rolling of leukocytes on activated endothelial cells, prior to activation of the white cell and firm adhesion mediated by static, more firm attachment (90). The presence of rolling receptors, rather than static interaction, might help to explain the neuropathological observations of 'margination' of PRBC in larger vessels. It also providea a mechanism whereby flow could be preserved in smaller vessels, allowing sequestration at the same time as maintained perfusion by normal erythrocytes.

Binding of PRBC to EC has not yet been conclusively proven to activate EC, which would be a possible pathway by which parasites could induce changes in the brain micro-environment. Morphological and immunophenotypic changes in endothelial cells, if

accompanied by changes in EC function, could explain how binding itself could influence neuronal function without producing permanent defects in the blood brain barrier. In contrast to the endothelial cell we know little of the reaction of the surrounding astrocytes and microglia to the adhesion of PRBC to vessels, although increased adhesion molecule expression is also noted on microglial cell during CM (98). These cells are likely to play an important role in mediating signalling through the blood brain barrier, perhaps by releasing cytokines which affect neuronal function.

The Parasite Ligands for Adhesion. A range of host molecules have been characterized as putative receptors for parasite adhesion *in vivo*. In contrast until recently very little has been known about the parasite ligand for adhesion. The molecules responsible were assumed to be localized at the knob protein on the infected erythrocyte surface. An immunoelectron microscopic study of affinity labelled TSP and CD36 binding to K+ parasitised erythrocytes showed that they concentrated over the knob proteins, giving an indication that the parasite ligand may well be expressed at these localized adhesion structures (62). A clone tree of laboratory parasite lines derived by micro manipulation from single cells showed that the adhesive phenotypes of binding to ICAM-1 and CD36 were separate and could vary independently (78). Antigenic phenotype of the parasites was co-modulated with changes in cytoadherence suggesting that the same molecule was responsible for both phenotypes. The cytoadherence properties of laboratory isolates have been investigated using biochemical techniques (16, 31) and suggest a role for the *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP-1) molecule. Long-term attempts to clone PfEMP-1 have recently come to fruition with the identification of a large family of *var* genes in the falciparum genome. These code for a large, antigenically variable protein expressed on the red cell surface during the later parasite stages. This PfEMP-1 molecule has several subunits encoding Duffy binding protein-like domains (6, 88, 91).

A number of *var* genes have now been cloned and research is continuing into their expression and functional role in binding host sequestration receptors. If different genes code for ligands with different binding specificity for particular receptors, then differences in expression within a parasite strain, or between clones of parasites generated within cycles of a particular infection, could theoretically influence patterns of adhesion in the body. Investigation of the *var* gene phenotype expressed by sequestered parasite populations within different organs, and their relationship to the pattern of host sequestration receptor expression within the brain, may indicate whether there is adhesive 'selection' of clones of parasites for binding in particular organs such as the brain. This is one potential link between the ability of different clinical parasite isolates to bind to the receptors *in vitro*, and their pathogenesis *in vivo*. Strain variation in PRBC induction of TNFα production has been well established, so theoretically selection of a virulent parasite clone by binding to a particular receptor in the brain, followed by local pathological mediator release in an individual genetically predisposed to more severe disease would form a combination of host and parasite factors predisposing to CM in that case.

Conclusion

Cerebral malaria is an important and complex neurological infection. It represents a disease where the role of neuropathology has had a great impact on our understanding of the pathophysiology of disease, and where pathology studies can continue to answer basic questions about the disease process. As in all pathology studies, our observations are biased to fatal cases and an insight into the course of pathological processes in living patients would be extremely helpful. The roles of cytokine release in the brain, parasite interactions with the blood brain barrier and parasite and host adhesion receptors are major areas of research which may provide us with more insight into the pathogenesis of the disease.

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