Pathogenesis of Cerebral Malaria: Recent Experimental Data and Possible Applications for Humans

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INTRODUCTION

Malaria is still the world’s most important parasitic disease and is responsible for the death of more people than any other communicable disease except tuberculosis. According to World Health Organization estimates (61), between 300 million and 500 million people are infected with malaria every year. The disease is a public health problem in more than 90 countries, which are home to some 2,400 million people, 40% of the world’s population. More than 90% of all malaria cases are in sub-Saharan Africa. Two-thirds of the remainder are concentrated in six countries, India, Brazil, Sri Lanka, Afghanistan, Vietnam, and Colombia, in decreasing order of prevalence. Mortality due to malaria is in the range of 1.5 million to 2.7 million deaths per year. Deaths occur mostly among young children in Africa, especially in remote rural areas with poor access to health services. Cerebral malaria (CM) is the most severe complication and the major cause of death. In some reports, CM accounts for up to 10% of all cases of Plasmodium falciparum malaria in hospitalized persons and for 80% of fatal cases.

The classic clinical presentation of malaria consists of bouts of fever accompanied by other symptoms such as headache, malaise, nausea, muscular pains, or mild diarrhea, often mistaken for influenza or gastrointestinal infection. Details of the diagnosis, outcome in different types of patients, and treatment recommendations have been extensively reviewed (118), as have the socioeconomic consequences of the malaria burden worldwide (72). Although various hypotheses have been proposed and some progress has been made using in vitro as well as in vivo models, the mechanisms of CM pathogenesis remain incompletely understood and are the subject of a continuing debate (9, 14, 32).

To investigate the pathogenesis of CM, several animal models have been established in which animals are infected with erythrocytes parasitized by various types of Plasmodium. Although these animal models do not exactly reproduce the human disease, they nevertheless exhibit some similarities to human CM, such as clinical signs of nervous system dysfunction and cerebral pathology. An elegant and exhaustive review on human CM pathology has been published recently (109). However, the assertion that “there is no model for CM,” based on the fact that murine CM shows leukocyte sequestration, can be modulated, since the presence of leukocyte sequestration in human CM, at least in pediatric patients, is now well substantiated (32, 37). Conversely, parasitized red blood cell (pRBC) sequestration also occurs in murine CM, although in a less prominent fashion than in humans (42a).

Several observations from the study of experimental CM have been extended and confirmed in human disease. First, cytokine overproduction was detected during experimental CM and was found to contribute to brain vascular pathology. Tumor necrosis factor TNF (33, 34) and gamma interferon (IFN-γ) were shown to be important mediators in the pathogenesis of CM. Second, helper T lymphocytes play a significant role in the development of murine CM (25, 38). In response to
malaria parasites, the host undergoes a Th1 rather than a Th2 response, in which susceptibility to CM is favored over resistance (11, 16). Third, cytokine-induced phenotypic changes of brain microvascular endothelial cells (MVEC) indeed represent a key event in the sequestration of both PRBC and parasitized leukocytes (22, 39, 85, 110).

Several recent results, from in vivo studies using gene-deficient mice and from in vitro studies using isolated brain MVEC led us to address other mechanisms of experimental CM. These results included (i) an unexpected role of platelets in TNF-induced microvascular pathology, (ii) protection from CM in TNF receptor p75-deficient mice but not p55-deficient mice, and (iii) the finding that brain MVEC derived from CM-susceptible and CM-resistant mice exhibit differential responsiveness to the cytokines TNF and IFN-γ. In this review, we will focus on these findings and their possible meaning in terms of pathogenic mechanisms and will discuss their applicability to human CM. The importance of other blood cells in the modulation of PRBC binding in the pathogenesis of CM is illustrated in Fig. 1.

**FIG. 1. Importance of other blood cells in the modulation of PRBC binding in the pathogenesis of CM.** The malarial parasite (PRBC) stimulated the host immune response, notably an expansion of Th1 clones, leading to overproduction of IFN-γ. Apart from upregulating some potential receptors, such as CD36, IFN-γ stimulates monocytes to produce soluble TNF (solTNF) and to express higher levels of the transmembrane form of the cytokine (memTNF). Both forms, but particularly the memTNF via an interaction with TNFR2 expressed in increased amounts, cause an upregulation of ICAM-1 on brain endothelial cells. In turn, high levels of ICAM-1 cause platelets to adhere and fuse to brain endothelial cells, with at least two important functional consequences: an increased adhesiveness for PRBC (via CD36) and leukocytes (via LFA-1, P-selectin, etc.), responsible for vessel obstruction, ischemia and possible neuronal dysfunction, and a potentiation of endothelial killing by TNF, leading to vessel disruption and brain hemorrhages.

**TABLE 1. Examples of in vivo models used for the study of CM**

<table>
<thead>
<tr>
<th>Malaria parasite</th>
<th>Animal</th>
<th>Characteristic</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. yoelii</em> 17XL</td>
<td>Swiss mice</td>
<td>pRBC sequestration</td>
<td>48, 123</td>
</tr>
<tr>
<td><em>P. berghei ANKA</em></td>
<td>CBA/Ca mice</td>
<td>Leukocyte sequestration</td>
<td>38, 63, 74, 87</td>
</tr>
<tr>
<td></td>
<td>CBA/T6 mice</td>
<td></td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>DBA/2 mice</td>
<td>Nonfatal CM</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>C57BL/6 mice</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td><em>P. berghei</em> K173</td>
<td>C57BL/6 mice</td>
<td></td>
<td>15, 19, 20</td>
</tr>
<tr>
<td><em>P. berghei</em> NK65</td>
<td>CBA/Ca mice</td>
<td></td>
<td>117</td>
</tr>
<tr>
<td><em>P. berghei</em> ANKA</td>
<td>Hamster</td>
<td></td>
<td>87</td>
</tr>
<tr>
<td><em>P. berghei</em> NK65</td>
<td>WM/M rat</td>
<td></td>
<td>45</td>
</tr>
<tr>
<td><em>P. knowlesi</em></td>
<td>Rhesus monkey</td>
<td></td>
<td>105</td>
</tr>
<tr>
<td><em>P. fragile</em></td>
<td>Rhesus monkey</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td><em>P. coatneyi</em></td>
<td>Rhesus monkey</td>
<td></td>
<td>1, 101</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>Saimiri monkey</td>
<td>pRBC sequestration</td>
<td>106</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>SCID mice</td>
<td></td>
<td>120</td>
</tr>
</tbody>
</table>

**IN VIVO MODELS OF CEREBRAL MALARIA**

Experimental animals models cannot reproduce all the features of human diseases; this is particularly true for CM. However, in CM the altered cells that play a pivotal role in human and experimental lesions are the same (the brain MVEC). In addition, the similarities between defined malaria antigens in rodents and human parasites and between immune response pathways in mice and humans justify the use of models. The concepts defined in experimental conditions might lead to further investigations in the human disease. Several animal models of CM have been established, each of which exhibits specific pathological characteristics depending on the malaria parasites and animal strains employed (Table 1).

The *Plasmodium yoelii* model has an advantage over the *Plasmodium berghei ANKA* (PbA) model in including an obvious PRBC sequestration. However, as discussed above, this cannot be taken as an argument to claim superiority for this...
model (48), since leukocytes are also sequestered in human CM. The leukocyte sequestration seen in the various murine models or in humans is never accompanied by transmigration, since leukocytes are also sequestered in human CM. The leukocyte sequestration seen in the various murine model (48), since leukocytes are also sequestered in human CM, especially since they can reproduce the sequestration of pRBC and brain vascular complications such as hemorrhages. Problems with the monkey model include the facts that the time point of CM onset is difficult to determine and that the expected incidence of this syndrome is low and variable whereas it is high and reproducible in mice. In spite of the cost and the lack of genetically modified animals, which are other limitations, the monkey models represent invaluable tools for the study of pRBC sequestration in vivo.

**P. BERGHEI ANKA MODEL**

Because of its high degree of reproducibility, easily manageable characteristics, brain histopathology (which is now validated further by recent observations in human CM [see “Perspectives” below]), and relevant clinical expression and the availability of susceptible and resistant strains, we have focused our attention on the neurovascular pathology induced by PbA (originally described by J. M. Bafort, Antwerp, Belgium [4]) asexual blood stages. The PbA model consists of a neurological syndrome occurring 6 to 14 days after infection and with a cumulative mortality of about 90% (33). Parasitemia at the time of death is consistently low. The neurological manifestations include hemi- or paraplegia, deviation of the head, tendency to roll over on stimulation, ataxia, and convulsions. The remaining 10% of infected CM-susceptible mice eventually die during week 3 or 4 of infection, with severe anemia and hyperparasitemia and without neurological signs (33).

**Susceptible versus Resistant Mice**

The reason why only a small percentage of the human population infected with malaria develops the neurological complication is still unclear. One of the features of the PbA model is to allow studies of the genetic control of susceptibility to CM. Following PbA infection, various mouse strains also exhibit a differential responsiveness to the malaria parasite. Some strains are highly susceptible to the development of CM, while others are resistant, in spite of identical levels of parasitemia during the acute phase of the infection (Table 2) (G. E. Grau et al., unpublished results).

These data clearly show that genes outside the H-2 complex are involved in the genetic control of susceptibility to CM. Other factors include the capacity to produce IFN-γ in response to malarial antigens (see below).

**Brain Histopathology**

The histopathology of experimental CM varies according to parasite-host combinations. The differential pathological changes in animal models were found to be related to different malaria parasites. For example, CBA mice clearly exhibit a brain vascular pathology when infected with PbA (33) but not with *P. yoelii* (33) or *P. vinckei* (13). However, *P. vinckei* causes other features of severe falciparum malaria. *P. yoelii* 17XL-infected Swiss mice show a significant sequestration of pRBC (123). In rhesus monkeys, *P. falciparum* infection is not associated with a sequestration of pRBC, whereas this sequestration does occur during *P. fragile* infection. Conversely, pathological changes induced by a given parasite may vary among different mouse strains. It has been shown that PbA-infected CBA mice develop a fatal cerebral malaria (33, 38, 74, 87) whereas DBA/2 mice develop a nonfatal cerebral syndrome (73) and BALB/c mice do not develop any cerebral pathology (33).

A particularly detailed analysis of brain histopathology has been performed with the CBA/T6 model (73). When inoculated with PbA, these mice exhibited cerebral symptoms and died from cerebral malaria 6 to 8 days after infection whereas DBA/2J mice developed (around day 6 to 9) a nonfatal CM, with milder cerebral symptoms and died between days 15 and 22 from other malaria-related complications. When inoculated with *P. berghei* K173, these mouse strains did not develop cerebral malaria. These mouse-parasite strain combinations were used, in conjunction with the retinal whole-mount technique, to elucidate factors critical in the pathology of murine CM. CBA/T6 mice infected with PbA (PbA-CBA mice) demonstrated mild changes in vascular permeability as early as 2 to 3 days before the appearance on day 5 of cerebral symptoms, whereas mice with noncerebral malaria did not show any vascular permeability changes until the very late stage of the disease (days 14 to 22). In PbA infections, progressive deterioration of endothelial barrier properties, demonstrated by Evans’ Blue leakage both generally and from specific focal areas, and developing monocytosis and adherence of mononuclear cells to the endothelium of the retinal vessels continued until death (in CBA/T6 mice) or resolution (in DBA/2J mice). Adherent monocytes, particularly in PbA-CBA mice, were associated with reduced Hoechst staining of individual endothelial cells and a banking up proximally of both parasitized and nonparasitized blood cells in the small blood vessels, often with accompanying focal leakage of Evans’ Blue from the retinal vessels. The occurrence and severity of these early changes in the microcirculation correlated with the subsequent development of cerebral symptoms. Monocyte margination appeared to be the most significant factor associated with the development of cerebral symptoms (73).

Table 3 summarizes some immunohistopathological features

**TABLE 2. Mouse strain susceptibility to PbA-induced CM**

<table>
<thead>
<tr>
<th>Strain</th>
<th>H-2 type</th>
<th>No. of mice</th>
<th>Cumulative incidence of CM (%)</th>
<th>Parasitemia on day 7b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/Ca</td>
<td>k</td>
<td>&gt;500</td>
<td>90</td>
<td>8.0 ± 3.4</td>
</tr>
<tr>
<td>CBA/HN</td>
<td>k</td>
<td>10</td>
<td>70</td>
<td>10.2 ± 3.3</td>
</tr>
<tr>
<td>C57BL/10</td>
<td>b</td>
<td>30</td>
<td>80</td>
<td>8.5 ± 3.2</td>
</tr>
<tr>
<td>DBA/1</td>
<td>q</td>
<td>30</td>
<td>80</td>
<td>17.0 ± 3.0</td>
</tr>
<tr>
<td>NMRI</td>
<td></td>
<td>30</td>
<td>90</td>
<td>6.5 ± 7.0</td>
</tr>
<tr>
<td>SJL/J</td>
<td>s</td>
<td>20</td>
<td>95</td>
<td>28.0 ± 6.0</td>
</tr>
<tr>
<td>BALB/c</td>
<td>d</td>
<td>&gt;100</td>
<td>0</td>
<td>10.5 ± 4.5</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>k</td>
<td>30</td>
<td>0</td>
<td>17.0 ± 5.2</td>
</tr>
<tr>
<td>C3H/HeN</td>
<td>k</td>
<td>10</td>
<td>0</td>
<td>16.0 ± 4.5</td>
</tr>
<tr>
<td>DBA/2</td>
<td>d</td>
<td>30</td>
<td>0</td>
<td>15.0 ± 3.5</td>
</tr>
<tr>
<td>(NZB×NZW)F1</td>
<td>z</td>
<td>10</td>
<td>0</td>
<td>6.3 ± 4.2</td>
</tr>
</tbody>
</table>

a In addition to these strains studied in our laboratory, the A/J mouse has recently been reported to be resistant to CM on PbA infection (46).

b Number of red blood cells which are parasitized (values are percentages, and standard deviations are shown).
of the CM model induced by PbA in CBA/J mice, the details of which have been described elsewhere (31). In this model, cerebrovascular pathological changes are the main features. A sequestration of pRBC, the hallmark of human CM, has been also found, albeit to a lesser extent, in CM-susceptible mice on infection with PbA (42a, 46; P. F. Piguet and G. E. Grau, unpublished data).

**Cytokine Interplay Leading to TNF Overproduction**

Previous results suggesting that TNF is a key element in the pathogenesis of experimental CM have been reviewed in detail elsewhere (36). These include the observation of high levels of this cytokine in serum at the time of CM, prevention of the neurological syndrome by the in vivo neutralization of the cytokine, and induction of a CM-like syndrome in CM-resistant mice by infusion of a cytokine. More recently, additional confirmation of the pathogenic role of TNF in CM has been provided in experiments with transgenic mice expressing high levels of TNF receptor 1 (TNFR1) (28) and in TNF/LT gene knockout mice (91). Clinical trials with anti-TNF monoclonal antibody (MAb) in humans have led to a significant drop in malarial fever, implying a role of TNF as an endogenous pyrogen (54), but mortality and morbidity were not reduced. Possible reasons for this lack of efficacy include late administration, well after irreversible lesions have occurred, and a carrier effect of the MAb (115).

While TNF production can be triggered directly by the malaria parasite (3), we propose that for CM to occur, there is a need for an amplification loop which involves T-cell activation. TNF release is triggered directly by malaria parasites via both protein kinase C and calmodulin-dependent protein kinase activation, with a regulation that differs from that of lipopolysaccharide (81). The receptor involved in the parasite-induced monocyte/macrophage stimulation has not been characterized. Malaria toxins are important molecules that are responsible for the direct induction by the parasite of TNF secretion by host monocytes (6, 51, 53, 92, 93; D. Kwiatkowski, Reply, Parasitol. Today 11:463, 1995). Similarly, malaria parasites might induce intercellular cell adhesion molecule 1 (ICAM-1) upregulation directly, in a TNF-independent fashion (21).

The requirement for T lymphocytes in CM pathogenesis has been suggested by observations that athymic nude mice do not develop this neurological syndrome on infection with PbA (25). The respective role of T-cell subsets in the triggering of CM has been analyzed: in the PbA model, anti-CD4 but not anti-CD8 monoclonal antibody significantly reduced serum TNF levels (33) and completely abrogated the occurrence of CM (38), while in the P. berghei NK65 model, anti-CD8 but not anti-CD4 antibody protected rats against brain pathology (45).

In experimental CM, the cytokine characteristic of Th1 cells, IFN-γ, plays a significant role. The treatment of PbA-CBA mice with neutralizing anti-IFN-γ MAbs significantly decreased serum TNF levels and prevented the development of cerebral pathology (34). Increased IFN-γ mRNA levels were found in CM-susceptible but not CM-resistant mice during the neurological syndrome. IFN-γ increases the TNF mRNA level (49) and upregulates the TNF receptors on the target cell surface (108). A synergy between IFN-γ and TNF, particularly with respect to the effects on endothelial cells, has also been demonstrated (83).

The IFN-γ production capacity appears to be one of the important parameters associated with the susceptibility to CM: CM-susceptible mice exhibit a preferential expansion of Th1-like clones that is characterized by a marked production of IFN-γ (16). Individuals at risk for severe malaria also produce more IFN-γ, in an antigen-specific manner, than do those who are immune and relatively protected against malaria complications (11). Although these findings may explain why cytokines such as TNF or IFN-γ are overproduced during CM and are sometimes detectable in high concentrations in mice or patients with CM, they cannot explain the phenomenon of tolerance to TNF. The recent observation that TNF differentially regulates the expression of its own receptors in CM-susceptible and CM-resistant brain endothelium (see below) may represent a complementary explanation. The kinetics and site of production of IFN-γ are other important parameters: a recent study indicates that a very early peak of IFN-γ production in the spleen is higher in nonlethal than in lethal murine malaria (17).

**Involvement of Cell Adhesion Molecules**

Since TNF can induce or upregulate various cell adhesion molecules (CAM) on endothelial cells, the expression of these molecules was analyzed by immunohistochemistry. Brain vessels from mice with CM showed a marked upregulation of ICAM-1 (22, 39) and vascular cell adhesion molecule 1 (VCAM-1) (unpublished data). We attempted to prevent CM by intravenous injection of MAbs directed against LFA-1, Mac-1, ICAM-1, VCAM-1, VLA-4 and P-selectin; only anti-LFA-1 MAb proved to be efficient as described below (see “Assessment of various effector cells in the neurovascular lesion”). The important role of ICAM-1 was confirmed using a SCID mouse model in which P. falciparum-infected human RBC adhere to brain ICAM-1 (120), and more recently using ICAM-1-deficient mice (23).

**IN VITRO MODELS OF CEREBRAL MALARIA**

In human CM, the attachment of RBC infected with mature-stage parasites to endothelial cells lining the postcapillary venules is not restricted to the brain. Microvessels of the heart, lungs, kidneys, small intestine, and liver are the principal sites of sequestration. This sequestration is important for the survival of the parasite but may have severe consequences for the host (32). Sequestered cells that clog the brain capillaries may
reduce blood flow sufficiently that confusion, lethargy, and unarousable coma (i.e., CM) result. The molecular characteristics of the surface proteins, that is, the RBC receptors and the endothelial cell ligands, involved in sequestration have been reviewed (99).

One of the specific features of human RBC on infection by *P. falciparum* is the formation of membrane knobs (12; reviewed in reference 98). Such structures are not found on mouse pRBC. Although pRBC with knobs indeed exhibit high adhesion capacities, the knobs are not essential for cytoadherence because pRBC without knobs were also found to bind efficiently in vitro (113). Thus, cytoadherence occurs irrespective of the presence of knobs, and, conversely, the knobby phenotype does not necessarily lead to a greater ability to cytoadhere (90). In addition, pRBC infected with *P. yoelii 17XL* adhered to mouse brain MVEC, indicating that the absence of knobs on mouse pRBC does not hinder their sequestration in mice with CM (48). The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), expressed on the surface of pRBC, specifically binds to CD36 and thrombospondin. In addition, *P. falciparum*-infected RBC can express a lymphocyte function antigen 1 (LFA-1)-like molecule that adheres to human umbilical vein endothelial cells (HUVEC) or human brain MVEC (104). Parasites isolated from patients with CM, severe malaria, or uncomplicated malaria exhibit similar adhesion properties: there does not seem to be a correlation between cytoadherence and disease severity (30, 88). These parasites exhibit a different capacity for rosette formation, suggesting that rosette formation may be another factor that contributes to the sequestration of pRBC (24); however, this has not been found by other authors (88).

Several adhesion molecules mediate the cytoadherence of pRBC to endothelial cells, including thrombospondin, CD36, ICAM-1, VCAM-1, endothelial cell leukocyte adhesion molecule 1 (ELAM-1, E-selectin), chondroitin sulfate A, and, more recently, P-selectin (43), αVβ3, and platelet-endothelial cell adhesion molecule 1 (CD31) (107). Synergism has been described between CD36 and ICAM-1. Furthermore, under flowing rather than static conditions, pRBC adhesion proves to be a multistep process, involving various CAMs (114). This aspect of CM pathogenesis has been reviewed (44).

Numerous cell types have been established to investigate the adhesion molecules for pRBC adherence in vitro. These models include HUVEC, C32 amelanotic melanoma cells, human monocytes, human platelets, U937 myelomonocytic cells (111) and Chinese hamster ovary (CHO) cells transfected with genes coding for CD36 or ICAM-1 (42). Recently, brain MVEC isolated from humans, mice, and monkeys have been used to study the cytoadherence mechanisms (Table 4). Several groups have compared the cytoadherence of pRBC using these models, and their results suggested that CD36 but not ICAM-1 is the principal receptor mediating the cytoadherence of pRBC. This has especially been clear when HB-MVEC and HUVEC were directly compared. HUVEC, with no or only low expression of CD36, display only a low binding capacity for pRBC (102). Interestingly, when comparing the expression of adhesion molecules on brain MVEC between human and murine CM, the main difference is the absence of a known equivalent for CD36 in murine brain MVEC. This may be one of the reasons why the sequestered cells are leukocytes rather than pRBC in experimental CM.

pRBC adhere not only to endothelial cells but also to platelets and monocytes, since the receptors which mediate cytoadherence for some pRBC (CD36 and ICAM-1) also exist on these cell types. This process may participate in microvessel plugging in CM.

### Retinal Whole-Mount Model

Changes in the cerebral microvasculature, such as breakdown of the blood-brain barrier, petechial hemorrhages, congestion, and edema, are observed in the later stages of murine CM and have been studied in an elegant retinal whole-mount model (10). The retinal vasculature offers a unique opportunity to study rheologic, barrier, and functional properties of the microvasculature within a normal spatial relationship with other tissues and as an intact vascular plexus. A combination of techniques, developed to examine the progressive microvascular changes in murine CM, detected phenomena such as monocyte adherence to endothelial cells, congestion, small haemorrhages, and breakdown of the blood-retina barrier, including details of the location of this leakage, earlier than was possible by studying brain sections. In addition, the covisualization of the blood elements, barrier properties, and vascular endothelial integrity that is possible with retinal whole mounts allowed a detailed analysis of the interaction of different cellular elements in the pathogenesis of CM. Except for the detection of edema, the retinal whole-mount technique offers a more powerful and less time-consuming technique for detecting early microvascular changes in murine CM.

The retinal whole-mount system has also been used to evaluate the pathogenic importance of glial cells in CM (67). Indeed, little attention had been given to the repercussion of microvascular damage on this important cell type during CM. The changes in astrocyte morphology and distribution were compared in three settings: a fatal model, a “resolving” model, and a non-CM model. In the fatal model, retinal astrocytes lost their even distribution from day 3 postinoculation (p.i.) with malaria parasites, progressing to gliosis (day 5 p.i.), well before the onset of cerebral symptoms on day 6 to 7 p.i. At the terminal stage of the disease, there was a loss of astrocyte...
processes contacting retinal vessels, often along vessel segments containing adherent monocytes. These features occurred in a mild form in the resolving model and were absent in the non-CM model. Manipulation of these experimental models indicates that astrocytes are involved in the pathogenesis of CM and that the initial changes in astrocyte distribution may be a consequence of the increase in blood-retina barrier permeability; the immune response triggered by the malaria parasite may also be responsible for the loss of astrocyte ensheathment of vessel segments. Moreover, glial cells, particularly microglia and astrocytes, in addition to monocytes, have been shown recently to represent an important source of TNF in CM (68).

**Brain MVEC**

Alterations in cerebral microvessels have long been recognized to be central in the pathology of CM. The first description of sequestration, i.e., the obstruction of cerebral microvessels by large numbers of mature pRBC, by Marchiafava and Bignami in 1894, has been confirmed by several authors (9, 32). The nonsequestering malaria parasites rarely cause severe illness or death, so that the association between histological and clinical parameters is clear. The causal relationship, however, is not established and remains a subject of debate (9, 14, 32). Descriptions of sequestration content vary. It has been claimed that only mature forms of the parasite would be sequestered, but it has been shown recently that all stages of *P. falciparum* are sequestered in the brain (100) and that immature forms may be particularly important (85a). Also, it has been claimed that only pRBC are present in brain microvessels, but macrophages and monocytes have also been described (18, 76, 78, 85). In a recent study of Malawian children with CM, sequestered pigmented macrophages were significantly more numerous in CM patients than in noncerebral malaria patients (C. D. Mackenzie, G. E. Grau, R. Carr, N. G. Liomba, M. E. Molynieux, and T. E. Taylor, Proc. 48th Meet. Am. Soc. Trop. Med. Hys., abstr. 781, p. 476, 1999).

Given this central role of the cerebral endothelium in CM pathogenesis, MVEC have been isolated from almost any organ or tissue (94). MVEC differ from large-vessel endothelial cells (LVEC) by various morphological and functional variables (35, 57). In addition, endothelial cells of arterial origin are different from those of venous origin (7) and show heterogeneity even within a tissue itself, e.g., the dermis (79). Moreover, MVEC derived from various organs also differ in some characteristics (8). Indeed, MVEC derived from different areas of the microcirculation exhibit differential adhesive properties for granulocytes (55). These data suggest that LVEC may not be adequate for the study of pathological events occurring in microvessels. In view of the organ specificity of endothelial cells, MVEC should be derived from the tissue involved in the diseases one wishes to study (94).

Only studies on human brain MVEC address this issue directly. Some studies have dealt with human brain MVEC (47, 102, 104), and have shown, among other things, that CD36 is a crucial molecule for *P. falciparum*-infected RBC binding. CD36 is indeed one of the differences between LVEC and MVEC. Human dermal also express CD36 but may be different from their brain counterparts (119). Studies using human brain MVEC have allowed the description of potentially new receptors for pRBC (121) and an evaluation of the effect of polyanions on cytoadherence as well as invasion (122). Prudhomme et al. have characterized an immortalized human brain capillary endothelial cell line, named BB19 (86). These cells, on transformation with the E6E7 genes of human papillomavirus, retained their endothelial nature, as shown by phenotypic and functional tests. Interestingly, the BB19 cells bound RBC infected with the FCR-3 and ITO4 strains of *P. falciparum*, making the BB19 cell line a useful system in the analysis of receptor-based cytoadherence and sequestration.

**RECENT EXPERIMENTAL RESULTS WITH THE *P. BERGHEI ANKA MODEL***

**Assessment of Various Effector Cells in the Neurovascular Lesion: Pathogenic Role of Platelets**

A possible role of platelets in CM pathogenesis was adduced from experiments with anti-integrin antibodies in PhA-infected animals. Treatment of infected mice with an antibody against LFA-1 prevented a fatal outcome even when given within minutes before death (22, 39). However, the mechanism of action of anti-LFA-1 MAb remained obscure, since it did not significantly diminish mononuclear cell sequestration. To investigate the mechanism of action of anti-LFA-1 MAb, we searched for other cell types involved in the neurovascular lesion. Given the known accumulation of blood platelets in the microvasculature of mice developing other types of immunopathological reactions (82), we investigated their possible role in experimental CM.

In this in vivo model, four lines of evidence suggest that platelets are critical effectors of the neurovascular injury. First, electron microscopic analysis during CM disclosed platelets in the lumen of brain venules between sequestered monocytes and infected RBC. Platelets were often adherent to damaged endothelial cells, and some appeared fused in the cytoplasm of endothelial cells (40). In contrast, blood mononuclear cells did not show evidence of fusion with brain endothelial cells while in close contact with these cells.

Second, radiolabeled platelet distribution studies indicated that platelets were sequestered in the brain and lung vasculature during CM. Noncerebral malaria was not associated with cerebral sequestration of platelets. Third, in vivo treatment with a MAb to LFA-1 (which is expressed on platelets) selectively abrogated the cerebral sequestration of platelets, and this correlated with prevention of CM. Fourth, the role of platelets in the development of neurovascular lesion of CM was more directly assessed by a depletion experiment. Malaria-infected animals rendered thrombocytopenic were significantly protected against CM, further indicating that platelets are central to the pathogenesis of CM. Thus, a CD11a-dependent interaction between platelets and endothelial cells appears pivotal to microvascular damage. These data suggest a novel mechanism of action for anti-LFA-1 MAb in vivo and illustrate an unexpected role for platelets, in addition to monocytes, in vascular pathology.

Thus, significant platelet sequestration in the brain occurs during CM and is abrogated by anti-LFA-1 MAb treatment. Surface LFA-1 might confer on platelets the ability to bind to...
ICAM-1 present on endothelial cells and to fuse with them. This markedly increased fusion, occurring at a late stage in CM, would then lead to irreversible endothelial cell damage and ensuing hemorrhages. This could also explain why delayed administration of anti-LFA-1 MAbs still be protective, since it would interfere with these late-occurring effector interactions. The fusion of platelets of EC has been described in vitro and in vivo as a physiological process, i.e., capable of exerting a trophic role for endothelial cells. In severe malaria, the fusion rate might be accelerated because of the increased amounts of ICAM-1 and would lead to endothelial damage.

Our present results suggest that the real effector of vascular damage might not be the most abundant cells (monocytes) in brain microvessels, since these cells are still sequestered after anti-LFA-1 MAbs treatment. Also, there is no direct evidence that pRBC inflict venular injury. It therefore seems possible that in mouse CM, the critical effector of venular damage is, in fact, the less obvious platelets. Platelets are generally considered to be involved in hemostasis, but there is also evidence of their toxicity, since they are capable of killing various cells including malaria parasites (84). Results obtained with the mouse CM model demonstrate that platelets can also act as effector of immunopathological reactions, notably by damaging endothelial cells, thus producing hemorrhages, the lesion they are commonly recognized to arrest. Recognition of this effector mechanism might have widespread application in other types of TNF-induced hemorrhagic necrosis and could represent an alternative mechanism of action of anti-LFA-1 MAbs in vivo.

Results obtained in vivo with the mouse CM model suggested that platelets may have a detrimental effect on endothelial functions. Since TNF and IFN-γ are important mediators in experimental CM, the relation between TNF/IFN-γ and platelet adhesion/fusion was further investigated in vitro using cocultures of brain MVEC and platelets (57).

The mechanisms of the fusion phenomenon were evaluated, with particular attention to the role of the adhesion molecules involved and to its functional consequences. TNF was found to induce the adhesion of radiolabeled platelets to MVEC in a dose-dependent manner. This effect was amplified by IFN-γ. Conversely, MAbs to ICAM-1 or to one of its ligands, LFA-1, abrogated the adhesion of platelets induced by these cytokines. Electron microscopic examination showed that platelets adhered and fused to endothelial cells. A fluorescent dye was found to be transferred from labeled platelets into endothelial cell cytoplasm, provided that these endothelial cells were pretreated with TNF. In addition, platelet surface markers, such as LFA-1 or the platelet antigen recognized by our anti-platelet MAbs, CV45-H7, were transferred to endothelial cell membranes. The addition of platelets to TNF-activated endothelial monolayers caused enhanced cytotoxicity, as shown by 51Cr release assays. Finally, binding studies indicated that adhesion and fusion of platelets to MVEC significantly increased their adhesiveness for blood leukocytes. These data indicate that fusion of platelets into MVEC, (i) is synergistically induced by TNF and IFN-γ, (ii) is dependent on the β2-integrin LFA-1 expressed on the platelet surface, (iii) participates in the platelet-mediated enhancement of endothelial injury, and (iv) critically modulates the cytoadherence capacity of MVEC for leukocytes, thereby representing an important mechanism in the modulation of TNF effects in microvascular pathology (Fig. 1).

Pathogenic Role of TNFR2 (p75) in CM Pathology

After studying the role of effector cells in TNF-induced endothelial alterations, we analyzed the respective roles of the two TNF receptors, TNFR1 (p55) and TNFR2 (p75) (59). The immunohistochemical examination of brain sections from PβA-infected CM-susceptible mice revealed a significant up-regulation of ICAM-1 and TNFR2, but not TNFR1, in capillaries and venules. To evaluate the respective role of each receptor in CM pathogenesis, we subsequently investigated the susceptibility of TNFR1- or TNFR2-deficient mice (TNfr1−/− and TNfr2−/−, respectively) to the neurological syndrome. Surprisingly, in contrast to most other infectious diseases in which TNF is involved that indicate a role for TNFR1 (26, 80, 89, 103, 116), protection from CM was found in TNfr2−/− mice but not in TNfr1−/− mice (60). To explain the resistance of the TNfr2−/− mice, we first investigated whether there were differences in the levels of critical mediators of CM, such as TNF and IFN-γ, in these mice. This was not the case: no significant differences were found in the levels of TNF. IFN-γ, soluble TNFR1 (sTNFR1), and sTNFR2 in blood, except for the absence of sTNFRs in the corresponding knockout mice. Second, we addressed the question of a different sensitivity to TNF in TNfr2−/− mice. Since brain MVEC isolated from the TNfr1−/− and TNfr2−/− mice both showed reduced sensitivity to the TNF-induced cytotoxicity compared to that shown by cells isolated from the wild-type mice, this result cannot explain the specific protection of the TNfr2−/− mice. We then examined the possibility that ICAM-1 could be differentially modulated in mice lacking one of the two TNFR. Indeed, in experimental CM, it has been shown that ICAM-1 upregulation in endothelial cells contributes to the subsequent adherence of leukocytes. Interestingly, the brain sections isolated from the CM-resistant PbA-infected TNfr2−/− mice did not show the leukocyte sequestration and ICAM-1 upregulation that occurred in the CM-susceptible wild type or TNfr1−/− mice.

Since the soluble TNF-mediated ICAM-1 upregulation on brain MVEC in vitro is exclusively mediated by the TNFR1, we then investigated whether membrane-bound TNF, which has recently been shown to preferentially interact with TNFR2, can explain the apparent discrepancy between the in vitro and the in vivo results (60). In experiments with MVEC isolated from wild-type, TNfr1−/−, or TNfr2−/− mice, we showed that soluble TNF requires the presence of both TNF receptors whereas membrane-bound TNF needs only TNFR2 for TNF-mediated ICAM-1 upregulation in brain MVEC. It was seen that in MVEC lacking TNFR2 only, neither membrane-bound nor soluble TNF can upregulate ICAM-1 in vitro. As controls, TNfr2−/− MVEC were still capable of upregulating ICAM-1 on stimulation by a stimulus different from TNF namely, CD40L (kindly provided by J. Y. Bonnefoy, Glaxo-Wellcome, Geneva Switzerland). Conversely, as another control, TNF was still able to increase the expression of E-selectin on TNfr2−/− MVEC. In conclusion, these results indicate that the interaction between membrane TNF and TNFR2 is crucial to the development of the neurological syndrome seen in severe malaria (59). More recently, TNFR2 was shown to be important in endo-
thelial cell apoptosis in the absence of sensitizing agents, i.e., under pathophysiologically relevant conditions (58).

**Role of Endothelial Cells in Genetic Susceptibility to CM: Comparison of Brain MVEC Derived from Susceptible and Resistant Mice**

The reasons why only a small number of individuals infected with *P. falciparum* develop cerebral malaria remain obscure. There is evidence that the nature of the malaria parasite as well as the host genetic background may partially explain this phenomenon (3, 66). In experimental models of CM, different mouse strains, for unknown reasons, also exhibit different sensitivities to CM (25, 38, 63) (Table 2).

It has been shown that overproduction of TNF contributes to the pathology of CM (33) and is related to the severity of the cerebral syndrome (41). In other studies, some patients with high levels of TNF in blood did not develop CM while some patients with low levels of TNF did (97), suggesting differential hosts sensitivity to TNF. Since brain MVEC are an important target for TNF action in experimental CM, we investigated whether these cells derived respectively from CM-susceptible and CM-resistant mice exhibit a different responsiveness to TNF.

Brain MVEC purified from CM-susceptible (CM-S) CBA/J mice and CM-resistant (CM-R) BALB/c mice indeed exhibit different sensitivities to TNF in terms of cytokine production, adhesion molecule expression and TNF receptor expression (56). CM-S brain MVEC displayed a higher capacity to produce interleukin-6 and to upregulate ICAM-1 and VCAM-1 in response to TNF than CM-R brain MVEC. In contrast, no difference was found in the induction of E-selectin after TNF challenge. CM-S brain MVEC were also significantly more sensitive to TNF-induced lysis. This differential reactivity to TNF was further substantiated by comparing TNFR expression on CM-S and CM-R brain MVEC. Although the constitutive expression of TNFRs was comparable on cells from the two origins, TNF induced an upregulation of both p55 and p75 TNFRs in CM-S but not in CM-R brain MVEC. A similar regulation was found at the level of TNFR mRNA but not at the level of receptor shedding. Although a protein kinase C inhibitor blocked the response to TNF in brain MVEC of both CM-S and CM-R mice, an inhibitor of protein kinase A selectively abolished the response to TNF in brain MVEC of both CM-S and CM-R mice, an inhibitor of protein kinase A selectively abolished the response to TNF in brain MVEC of both CM-S and CM-R mice, indicating the importance of MHC class II molecules in CM pathogenesis. These data demonstrate that differential inducibility of MHC class II expression on brain MVEC is correlated with a genetic susceptibility to CM (69).

**PERSPECTIVES**

**Overall Relevance of Models: Human versus Murine Pathology**

Experimental CM cannot reproduce exactly the brain pathology of this complication in humans, but there is a growing number of similarities between several models and the human disease (Table 3). In particular, in the PbA model, the presence of mononuclear cells in brain venules finds a parallel in pediatric CM, since there is a substantial accumulation of monocytes and pigmented macrophages (62) in brain vessels. Furthermore, the number of these leukocytes is significantly greater in CM than in noncerebral malaria patients. Another parameter in common between human and murine CM is the upregulation of TNFR2 on brain microvessels (62) (Table 3).

The accumulation of platelets in the brain vessels of these children is also significantly more important in CM than in noncerebral malaria patients (G. E. Grau, C. D. Mackenzie, R. Carr, M. Redard, G. P. Pizzolato, P. Moulin, C. Cataldo, N. G. Liomba, M. E. Molynieux, and T. E. Taylor, Proc. 48th Meet. Am. Soc. Trop. Med. Hyg., abstr. 780, p. 475, 1999), this represents another new factor in common with the PbA model. Conversely, as mentioned above, there is also pRBC accumulation in the brain of PbA-infected mice, although this is less marked than in other murine models.

**Platelet-Endothelial Interactions in Microvascular Pathology Beyond CM**

The effects of anti-LFA-1 MAb on CM and on platelet accumulation in brain vessels may offer insight into the mechanism of action of this antibody in vivo. Besides CM, in vivo treatment of mice with anti-integrin MAb (Aa0) from their beneficial role in hemostasis, platelets also be viewed as pathogenic effectors in vascular lesions. More recently, in vitro experiments with human cells have indicated a role for LFA-1 in platelet-endothelium interactions, substantiated the fusion phenomenon, and confirmed that platelets can potentiate the TNF-induced endothelial killing (L. Camoin et al., submitted for publication).

A pathogenic role for platelets is also suspected in disorders other than CM: gram-negative bacterial septic shock and acute respiratory distress syndrome, vasculitides (e.g., systemic lupus
erythematous, pulmonary fibrosis, tumor metastasis, transplant rejection, and stroke, brain hypoxia, and related conditions (reviewed in reference 64). Indeed, platelets have been detected during rejection episodes (70); particularly bound to ischemic kidney allografts (50). While both condition seem to involve platelets, ischemia-reperfusion (IR) may differ from CM in some aspects. In murine CM, the adhesion of platelets can be regarded as a particular type of thrombosis. Indeed, in this model, platelet depletion does not correlate with fibrinogen consumption (96), while fibrinogen deposition is a prerequisite for platelet adhesion in IR injury (65). Interestingly, in human CM, fibrinogen is deposited inside the vessel lumen, i.e., among sequestered pRBC, leukocytes, and platelets, rather than along the endothelial lining, the pattern seen in IR (Grau et al., Proc. 48th Meet. ASTMH; Mackenzie et al., Proc. 48th Meet. ASTMH). This pattern is consistent with the data of murine CM and is compatible with a less important role of fibrinogen in CM.

Recently, additional in vivo evidence of a pathogenic role of platelets has been obtained in experiments with uPAR (CD87) KO mice (P. F. Pigue et al., Infect. Immun., in press). These mice are resistant to CM when infected with Quantitative immunohistochemistry for GPIIb-IIIa revealed that the platelet sequestration occurring in wild-type mice which develop neurological signs was prevented in uPAR KO mice. The uPAR deficiency did not disturb the immune response leading to TNF overproduction, supporting the hypothesis that platelets are acting as effectors of the neurovascular lesion.

The mechanisms by which platelets act as effectors are potentially numerous: a pathogenic role of platelets can be envisaged at several levels. Platelets can alter endothelial functions in numerous ways directly and/or indirectly via a modulation of leukocyte functions and even via effects on normal RBC and pRBC (reviewed in reference 64).

The relative importance of these numerous effects of platelets in CM pathogenesis remains to be established. Based on reports describing only the pRBC sequestration in human CM (2, 95), attention has focused on developing a model of pRBC binding. The presence of leukocytes and platelets in human CM lesions, however, prompts the need for studying tripeptide and even four-party-interaction models in more depth. A better understanding of these complex interactions leading to vascular injury can help us improve the outcome of this disease.

REFERENCES

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