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Experimental cerebral malaria: Possible new mechanisms in the TNF-induced microvascular pathology

Summary

In order to contribute to the prevention of malaria morbidity and mortality, especially in endemic zones, we have carried out a series of studies on cytokine interactions in an experimental model of cerebral malaria (CM). This rapidly lethal syndrome develops, in some strains of mice, upon infection with Plasmodium berghei ANKA (PbA). A crucial mediator of neurovascular lesions appears to be TNF, found in high amounts in relation with cerebral complications, in both experimental and human CM[1]. In experimental CM, in vivo injections of anti-cytokine antibodies have been used to analyze the cascade of reactions leading to brain vascular damage[1]. In this review, we fill focus on the interplay of cytokines responsible for TNF overproduction in experimental malaria, therefore delineating the subset of T cells whose activation can lead to pathology, and effector mechanisms of neurovascular lesions characteristic of mouse cerebral malaria, with recent findings that appear to involve an unexpected cell type, the blood platelet.

Malaria remains a major problem of public health at the world level. Morbidity and mortality of malaria result from complications that include cerebral malaria (CM) and severe anemia.

In addition to clinical and epidemiological studies allowing for the asessment of markers of disease activity, an experimental approach towards malarial pathology is required. One of the interests of the experimental approach is the possibility to gain knowledge in the fine mechanisms of disease, and to define new markers of disease severity.

In order to understand better the mechanisms of CM, we have used both *in vivo* and *in vitro* models. The *in vivo* model is suitable for direct intervention studies, while the *in vitro* studies, dealing with the target cell of neurovascular lesions, namely the endothelial cell, allows for a functional dissection of the mechanisms of tissue lesion.

We have studied cytokine interactions in an experimental model of vascular pathology: cerebral malaria (CM). This rapidly lethal syndrome develops, in some strains of mice, upon infection with *Plasmodium berghei* ANKA (PbA). A crucial mediator of neurovascular lesions appears to be TNF, found in high amounts in relation with cerebral complications, in both experimental and human CM[1]. In experimental CM, *in vivo* injections of anti-cytokine antibodies have been used to analyze the cascade of reactions leading to brain vascular damage[1].

In this review, we will focus on 1.) the interplay of cytokines responsible for TNF overproduction in experimental malaria, therefore delineating the subset of T cells whose activation can load to pathology, and 2.) effector mechanisms of neurovascular lesions characteristic of mouse cerebral malaria, with recent findings that appear to involve an unexpected cell type, the blood platelet.

Cytokine interplay in cerebral malaria: Evidence for preferential expansion of T cells of the Th1 subset in genetically susceptible mice

The immunological responses that contribute to resistance *versus* susceptibility to bacterial and parasitic infections seem to depend upon the presence of functionally distinct $CD4^+$ T cells: T helper (Th)1 and Th2 cells[2]. Th1 cells, which release

interferon-gamma (IFN-γ) and IL-2, appear to participate in protective responses, whereas Th2 cells, which release IL-4, IL-5, IL-6 and IL-10, are more often associated with pathology (for review see[3]). In the murine model of leishmaniasis, for example, it has been demonstrated that strains susceptible to lesions show a predominant Th2 response while resistant strains of mice display a Th1 response[4]. Th1 cells also participate in resistance to *Trichinella* infection via release of IFN-γ while Th2 cells contribute to susceptibility by production of IL-4[5]. In malaria, using the mouse model of *Plasmodium chabaudi* infection, protective immune responses involving IFN-γ were shown to be a result of a predominant Th1 response[6]. Conversely, in the mouse model for CM induced by infection with *Plasmodium berghei* ANKA (PbA), IFN-γ seems to play a role in susceptibility inasmuch as anti-IFN-γ monoclonal antibody treatment *in vivo* leads to protection against CM[7]. In previous experiments we showed that CM is strictly dependent upon the presence of $CD4^+$ T cells[8]: indeed, depletion of $CD4^+$ T cells completely protects infected CM-susceptible (CM-S) mice against the cerebral complications associated to CM. A crucial mediator of these neurovascular lesions appears to be tumor necrosis factor/cachectin (TNF)[9], released by macrophages stimulated by $CD4^+$ T cells via a cascade of cytokines that includes IFN-γ[7], IL-3 and GM-CSF[10].

In order to examine the relationship between susceptibility and resistance to CM and Th1 *versus* Th2 responses, we examined cytokine mRNA expression *in vivo* in brain and spleen from mice that are susceptible (CM-S) or resistant (CM-R) to cerebral malaria. CBA/J (CM-S) and BALB/c (CM-R) mice were compared before and 7 d after infection with PbA. Expression of cytokine mRNAs was correlated to *in vitro* cytokine production profiles in lymph node and spleen cells from uninfected and infected mice in response to parasitized red blood cells and crude malarial antigens. Our results provide evidence that susceptibility to cerebral malaria is accompanied by the up-regulation of IFN-γ gene expression and *in vitro* production, in response to specific malarial antigens. The expression of TNF-α, IL-1, and IL-6 mRNAs was also found to be increased in the spleen of infected mice as compared to that of uninfected mice. There was no difference between CM-S and CM-R mice, except for TNF-α mRNA, which accumulated in higher amounts in the brain of infected animals with CM than in those with similar level of infection but without CM. Conversely, we found that the expression of two cytokines that are potentially able to antagonize TNF-α effects, IL-4 and TGF-β, was significantly down-regulated at the time of CM. IL-4 gene expression *in vivo* appeared to be turned off, as no transcript was detected even when assayed by PCR. Upon restimulation by crude malarial antigens *in vitro,* production of IL-4 was significantly decreased in infected CM-S mice. The levels of IL-3 produced in response to malarial antigens were found to be comparable between both infected CM-R and CM-S mice and no detectable IL-5 was found in spleen cell cultures, either unstimulated or stimulated with parasitized red blood cells or malarial antigens[11].

It had previously been shown that treatment *in vivo* with anti-IFN-γ mAb was able to protect PbA-infected mice from CM and to prevent the associated TNF overproduction[7]. Thus, beyond the question of the requirement of IFN-γ in CM, there remained the question of whether susceptibility to CM correlated with a particular pattern of cytokine production. Genetic susceptibility to CM was found to be linked to a higher IFN-γ production capacity. Moreover, at the onset of cerebral complications, IFN-γ mRNA significantly accumulated in the brain. These data correlate with the results obtained by Waki *et al.*[12] who showed, in a mouse model of malaria induced by a virulent strain of *P. berghei* NK 65, that $CD8^+$ T cells were capable of producing IFN-γ and thus induce the production of TNF-α in the liver. In this particular model, an anti-$CD8^+$ T cell treatment led to prolonged survival while anti-$CD4^+$ T cell treatment had no effect. In our model, the $CD8^+$ T cell subset does not appear to exert a protective role since depletion of $CD8^+$ T cells in CM-R mice did not lead to the development of CM (unpublished data). These results should be discussed in relation with previous observations of a higher capacity of malaria-specific IFN-γ production capacity in non-immune individuals compared to that of immune subjects[13]. Since it is known that non-immune individuals, such as children in endemic areas or adult visitors from non-endemic areas, are particularly prone to CM, these data suggested that a naive immune system is associated with a higher susceptibility to develop CM. If IFN-γ over-production is indeed associated to susceptibility, this would hint at the involvement of a Th1-like response. A Th1-like pattern of cytoline synthesis associated to susceptibility has also been suspected in other infectious diseases such as Theiler's murine encephalomyelitis[14]. This is in sharp contrast to the situation observed in other parasitic diseases such as schistosomiasis and leishmaniasis in which Th2 rather than Th1 responses correlate with pathology. In the murine model of schistosomiasis, experiments had suggested that T cells from vaccinated mice, after stimulation with specific antigen or mitogen, responded primarily with Th1 cytokines, whereas lymphocytes from chronically

infected mice instead produced Th2 cytokines[15]. In the murine model of leishmaniasis induced by infection with *Leishmania major* promastigotes, it had been shown that susceptibility to lesions was associated with a predominant Th2 response, whereas resistant strains of mice displayed a Th1 response[3,4]. Overproduction of IFN-γ may have direct significance in the development of CM: it is known to active macrophages and to induce the up-regulation of TNF receptors[16]. It is likely that over-production of IFN-γ in the brain of PbA-infected mice leads to the activation of endothelial cells, macrophages and possibly glial cells. Endothelial cells would then up-regulate various sets of adhesion molecules, notably ICAM-1, which has been shown to be involved in the pathogenesis of CM[17]. IFN-γ could also be responsible for TNF receptors' up-regulation[16] on brain endothelial cells, rendering them more sensitive to TNF. In the presence of IFN-γ, macrophages would be primed to release further amounts of TNF-α. Also, one can envisage that a synergy between TNF-α and IFN-γ, particularly with respect to effects on endothelial cells (see below), is relevant to the pathogenesis of CM.

Taken together, these data are consistent with a predominant Th1 response in mice developing cerebral complications of malaria (summarized in Table 1). However, the pattern of cytokines preferentially produced by cells from CM-R mice does not allow to assign it to a Th2 response. Such a difference in T-cell proliferation and IFN-γ production was also seen in relation with immune status in human malaria. It was found that $CD4^+$ T cells from non-immune individuals produced significantly more IFN-γ and proliferated more than malaria-immune subjects in the presence of malarial antigens[13]. Since non-immune subjects are particularly prone to the development of CM, these data suggest that, akin to the mouse model, a predominant Th1 response could be associated with susceptibility to neurological complications of malaria in man.

Effector mechanisms in cerebral malaria: Role of interactions at the microvascular endothelial cell level

In view of the role for TNF in cerebral malaria (both human and murine, summarized in[18]) and of the potent effects of TNF on endothelial cells, we investigated the respective role of adhesion molecules in experimental CM. Treatment with anti-LFA-1 mAb, even given at a late stage of the disease, when mice had developed a full-blown neurological syndrome, was able to prevent cerebral lesions and death. The mechanism of action of anti-LFA-1 mAB remained incompletely understood, until the sequestration of another cell type was envisaged in brain vessels (see below). We found that ICAM-1 expression was markedly upregulated in mice with CM, but not in mice with uncomplicated malaria. Thus, ICAM-1 might be a central structure in sequestration, mediating the adherence of mostly leukocytes in mouse CM and mostly parasitized erythrocytes in human CM. In contrast to anti-LFA-1, treatment of PbA-infected mice with anti-ICAM-1 mAb precipitated death with massive hemoptysis, suggesting that therapies interacting with adhesion molecules should be against carefully selected targets[17].

Among the numerous targets of TNF, endothelial cells (EC) represent a major element. These cells are not only a crucial interface in local inflammation, but also act as fine regulatory components of the expression of immunity, coagulation, and homeostasis. These phenomena, once thought to occur passively, are now recognized to depend upon a complex set of active mechanisms, involving interactions with blood components. Because of their potent functions, these cells are also critical in the development of diverse types of tissue lesions.

In the context of the analysis of potential effector mechanisms in the model of cerebral malaria, the role of nitric oxide (NO) was evaluated. NO production has been shown to be triggered by various cytokines, particularly TNF[19], but diminished by LPS[20]. The involvement of NO in TNF pathology is therefore complex. NO can undoubtedly explain some of the early changes of cerebral malaria[21], but probably not the end stage lesions of the disease, which include brain hemorrhages due to microvasculur EC lesions, since treatment with L-NMMA treatment had an aggravating rather than protecting effect in mouse CM[22, 23]. Because the relevance of this model has been questioned, it seems important to stress the point that the mouse model is indeed a model of lethal cerebral malaria, with brain hemorrhages, a situation which is identical to human CM[24]. In view of what is discussed below, namely the role of platelets in CM, two other argu-

In genetically susceptible mice:
- Prevention of the syndrome by treatment with anti-IFN-γ mAb *in vivo*
- High IFN-γ production capacity *in vitro*
- Upregulation of brain IFN-γ mRNA
- Suppression of brain and spleen IL-4 mRNA

Table 1. *Evidence for an involvement of Th1 cells in cerebral malaria.*

ments can suggest that NO has a protective rather than pathogenic role in CM: NO can reduce platelet aggregation[25] and L-NMMA treatment increases platelet deposition on damaged endothelium in vivo[26]. It is quite conceivable that NO mediates early changes of cerebral malaria, such as neurotransmission disturbances, when the neurological syndrome is still reversible, but this mediator is not likely to be involved in the process of the vascular damage itself, *i.e.,* the brain hemorrhage.

Recent investigations in the murine model of cerebral malaria allowed us to propose a new effector mechanism of TNF-induced EC lesion. It consists in the adhesion of platelets to the surface of EC and subsequent fusion of these platelets into the cytoplasm of EC. This physiological process *in vivo*[27, 28], has been characterized *in vitro*. Evidence for fusion have been described[29]. Platelets were envisaged in the pathogenesis of CM because there was no clear explanation for the dramatic protective effect of anti-LFA-1 mAb, even when this mAb was given shortly before death: this mAb protected malaria-infected mice without significantly reducing the number of mononuclear cells in brain vessels[17, 30]. We demonstrated that platelets are in fact the critical effector of the neurovascular injury of CM[31]. First, electron microscopical analysis of brains from mice with CM revealed that during CM platelets adhere to and probably damage brain endothelial cells. Interestingly, platelets have also been disclosed in brain vessels in fatal cases of human cerebral malaria[32]. Second, radiolabelled platelet distribution studies indicated that platelets significantly sequestered in the brain and lung vasculature during CM. Non-cerebral malaria was not associated with cerebral sequestration of platelets. Third, treatment *in vivo* with anti-LFA-1 mAb selectively abrogated the cerebral sequestration of platelets; moreover, this correlated with prevention of the neurological syndrome. The α (CD11a) and, to a lesser extent, the β (CD18) chain of the integrin LFA-1 were found to be expressed on platelet membranes. Fourth, 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. Microvascular lesions of CM would thus depend on an integrin-mediated platelet, TNF-induced EC damage (schematized in Fig. 1) (discussed in[29]). These data suggest a novel mechanism of action for anti-LFA-1 mAb in the neurovascular complications of murine malaria and illustrate an unexpected role of platelets in vascular pathology.

Platelet-endothelium interactions in other models of experimental pathology

Piguet *et al.*[33] have originally described that platelets accumulate in increased amounts in an experimental model of pulmonary fibrosis. It has been shown that, in this context, treatment of mice with anti-LFA-1 mAb prevents this accumulation as well as tissue damage. More recently, an involvement of platelets in sepsis-related pathology was also shown[34]. The toxicity of LPS was analyzed both at the systemic (lethality) and at the localized level (cutaneous Shwartzman reaction). Interestingly, in this setting, platelets were shown to accumulate in the skin before the triggering of hemorrhagic necrosis. The arguments in favor of a role of platelets as effectors of these lesions are summarized in Table 2.

1. Increased amounts of platelets sequester in damaged organs
2. This sequestration is prevented by *in vivo* treatment with anti-LFA-1 mAb
3. Microvascular damage is prevented by *in vivo* treatment with anti-platelet mAb
(*) Pulmonary fibrosis, cerebral malaria and LPS toxicity

Table 2. *Arguments for a role of platelets in TNF-induced pathology (experimental mouse models) (*)*

Conditions	Rate of fusion	Mechanism of fusion	Effect of platelets on endothelium
Normal	+/–	?	Trophicity
Non-specific EC alteration	+	Decreased surface charge?	Repair
TNF-induced pathology	+++	Upregulation of ICAM-1 and others	Damage

Table 3. *Platelet/endothelium interactions in physiology and pathology.*

The mechanism of fusion may be the same in physiological and pathological situations, and the main difference between these two conditions may be the intensity of the phenomenon (Table 3). Morphological data should orientate towards the precise mechanisms involved, which remain incompletely understood. In experimental CM, cerebral capillaries and venules contain platelets in dense contact to the endothelium or fusing to damaged brain endothelial cell. The endothelium is damaged and interrupted with the presence of granules, suggesting the fusion of platelets with endothelium. Monocytes also adhere to brain endothelial cells but, at variance with platelets, do not show any dense fusion of the membranes and the endothelium is unaffected. These morphological features of platelet-endothelium interactions are reminiscent of those reported in the context of the endothelium-supporting role of platelets[35,36]. We propose that TNF-induced microvascular injury may be due to an exaggeration of the fusion phenomenon, caused by ICAM-1 up-regulation on endothelial membranes. Platelets carrying the LFA-1 molecule would then be bound in higher amounts on these endothelial surfaces, and cell fusion would occur at a higher rate (Table 3 and Fig. 1). The role of other CAM on the surface of both EC and platelets is under current investigation.

The involvement of platelets as effectors of vascular lesions may not be restricted to experimental pathology (Table 4). In man, cerebral malaria has been associated with accumulation of platelets in cerebral microvessels, although the nature of the platelet/endothelial interactions in this setting have not been investigated in details[32]. Also, platelet trapping in lesions of brain vasculitis in patients with systemic lupus erythematosus (SLE) has been reported[37]. However, differences between human and murine platelets suggest that the involvement of platelets should be different in human *versus* mouse pathology (Lou *et al.*, in preparation).

Further analysis of the fine mechanisms of this interaction between platelets and endothelial cells is now performed *in vitro*. Cultures of microvascular EC derived from brain, lungs and other organs have allowed the identification of several pathways. Microvascular endothelial cells have indeed been shown to differ substantially from the classically studied umbilical vein endothelial cells (reviewed in[29]). Platelets modify a number of functions of TNF-activated MVEC (Lou et al., ms. in preparation). The regulation of the fusion phenomenon is under current investigation.

Pathological situation	Species	Reference
Cerebral malaria	man	32
Pulmonary fibrosis	mouse	33
Brain vasculitis in SLE	man	37
Cerebral malaria	mouse	31
LPS toxicity (systemic and local: Shwartzmann reaction)	mouse	34

Table 4. *Microvascular pathology: importance of platelet-endothelium interactions.*

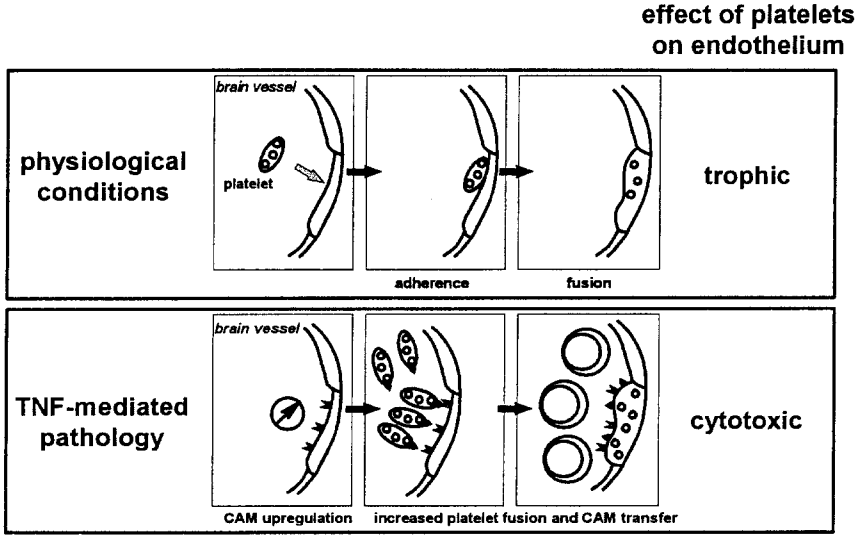

Figure 1. *Comparison of the role of platelets in normal and pathological conditions (e.g., in experimental cerebral malaria).*

Conclusions

Malarial pathology, at least cerebral malaria, seems to depend upon the very T-cell subset, Th1, that is capable of mediating protection against infection by the parasite. Conditions and intensity of stimulation (type of antigen-presenting cells, nature of the antigen, etc.), duration of activation, as well as site of inappropriate activation of these cells may be among the crucial parameters that will determine the balance between protection and pathology. A critical issue

orienting towards pathology might indeed be a preferential homing of some activated Th1 cells in certain microvascular beds. Therefore, the balance between Th1 and Th2 cells, as well as the expression of diverse cell adhesion molecules, are among the deciding parameters that can determine the outcome of the complex host-parasite relationship seen in malaria.

The abnormally activated T cells result in various cascades of cytokines, some of which have direct toxic effects on vital host structures. TNF-mediated vascular damage certainly involves intricate pathways, utilizing almost all the properties of this cytoline. In addition, the role of platelets in the vascular pathology of cerebral malaria is an illustration of unexpected TNF properties. Alternatively, the involvement of this cell type also helps explaining the efficacy of late administration of anti-LFA-1 antibody in cerebral malaria and represents a new mechanism of action of anti-integrin antibodies *in vivo*. The way platelets interact with EC and modifies the physiology of these cells represents a possibly important effector pathway of TNF-induced vascular pathology.

Much remains to be determined on how some malaria vaccines will modulate T cell functions and, more critically, how they will alter the Th1/Th2 balance. Likewise, even though *in vitro* and especially *in vivo* studies have provided some insights in the extraordinary complexity of lesions, much remains to be learned about fine malarial pathology. Understanding better the distinct mechanisms of lesions remains important in order to define targets, design relevant parameters to study, and evaluate side effects of new therapeutic measures. Animal experimentation, particularly in mice, remains an irreplaceable tool to address these major questions and, thereby, to help improve the status of patients who suffer from severe malaria.

Zusammenfassung

Experimentell induzierte zerebrale Malaria: Mögliche Mechanismen microvasculärer Veränderungen durch TNF

Mit dem Ziel zur Prävention von Malaria-Morbidität und -Mortalität beizutragen, wurde eine Serie von Studien über die Interaktionen von Cytokinen in einem experimentellen Modell für cerebrale Malaria (CM) durchgeführt. Dieses in kurzer Zeit tödliche Syndrom entwickelt sich, in einigen Mausstämmen, nach einer Infektion mit Plasmodium berghei ANKA (PbA). TNF scheint ein entscheidender Vermittler für neurovasculäre Läsionen zu sein, der im Zusammenhang mit cerebralen Komplikationen sowohl bei experimenteller als auch humaner CM in grosser Menge gefunden wird. Bei experimenteller CM wurden in vivo Injektionen von anti-Cytokine Antikörpern verwendet um die Kaskade von Reaktionen zu analysieren, die zur Schädigung von Hirngefässen führen. In diesem Überblick werden wir uns auf das Zusammenspiel der bei experimenteller Malaria für die Überproduktion von TNF verantwortlichen Cytokine konzentrieren, und dazu die T-Zellpopulation darstellen, deren Aktivierung zur Pathologie führen kann, und auf die Effektormechanismen von neurovasculären, für die cerebrale Malaria der Maus typischen Läsionen, mit neuen Befunden, die eine Beteiligung eines unerwarteten Zelltyps, der Blutplättchen, zu ergeben scheinen.

Résumé

Neuropaludisme expérimental: nouveaux mécanismes possibles dans la pathologie microvasculaire induite par le TNF

Dans le but de contribuer à la prévention de la morbidité et de la mortalité due au paludisme, en particulier dans les zones endémiques, nous avons mené une série d'études sur les interactions entre cytokines dans un modèle expérimental de paludisme cérébral (CM pour cerebral malaria). Ce syndrôme rapidement mortel survient chez certaines souches de souris à la suite d'une infection par Plasmodium berghei ANKA (PbA). Un médiateur particulièrement important des lésions neuro-vasculaires semble être le Tumor Necrosis Factor (TNF) que l'on retrouve en quantité importante dans les cas présentant des complications cérébrales (CM), aussi bien chez l'homme que chez l'animal d'expérience. Dans les CM expérimentales, des injections in vivo d'anticorps anti-cytokines ont été utilisées pour analyser la séquence des événements menant aux lésions vasculaires cérébrales. Dans cette revue, nous nous concentrons sur les interactions entre cytokines responsables de la surproduction de TNF dans le paludisme expérimental qui, de ce fait, définissent le sous-groupe de cellules T dont l'activation peut conduire à des manifestations pathologiques, et les mécanismes effecteurs des lésions neuro-vasculaires caractéristiques du paludisme cérébral chez la souris, avec la découverte récente du rôle d'une cellule inattendue, la plaquette sanguine.

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