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REVIEW

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Nitric Oxide Involvement during Malaria Infection; Immunological Concepts, Mechanisms and Complexities: a Novel Review

Hossein Nahrevanian

Malaria Research Group, Department of Parasitology, Pasteur Institute of Iran, Pasteur Ave, Tehran 13164, Iran

Abstract

Malaria is a chronic disaster and still remains on the agenda for its top pathogenesis and morbidity. The exact mechanism of host immunity during malaria infection has not been fully described. A number of cytokines have been shown to be produced in malaria, but one of the key molecules is nitric oxide (NO). This tiny molecule acts as a signal in cells to regulate some functions, transmit messages, kill pathogens and induce cell death. There are different ideas about the role of NO and its related molecules in the physiopathology of malaria. NO is implicated in the immune response and the pathophysiology associated with malaria and it may cause some symptoms in severe malaria. NO and its related molecules are produced in the host during malaria to resist infection or cause tissue damage in low or high concentrations, respectively. The NO theory of malaria pathology may lead to novel ideas for immunization, therapy and prevention.

Keywords: malaria, cytokine, nitric oxide, NO

Introduction

Malaria still presents a major threat to the well being of mankind, in much the same way it has since prehistoric times [1]. Despite many attempts to control malaria, including parasite treatment, vector control and environmental sanitation, there has been no reduction in the number of infected cases. During the past few decades, it has become clear that malaria eradication in endemic areas is unlikely to be achieved; therefore, more focus has turned to immunoprotective agents, especially vaccines [2-3]. Despite intensive research efforts, no vaccine against malaria, which is both effective and suitable for mass production, is yet available [4]. A recent hypothesis suspects that a vaccine for malaria might be aimed at neutralizing the effects of toxins rather than eliminating the parasite [5]. Therefore, clinical trials of a new generation of vaccines are underway [6].

Although immunity to malaria is complex and improperly understood, a number of different effector mechanisms have been implicated, including nitric oxide (NO) [7]. It is suggested that a cascade of reactions leading to NO production are involved in malaria [8]. There are some contradictory reports about the role of NO and related molecules in malaria. Some researchers propose NO is involved in the development of severe malaria, whereas others argue a protective role for NO [9]. Due to its contradictory actions,

Correspondence:
Hossein Nahrevanian
E-mail: mobcghn@yahoo.co.uk
Tel: +98 21 6968855, Fax: +98 21 6465132

it is still an open question as to whether NO effects are protective or damaging [10]. Although knowledge about the cytotoxic effects of NO is steadily increasing, we are still at the beginning of understanding how, why, when and where cells are affected by NO. Consequently, the NO theory of malaria pathology may lead to novel ideas for therapy and prevention [5].

The immune system of the host acts in a complicated fashion during malaria infection. Major immuno-pathological syndromes occur, including: cerebral malaria (CM), hyper-reactive malarial splenomegaly (HMS), malaria nephropathy, depositions of pigments, excessive anemia, hypoglycemia and hypotension [8, 11-12]. Anemia is an inevitable consequence of malaria and the degree of anemia corresponds with the duration and severity of infection with a multifactorial pathogenesis [13], which may be related to the degree of parasitemia and erythrocyte destruction [8]. Splenomegaly and hepatomegaly are frequent manifestations of acute malaria in both humans and rodents [13]. The primary induction of immunity to blood stage parasites may occur in the spleen, although the liver may assume this function in the absence of the spleen [14]. Jaundice, prolonged coagulation, failure of gluconeogenesis and sequestration of parasitized red blood cells (PRBC) in the portal and hepatic vasculature, all indicate liver disfunction in malaria [15].

Cerebral malaria (CM)

CM is a neurological syndrome and a severe complication of *P. falciparum* malaria occurring 6-14 days after infection, which generally leads to death even after treatment [8]. One million victims of CM are reported annually in African children [16]. The majority of animals in experimental rodent CM die early in week two after infection, with progressive hypothermia, histological observations of brain hemorrhages, mental disturbances and adherence of WBC to the endothelial lining [17-18]. In *P. falciparum* CM, PRBC are sequestered in the brain capillaries, leading to macrophage activation and NO release [19]. Electron microscopy shows multiple electron-dense knobs protruding from the membrane of the PRBC in capillaries [20], which is attached to the cerebral capillary endothelial cells by the knobs. In addition, knobs contain proteins produced by the parasite, on the surface of PRBC, which have a key role in cytoadherence [21]. A variety of cytoadherent receptor molecules have been recognized, including cluster differentiation 36 (CD36), intercellular adhesion molecule-1 (ICAM-1), thrombospondin (TSP), E-selectin, P-selectin, vascular cellular adhesion molecule-1 (VCAM-1), platelet endothelial cellular adhesion molecule-1 (PECAM-1)/CD31, $\alpha_v\beta_3$ and chondritin sulfate [22-23]. Moreover, at least 4 malarial proteins have been identified on the surfaces of *P. falciparum* PRBC, including histidine-rich protein 1 (HRP1), HRP2, erythrocyte membrane protein 1 (EMP1), and EMP2 [20].

Hypotheses that describe the etiology of CM include micro-vascular obstruction by coagulation induced thrombus formation, deposition of immune complexes and local inflammation leading to alteration of cerebral permeability and edema [2, 8]. The current hypothesis defines a central role for intracapillary sequestration of PRBC cytoadherence to endothelial receptors [8]. The explanation for the mechanism of coma in CM may be the association between cytokines, *eg* TNF-α and free diffuseable NO [24].

Immunity to malaria and host resistance

Different effector mechanisms play variable roles in immunity to malaria, including antibodies, mononuclear cells, cytokines and mediators. Two aspects are mainly responsible for the ability of the host to resist malaria infection; immunological mechanisms and innate characteristics. Some key concepts have to be considered for immunological studies of malaria, including natural resistance, species- and stage-specificity, antigenic variation and immune suppression [2, 3].

The presence of malarial antigens on the surface of PRBC and their role in the immune reaction have been demonstrated earlier [20, 22-23]. The release of antigenic material by the parasite may act directly on B-cells and could lead to polyclonal activation. Both macrophages and non-specific T-suppressor cells appear to be

involved in malaria immune depression, because they act as target cells for parasite released soluble factors [22]. It is suggested that multifactorial protection is responsible for immune responses in malaria infection [25].

Cell-mediated immunity (CMI)

T-cells are crucial for malaria immunity, especially during the erythrocytic stage of infection. T-cells help produce immunoglobulins (IgG1, IgG2a, IgM, IgE) [26-27] to activate macrophages and Th1 immune response, which are essential in the early stage of malaria. Later, there is an immunity switch to a Th2 response with antibody-mediated mechanisms to eliminate parasites [22]. Macrophages, neutrophils and other phagocytic cells are key components of the antimicrobial and tumoricidal immune responses, because these cells are capable of generating large amounts of highly toxic molecules, reactive oxygen and reactive nitrogen intermediates (ROI, RNI) [28].

Both Th1 and Th2 cells can protect the host from malaria infection. Th1 cells protect, in part at least, by the NO pathway, whereas Th2 cells protect by enhancing a specific IgG1 antibody response [29]. Both $CD4^+$ and $CD8^+$ T-cells appear to serve a protective role with IFN-γ [30]. In addition, early NO production may promote the proliferation of specific $CD8^+$ T-cells, or perhaps a subset required to eliminate parasites [31]. Both $CD4^+$ T-cells and IFN-γ are necessary to induce NO synthesis in infected hepatocytes or hepatic iNOS [32].

The effector functions of macrophages include release of H_2O_2, ROI, RNI, NO, TNF and production at least 80 other cytokines and enzymes [33]. Macrophages can be stimulated by IFN-γ and subsequently TNF-α to produce high levels of NO. They can kill erythrocytic-stage of malaria parasites by different mechanisms, including phagocytosis of smaller parasites and secretion of many cytotoxic factors. Macrophages also act as killer cells by antibody dependent cell-mediated cytotoxicity (ADCC) [34]. In addition, TNF-α and IFN-γ can induce production of RNI by neutrophils, Kupffer cells and hepatocytes [35]. There are some possible effects of Ab action during parasite development, including blockage of merozoite dispersion, inhibition of cell invasion, intracellular killing of erythrocytic stages, inhibition of reverse cytoadherence and cooperation with various cells to increase cell-mediated killing [8].

Cytokines and soluble mediators

Cytokines play an important role in the defence against malaria and some have long been recognized to have anti-parasitic effects on different stages of malaria. This protective effect was further demonstrated by administration *in vivo* of some key cytokines [16]. A large number of cytokines appear to be involved in malaria, *ie* TNF-α, IFN-γ, GM-CSF, IL-1, IL-4, IL-6, IL-8, and IL-10 [8]. NO production during murine malaria is regulated *in vivo* by the Th1- cytokines (TNF-α and IFN-γ), but not by IL-4, which is a Th2 cytokine. TNF-α and IFN-γ induce high amounts of NO involved in controlling the peak level of parasitemia [36]. To date, NO is known to affect the production of more than 20 cytokines, including IL-1, IL-6, IL-10, IL-12, IFN-γ, TNF-α, and TGF-β by various immune cells, *eg* macrophages, T-lymphocytes, natural killer cells (NKC) and endothelial cells [28]. Conversely, more than 30 cytokines or cytokine-like factors have been described that increase or inhibit the expression of iNOS activity in cells participating in the immune response: macrophages, microglia, Kupffer cells, neutrophils, eosinophils, mast cells and NKC [28]. The importance of a balance in the cytokine network to achieve protective immunity has been emphasized; eventual effects will depend on the amount of these cytokines released and the rate, time and site of production [16].

Plasmodia have the ability to promote the secretion of TNF-α, IL-1, LT with some overlapping functions [24]. TNF-α and LT are able to increase RNI production, which may serve as anti-microbial cooperation between them [37]. IFN-γ and TNF-α transmit a series of immune signals leading to expression of NOS [38] or activation of iNOS [7]. IFN-γ, IL-1, IL-6, TNF-α, c-reactive protein (CRP) and NO have all been implicated in killing exo-erythrocytic stage *Plasmodium* [22]. A close association was found between expression of

spleen IFN-γ and iNOS mRNA and levels of IFN-γ and NO in serum [39]. NO produced in high concentrations by iNOS can inhibit Th1 cell proliferation, which may act by blocking the synthesis of IL-2, a major Th1 cell factor [29]. IL-6 and TNF-α increase acute phase proteins and these molecules may trap RNI and ROI [40]. Levels of IL-10 also rise in CM and other severe forms of malaria [41]. It is likely that the effect of NO is highly specific, since it has little or no effect on the secretion of IL-4 and IL-10 [29], whereas, IL-4 does not appear to be involved in regulating NO production *in vivo* [36]. NO synthesis by macrophages is induced by IFN-γ and TNF-α; therefore, when they act together it is greatly increased [34]. TGF-β can also inhibit NO synthesis, whereas migration inhibitory factor (MIF) activates macrophages to produce NO [42]. It is indicated that TGF-β production is the key event in failure of resistance to mouse malaria [39]. Importantly, TGF-β suppresses both macrophage products (TNF-α, NO) and NKC products (IFN-γ, TNF-α) [1].

Altogether, the data in published reports outline the importance of balance in the cytokine network for protective immunity against malaria infection. The balance includes the amounts of cytokines released, the rate, time and site of production [16]. Nevertheless, the factors responsible for acting against the parasite need further investigations [3].

Cytokines and CM

During malaria infection, it appears that immunopathological reactions, in addition to complement induction by immune complexes, leads to excessive release of cytokines, which are actively involved in the defence against malaria infection [16]. Apparently, immuno-pathological T-cell dependent reactions are involved in rodent CM. In some non-fatal cases, some CM signs, including coma, seizures and high levels of circulating cytokines, *eg* TNF-α and IL-1, have been observed [17]. Although high serum concentrations of TNF-α were detected in human CM, they were not alone sufficient for pathology [41], which indicates the failure of anti-TNF-α administration to prevent the development of murine CM [18].

NO in CM

The interaction between the parasite and cytokines is the most important factor during CM, and is known to modulate the expression of adhesion molecules and NO on the surface of endothelial cells [23]. Cytokines could induce the synthesis of NO in the cerebral vascular wall, which could then affect nearby neurones or cross the blood brain barrier (BBB) to act as a neuromodulator, causing functional changes such as inhibition of glutamate-induced calcium entry, reduction in calcium dependent NOS activity or a decrease in NO formation by post-synaptic neurones [43-44]. NO blocks glutamate binding to its post-synaptic receptor and inhibits excitatory neurotransmission [24]. It is hypothesized that NO, released by vascular endothelial cells stimulated by TNF-α, diffuses into the brain, where it disrupts the regulation of glutamate-induced nNOS, resulting in neurotransmission alteration and consequent coma [45]. There are two opposing hypotheses for NO involvement in CM. The first states that NO has a protective role in the host during malaria [46], since plasmodial growth, leucocyte adhesion to vascular endothelium, and platelet aggregation, are all inhibited by NO [47]. The second theory states that NO diffuses into the brain and causes damage as a cytotoxic agent by various means, including lipoperoxidation, inhibition of glycolysis and respiration, neuronal death after ischemia, hypoglycemia, disturbance to neuronal functions, increase in intracranial pressure and systemic hypotension [48]. In malaria-tolerant people, the RNI level was higher than in CM cases [33]. In contrast, in African children with CM, the average levels of RNI were higher in deepest coma than lighter coma, and it was concluded that high levels of RNI are correlated with malaria severity and CM [44].

In conclusion, the mechanism of coma in CM remains unknown. The old view based on obstruction of cerebral blood flow by sequestered PRBC, is not an adequate explanation, because patients who had recovered from CM did not have a high residual neurological deficit, which is the usual result of cerebral ischemia [43]. The recent proposal by Mazier, *et al* [23] and Taylor-Robinson [45], based on a role for cytokines and NO in CM, requires more study.

Antimalarial effect of NO and RNI

NO in the serum of malaria-tolerant people is generated by macrophages and appears to be responsible for the malaria tolerance [33]. NO also is reported to inhibit both the liver and blood forms of malaria parasites [24]. In murine malaria, NO has been shown to have a significant protective role in the early non-specific response [38]. It is suggested a cascade of reactions leading to NO production are involved in killing infected hepatocytes [8]. High concentrations of NO have an antiproliferative effect on lymphocytes during the blood stage of rodent malaria, indicating that NO can act as an autoregulatory molecule preventing the overexpression of Th1 and $CD8^+$ T-cells [31]. The sensitivity of different strains of *Plasmodia* to non-specific mediators varies, *eg P. berghei* is considerably less sensitive than *P. vinckei* [38] and *P. yoelli* [40]. *P. falciparum* has also been shown to be susceptible to RNI toxicity *in vitro*, even in μM concentrations [37], whereas, NO inhibits the asexual blood stage of *P. falciparum in vitro* and the exoerythrocytic stage of *P. berghei* and *P. yoelii*. However, excessive NO production may lead to negative side effects in malaria [48].

In contrast, data reported by some researchers do not support a potent role for NO during malaria; NO does not play an anti-parasitic role in *P. yoelii* infection [49]. It is not a crucial factor for the development of murine CM [19]; it did not alter the course of nonlethal *P. yoelli* infection and mortality [49]. It can not inhibit high parasitemia in *P. chabaudi* [50], and little difference was found in parasitemia throughout lethal *P. berghei* ANKA and nonlethal *P. vinckei prtteri* infection after treatment with N^G-monomethyl-L-arginine (N^G-MMLA or L-NMMA) [38, 51], or aminoguanidine (AG) [52].

NO production in resistance to malaria might be strain specific. This might explain some of the contradictory data obtained with different human malaria studies; NO correlated with CM in Papua New Guinea [53], but an inverse correlation was observed in Tanzania [46]. A biological link was predicted between malaria and NO that could result in treatment for severe malaria. Asymptomatic children with the mildest form of malaria parasites (*P. vivax*) in their blood, had increased NO production. Conversely, the sickest children had the lowest level of NO, which suggests that treatment with a NO inducer might be useful for CM [54]. Increased NO synthesis in *P. falciparum* malaria can be promoted directly by parasite soluble factors, without cytokine [48]. Some data from *in vitro* studies on the asexual stage of *P. falciparum* indicate NO itself cannot inhibit parasite development, whereas RNI do have some antiplasmodial activity [38]. An increase in NO level was reported during infection with *P. chabaudi,* but not with *P. berghei* or *P. vinckei* [55]. Therefore, the requirement of NO production for parasite killing in murine malaria might be strain-dependent [19]. In addition, there is an indication of host specifity and NO. Although NO production may not be essential in protection against *P. berghei* ANKA in iNOS deficient mice, NO is absolutely essential for the protection of wild-type mice as it boosts the survival rate, which correlates with an increase in the level of NO [56]. Thus, the protective role of NO in mice is dependent on genetic background and alternative effector mechanisms, when NO production is prevented [57].

NO and RNI in malaria target organs

The changes in NO may be pathogenic rather than protective in the malaria host. In nonlethal *Plasmodia*, an early NO increase may stimulate Th1 cell types to produce more mediators (RNI, ROI, O_2) to control parasitemia later during infection, which could be similar to natural immunization. Late increases in NO production in the liver and spleen appeared to have pathological consequences and were associated with hepato-splenomegaly. Increased NO production in the spleen was common in mice infected with both lethal and nonlethal *Plasmodia*, whereas increased NO production in the brain and liver was seen only in lethal malaria. Increases in brain and liver NO may contribute to the pathology of severe malaria, rather than protection of the murine host [58].

NO variation in malaria: dependency on *Plasmodium* strain and host species

The pathology and prognosis of malaria depends not only on the strain of *Plasmodium*, but also on the species and strain of the vertebrate

host [59-61]. Differences in the pathologies of *Plasmodia* appeared to depend more on the strain of parasite than the strain of mouse, indicating the nature of the parasite and/or its antigens was more important than the mouse strain in determining host defence response [10]. Different levels of resistance to *P. chabaudi* have been reported in different strains of mouse [52, 62]. The data revealed that RNI correlated with the stage of disease and degree of parasitemia. RNI appeared to be unable to act against lethal *Plasmodia,* because the parasite may release toxins to inhibit NO and/or RNI production. In contrast, RNI did appear to resolve nonlethal *Plasmodia,* which suggests that high levels of NO in the early and mid-stages of infection can reduce the amount of NO production in the late stage [63]. Therefore, the contribution of NO in the lethal strain might be pathological rather than preventive [61].

Host-parasite combination and NO involvement

The results of studies in man remain conflicting and in rodent malaria the principal protector mechanisms vary between different host-parasite combinations [50]. Therefore, the importance of NO in killing *Plasmodia* might be different between mice and humans [52]. Whilst murine malaria models, such as *P. berghei* as a fatal and *P. chabaudi* as a resolved strain, are useful in exploring the role of NO and its metabolites in malaria, different patterns for involvement in such malaria models are evident in published reports. These differences may be attributable to such factors as tissue sampled, target organs, day of infection, degree of parasitemia, assay method, host species and strain of *Plasmodia*. The presence of parasites resistant to the microbicidal action of NO may result in high levels of NO being generated, which may then play a role in host pathology rather than controlling parasitemia [13, 64]. Consequently, the host-parasite combination will be a determining factor as to whether the parasite is capable of stimulating NO production [50].

Conclusion

Taken together, the data provided by researchers highlight the fact that NO and/or its up/downstream molecules are involved in malaria, but the involvement is not independent of other immune events. NO appears to be an important, but possibly non-essential contributor in controlling acute-phase malaria infection. Although the protective immune responses against malaria parasite are multifactorial, and the final effector molecules that mediate parasite death are not known, NOS, NO and RNI have been significantly implicated [10, 65]. It is concluded that NO is only part of an immunopathological chain against malaria infection and the anti-parasitic function against *Plasmodia* did not relate only to NO action; therefore, a combination of NO and other immune factors is required to resolve *Plasmodia* and to eliminate malaria in the host. A combination of some key cytokines appears to be essential for iNOS gene regulation. Perhaps NO comes from several cellular sources; further investigation in defining these sources will be important for understanding cell-mediated defence mechanism(s) in malaria. Selective delivery of inhibitors and donors of NO synthesis in tissues of the malaria host is also indicated as potential novel therapies to inhibit parasites or prevent pathological symptoms [66].

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