Malarial anaemia and nitric oxide induced megaloblastic anaemia: a review on the causes of malarial anaemia

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Abstract

Direct destruction and ineffective erythropoiesis does not adequately explain the cause of anaemia in malaria. It is possible that there are more other mechanisms involved besides the causes described till date in malarial anaemia. The effect of NO on erythropoiesis and a major haematological abnormality (microcytic/normocytic/megaloblastic picture) can significantly be observed on repeated exposure. In addition, NO can inhibit the enzyme methionine synthase so functional vit B₁₂ deficiency state may occur which can lead to megaloblastic anaemia. This review will focus on causation of malarial anaemia and nitric oxide induced megaloblastic anaemia.

Key words Dyserthropoiesis – immunological destruction of RBC – malarial anaemia – megaloblastic anaemia – methionine synthase inhibition – NO induced anaemia – vit B₁₂ deficiency

Introduction

Malaria is caused by obligate intracellular parasites, which live in host erythrocytes and remodel these cells to provide optimally for their own needs. It is a major public health problem in tropical areas, and it is estimated that malaria is responsible for 1 to 3 million deaths and 300–500 million infections annually. The vast majority of morbidity and mortality from malaria is caused by infection with *Plasmodium falciparum*, although *P. vivax, P. ovale, and P. malariae* are also responsible for human infections. A lack of acquired immunity to *P. falciparum* malaria in young children appears to underlie the high rates of morbidity and mortality from malaria in areas of sub-Saharan Africa where malaria is endemic¹. Although the molecular mechanisms responsible for effective malarial immunity remain elusive, production of nitric oxide (NO) appears to be an important marker and potential mediator of disease severity². Also, NO mediates a diverse array of physiologic and pathologic processes, and appears to be an important mediator of the protective immune response to all stages of *Plasmodium* infections³. This review will focus on malarial anaemia and the relationship of nitric oxide with malarial anaemia.

Anaemia in malaria

Malarial anaemia is thought to arise from both decreased red blood cell (RBC) production and increased RBC destruction. Destruction of RBCs can occur as a result of parasite invasion and replications. The pathophysiology of severe anaemia is a complex but relatively neglected area of study. Certainly, malaria gives ample reasons for both increased destruction and reduced production of red cells.

Direct and immunological destruction: Red blood cells are destroyed as parasites complete their growth cycle, although some parasites may be removed from erythrocytes as immature ring forms by phagocytic cells⁴. Infected erythrocytes may also be phagocytosed by macrophages following opsonization by immunoglobulins and/or complement components. Other effector cells and mechanisms are less well-defined but may include antibody-dependent cytotoxicity and natural killer (NK) cells. The survival of
uninfected erythrocytes is also reduced. Mathematical modeling of haematologic data from experimental human *P. falciparum* infections as well as analysis of clinical data from endemic areas, have suggested that up to 12 uninfected RBCs (uRBCs) are lost for every infected RBC.

Thus, it is widely accepted that direct destruction of RBCs following parasitization cannot account for the degree of anaemia observed during malaria infection, suggesting that the destruction of uRBCs is the major cause of haemoglobin (Hb) loss. Malarial anaemia may be mediated in part by immunopathologic processes. The activity and the number of macrophages are increased in malarial infection. Moreover, the signals for recognition of uninfected erythrocytes for removal by macrophages are enhanced. Uninfected erythrocytes bind increased amount of immunoglobulin and/or complement as detected in the direct antiglobulin test (DAT or Coomb’s Test). The specificity of the immunoglobulins on the surface of the red cells has remained controversial. These antibodies do not have a particular specificity but are more likely to represent immune complexes absorbed onto the surface of red blood cells by complement receptors including CR1 (CD35). Reticulocytopenia has been observed in numerous clinical studies of malarial anaemia.

Sometimes in asymptomatic malaria, anaemia is frequently out of proportion to the low level of parasitaemia found, suggesting that it is not mediated simply by direct destruction/haemolysis of parasitized red blood cells. The anaemia of asymptomatic parasitaemia is important since children may later develop severe anaemia, both with and without subsequent episodes of acute clinical malaria.

**Due to suppression of erythropoiesis:** The histopathological study of the bone marrow of children with malarial anaemia shows impaired bone marrow response, erythroid hyperplasia, with dyserythropoiesis, cytoplasmic and nuclear bridging, and irregular nuclear outline. The functional abnormality has not been defined, but an increased proportion of the erythroid progenitors are found in the G2 phase compared with normal controls. The prime candidates for the host factors mediating dyserythropoiesis have been growth factors and cytokines. Serum erythropoietin (Epo) was appropriately raised in a study and EPO production is robust and correlates inversely with the degree of anaemia. Acute haemolysis and impaired bone marrow response are important in this setting. Parasitaemia and NO production measured in cross-sectional studies on any one day will not necessarily reflect mean parasitaemia and mean levels of NO production to which the bone marrow has been exposed in preceding weeks. Although there was no history of fever in the two weeks prior to recruitment, it is possible that the haemoglobin levels measured could have also been influenced by past intercurrent episodes of acute clinical malaria.

The concentrations of tumor necrosis factor-α (TNF-α) and interferon (IFN)-γ have been correlated with the severity of the disease and high levels of TNF-α have been shown to suppress erythropoiesis. These cytokines may also contribute to reduced production of Epo and to increased erythrophagocytosis. The possibility has been raised that high levels of the Th2-type cytokine interleukin-10 (IL-10) might prevent the development of severe malarial anaemia. Low levels of IL-10 have been described in African children with severe malarial anaemia. However, the mechanism of protection from anaemia by IL-10 is unclear. The hypothesis that parasite products directly stimulate the production of inflammatory cytokines, including TNF-α, has been widely promoted.

Nitric oxide is an inhibitor of erythropoiesis. Cytokine-induced NO is known to decrease human erythropoiesis, and NO is likely an important mediator of the anaemia of chronic disease in humans. Although increased NO production appears to be associated with protection against malaria in the Gabonese children that were previously investigated, elevated levels of NO can suppress erythropoiesis and induce apoptosis in cultured CD34+ cells. *In vitro* studies show that tumor necrosis factor TNF-
α and interferon-γ induced suppression of human haematopoiesis is in part mediated by NO\textsuperscript{25}.

The glycosylphosphatidylinositol (GPI) anchor of malarial membrane proteins may cause cellular dysfunction, but a role for this toxin in dyserythropoiesis remains to be established\textsuperscript{26}. Other toxic products may exist. During its blood stage, the malaria parasite proteolyses host haemoglobin, releasing heme as a by-product. β-Haematin forms as a crystalline cyclic dimer of oxidized heme and is complexed with protein and lipid products as malarial pigment or haemozoin. The function of monocytes and macrophages is severely inhibited after ingestion of haemozoin. Here, the biologically active moieties may be lipoperoxides such as 4-hydroxynonenal (4-HNE) and 15(S, R) hydroxyeicosatetraenoic acid (HETE) produced by oxidation of membrane lipids\textsuperscript{27}. Their effect on other cellular functions, such as erythropoiesis, has not been established. Anaemia in falciparum malaria is clearly multifactorial and there is a strong argument that erythrocyte destruction and ineffective erythropoiesis play equal parts in the etiology of malarial anaemia.

In the above conditions, anaemia is typically normocytic and normochromic, with a notable absence of reticulocytes, although microcytosis and hypochromia may be present due to the very high frequency of alpha and beta thalassemia traits and/or iron deficiency in many endemic areas\textsuperscript{28}.

Due to defect in one carbon transfer by NO: Nitric oxide (NO) is produced by most cell types and regulates a diverse array of biological functions\textsuperscript{29–31}. NO is known to react with heme proteins, porphyrins, and cobalamins to form nitrosyl-metal complexes\textsuperscript{32–36}. NO has been reported to inhibit methionine synthase activity \textit{in vitro}\textsuperscript{37–39}, and it might be expected to bind to the cobalamin in the first case, because, first, NO binds tightly to the iron in heme\textsuperscript{40}; second, ferrous heme and cbl(III) are isoelectronically; and third, in both heme and cobalamin, the metal ion is coordinated to four in-plane nitrogen atoms of a tetrapyrrole ring and has two out-of-plane ligands\textsuperscript{41}. NO has a remarkably high affinity for ferrous heme with a binding constant on the order of 10\textsuperscript{12} to 10\textsuperscript{14} M\textsuperscript{–1}, and NO also binds to ferric heme\textsuperscript{42}. Iron and cobalt are transition metals adjacent in the periodic table, and the porphyrin ring of heme and the corrin ring of cobalamin are both substituted tetrapyrrole rings\textsuperscript{43}. Thus, it is not surprising that NO binds to the cobalt in cobalamin, and it is observed that NO reacts with all three valency states of cobalamin\textsuperscript{44}.

Cobalamin exists in two metabolically active forms, identified by alkali group attached to sixth co-ordinated position of cobalt atom: methylcobalamin and adenosylcobalamin. The therapeutic preparation is Vitamin B\textsubscript{12} (cyanocobalamin) which has no known physiologic role and must be converted to biologically active form. Methylcobalamin is the form required for methionine synthase in folate metabolism\textsuperscript{45}. In methylcobalamin, a methyl group is bonded to cobalt in the upper axial position, and the lower axial position is occupied by a nitrogen of the dimethylbenzimidazole nucleotide substituent of the corrin ring. During turnover, the cobalamin cofactor of methionine synthase shuttles between methylcobalamin and cob(I) alamin which contains a pair of electrons in the dz\textsubscript{2} orbital oriented perpendicularly to the plane of the corrin ring. The enzyme-bound cob(I) alamin can be oxidized to cob(II) alamin, with a single electron in the dz\textsubscript{2} orbital, or to cob(III) alamin\textsuperscript{46}. Adenosylcobalamin is required for conversion of methylmalonyl CoA to succinyl CoA and abnormality in this will lead to fatty acid accumulation\textsuperscript{45}. Methionine synthase is poised at the point of convergence of two major biosynthetic pathways: the tetrahydrofolate-dependent pathway for biosynthesis of methyl groups and the homocysteine biosynthetic pathway in prokaryotes. However, in mammals on the other hand, are unable to synthesize homocysteine \textit{de novo}; rather they use methionine synthase to regenerate methionine from homocysteine to provide one-carbon units for S-adenosyl-methionine (AdoMet)-dependent methylation reactions (Fig. 1)\textsuperscript{46}.

Some researchers have proved that NO reacts rapidly and irreversibly with cbl(I)\textsuperscript{44} which could oxidize
cbl(I) to cbl(II) and therefore, interfere with the cbl(I)→cbl(III) cycle that is an essential part of the methionine synthase reaction\textsuperscript{47}. Marius \textit{et al}\textsuperscript{48} have observed that NO is able to diminish the cofactor activity of cobalamin for methionine synthase. Inhibition is mediated by NO-cobalt interactions. Because nitrosylcobalamin can transfer NO to glutathione. It is also possible that the decrement in cofactor ability of cobalamin is caused by modification of the substrate homocysteine to S-nitroso-homocysteine formation (by NO transferred from nitrosylcobalamin to homocysteine)\textsuperscript{48}. Nicolaou \textit{et al}\textsuperscript{49} have reported NO-mediated inhibition of methionine synthase (Fig. 2). In some experiments it has been seen that NO inhibits methionine synthase activity \textit{in vivo} and that NO produced by three different pharmacological agents or produced physiologically by rat C6 glioma cells inhibits carbon flow through the folate pathway\textsuperscript{44}. Surprisingly, it binds to all three valency states of cobalamin and mechanism of methionine synthase inhibition appears to be similar to that of N\textsubscript{2}O\textsuperscript{44}. However, conflicting results have been published concerning NO binding to cobalamin like, NO binds only to divalent cobalamin (\textit{i.e.} cbl[II])\textsuperscript{50}, and some found that NO binds to both cbl(II) and (III)\textsuperscript{48}.

**Fig. 1:** Transfer of the methyl group of methyltetrahydrofolate (CH\textsubscript{3}-THF) to homocysteine via methionine synthase-methylcobalamin [MetSyn-CH\textsubscript{3}-Co(III)] as an intermediate methyl carrier. The reductive methylation in the lower part of the scheme is the mechanism by which S-adenosylmethionine (Ado-Met) together with an electron reactivates the enzyme after oxidative inactivation. Ado-Hcy, S-adenosylhomocysteine.

**Fig. 2:** \textbf{Folate-methionine reactions.} Folic acid (FA) is reduced to tetrahydrofolate (THF) via dihydrofolate (DHF). Formate enters the folate pathway by combining with tetrahydrofolate to form 10-formyltetrahydrofolate, which can be reversibly converted to 5, 10-methyltetrahydrofolate and to 5,10-methylene tetrahydrofolate. The latter compound is converted irreversibly to 5-methyltetrahydrofolate, the major intracellular storage form of folates. Methionine synthase transfers the methyl group of 5-methyltetrahydrofolate to homocysteine (Hcy) via a cobalamin intermediate and thereby regenerates free tetrahydrofolate. Methionine (Met) can be converted to AdoMet, the main intracellular source of methyl groups for transmethylation reactions. A transsulfuration cycle is completed when S-adenosylhomocysteine (AdoHcy) is converted to homocysteine. Carbons in positions 2 and 8 of the purine ring are derived from 10-formyltetrahydrofolate during \textit{de novo} purine synthesis, and serine (Ser) can be synthesized from glycine (Gly) using a carbon from 5,10-methylene tetrahydrofolate.
And some, initially reported that NO binds to cbl(III) and oxidizes cbl(II) to cbl(III) but subsequently concluded that NO does not react with either species\textsuperscript{51}.

In an experiment by Idrees et al\textsuperscript{44} NO donors decreased $[14C]$ methyl tetrahydrofolate incorporation into protein; and homocysteine reduced the effectiveness of PAPA-NONOate (propylamine propylamine NONOate) as an inhibitor of de novo purine nucleotide synthesis. NO, is known to inhibit ribonucleotide reductase\textsuperscript{52}, which could decrease DNA synthesis and thereby cause purine nucleotides to accumulate; the latter could inhibit de novo purine synthesis by a feedback mechanism.

Relative high levels of NO may be produced in vivo in humans in various conditions, including infection, septic shock, and trauma. It is clear that humans can be induced to produce increased amounts of NO in vivo with infection and shock\textsuperscript{53} and when receiving IL-2 treatment for cancer\textsuperscript{54,55}. The cells producing the excess NO in vivo in these conditions are not known. Mononuclear phagocytes, hepatocytes, smooth muscle cells, endothelial cells, and/or other cells could be overproducing NO. In these circumstances, it is possible that the NO might diminish the enzyme cofactor abilities of cobalamins and produce a functional vitamin B\textsubscript{12} deficiency state.

Nitric oxide also alters cellular iron metabolism, and it likely contributes (through its effects on iron metabolism) to the anaemia of chronic diseases\textsuperscript{22}. Iron deficiency itself is a major cause of anaemia in malaria-endemic areas\textsuperscript{56}.

**Discussion**

Some workers defined two forms of malaria-associated anaemia predominate: anaemia associated with (a) acute clinical episodes of malaria (or a history of such episodes), and (b) anaemia associated with the chronic, intermittent, asymptomatic, low-grade parasitaemia found in 100\% of children in endemic areas\textsuperscript{57–60}. In this latter group with asymptomatic parasitaemia, the anaemia is frequently out of proportion to the low level of parasitaemia found, suggesting that it is not mediated simply by direct destruction/haemolysis of parasitized red blood cells. The anaemia of asymptomatic parasitaemia is important since children may later develop severe anaemia, both with and without subsequent episodes of acute clinical malaria. So direct destruction and ineffective erythropoiesis does not adequately explain the cause of anaemia in malaria. It is possible that there are more other mechanisms involved besides the above described for causation of malarial anaemia.

NO is rapidly oxidized to the stable inorganic nitrogen oxides, nitrite and nitrate in vivo\textsuperscript{47,61}. Nitrite, rather than nitrite plus nitrate, is believed to be the product of NO in oxygenated water\textsuperscript{62}. Haemoglobin possesses anion binding sites that may retain nitrite, raising concerns that the measured NO levels may be overestimated as a result of conversion of haemoglobin-bound nitrite to NO\textsuperscript{63}. The majority of studies examining NO production in malaria-exposed adults have reported NO metabolite levels in the setting of clinical disease\textsuperscript{64–67}. Most of these studies did not control for dietary nitrate ingestion\textsuperscript{64,65} or altered nitrate handling in renal impairment\textsuperscript{64,67}. Very few studies have reported NO metabolite levels in asymptomatic malaria-exposed adults\textsuperscript{64,68}, and none of these controlled for the confounding effect of dietary nitrite-plus-nitrate (NO\textsubscript{X}) ingestion. Also NO is produced in response to severity and some other cause (inflammation, super added infection, shock etc)\textsuperscript{53} as the disease progress. So it is not specific that only malaria induced NO will inhibit methionine synthase and direct cause of ineffective erythropoiesis in malaria endemic area. Also we can infer that in chronic inflammatory conditions or infections, megaloblastic anaemia can be seen.

Any adverse influence of NO on haematopoiesis is likely to result from the effects of sustained NO production over days-weeks in response to chronic parasitaemia. Production of NO is likely to fluctuate longitudinally in response to the longitudinal fluctuations in parasite density known to occur in asymptomatic parasitaemia\textsuperscript{69}. Parasitaemia and NO
production measured in cross-sectional studies on any one day will not necessarily reflect mean parasitaemia and mean levels of NO production to which the bone marrow has been exposed in preceding weeks. Although there was no history of fever in the two weeks prior to recruitment, it is possible that the haemoglobin levels measured could also have been influenced by past intercurrent episodes of acute clinical malaria. Thus the effect of NO on erythropoiesis and a major haematological abnormality (microcytic/normocytic/megaloblastic picture) can significantly be observed on repeated exposure and in asymptomatic cases after repeated exposure in endemic areas. And sudden burst of NO in 4–5 days may not predispose to megaloblastic or microcytic picture.

The majority of studies examining NO production in malaria-exposed adults have reported NO metabolite levels in the setting of clinical disease, reflecting altered NO production in clinical malaria. NO could bind to the methyl ligand binding site of cbl(III), thereby preventing or diminishing formation of the CH3-cbl(III) intermediate in the methionine synthase reaction. Second, NO could oxidize cbl(I) to cbl(II) and therefore, interfere with the cbl(I) → cbl(III) cycle that is an essential part of the methionine synthase reaction. Although NO, like N2O, appears to inhibit methionine synthase activity by interacting with cbl(I), the nature of enzyme inhibition may be quite different between the two gases. While N2O damages the protein by producing OH-radical, with depletion of cobalamin being a secondary cause, NO should prevent only regeneration of the CH3-cbl(III) prosthetic group. If rapid burst of NO by iNOS occurs then it is possible that like N2O acute megaloblastosis with normocytic normochromic anaemia like features can be observed. But some researchers found no evidence of NO binding to either cbl(II) or (III) and some initially reported that NO binds to cbl(III) and oxidizes cbl(II) to cbl(III) but subsequently concluded that NO does not react with either species. Again it has been seen that aquocobalamin (H2O-Cbl) but not cyanocobalamin (CN-Cbl), methylcobalamin (Me-Cbl), or adenosyl-cobalamin (Ado-Cbl)) reacts with NO. So the causation of megaloblastic anaemia in malaria remains elusive and can be confirmed by measuring methionine synthase level in blood and peripheral blood smear in chronic asymptomatic cases where sustained NO production is evident.

References


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