Reactive Nitrogen Intermediate (RNI) Levels Inside and Outside *Plasmodium* Infected Red Blood Cells in Murine Malaria

Hossein Nahrevanian[1], Michael J Dascombe[2]

[1]*Department of Parasitology, Pasteur Institute of Iran, Pasteur Ave. 13164, Tehran, Iran*
[2]*University of Manchester,1.124, Stopford Building, Biological Sciences, Manchester M13 9PT, UK*

Abstract

Malaria is one of the most important parasitic diseases in the world. In addition to numerous cytokines, nitric oxide (NO) is thought to be an important mediator and critical signaling molecule of malaria pathology and a target for novel drug therapy. This study has investigated the involvement of reactive nitrogen intermediates (RNI) in murine malaria, *P. berghei* parasitized and uninfected red blood cells (PRBC, URBC), in order to evaluate the actual level of RNI relating to intracellular and extracellular activities of *Plasmodium*.

In PRBC, nitrite reacts with Hb to form toxic nitrosothiol-Hb, *met*-Hb and hydrogen peroxide. NO may be the precursor of chemical species that are even more toxic, such as peroxinitrite or hydroxyl radicals. The RNI levels are measured in plasma as the surrounding medium for PRBC, however, understanding the quantitative level of NO production inside the PRBC seems to be important to elucidating the mechanism of parasite penetration into the RBC.

The objective of this study was to measure RNI levels in both inside and outside the RBC during infection with *Plasmodium* as an intracellular parasite. It is suggested that NO or RNI is critical for intracellular parasites such as plasmodia. This experiment was carried out in two parts; in part A, the RNI levels were measured inside the PRBC and URBC of the host, and in part B, it was studied in plasma as an extracellular level of NO production. The results showed that the amounts of RNI levels inside the RBC were low at the baseline detection of Griess microassay (GMA). However, the RNI levels in the plasma of the malarial (47.7 ± 9.1 μM) and control groups (26.8 ± 2.1 μM) showed a significant difference ($n = 5$, $P < 0.05$). The exact mechanism(s) by which NO acts during malaria infection is not fully understood and requires more investigation.

Keywords: nitric oxide, RNI, malaria, *Plasmodium,* RBC, hemoglobin

Introduction

Nitric oxide (NO) is produced by many cells and released into the circulation or trapped by other cells. NO and RNI may react in several ways to cause cell death by diffusing into cells, as well as forming nitrosothiol groups, leading to the inactivation of enzymes or changes in protein function [1].

Mononuclear cells and several other types of mammalian cells produce NO after stimulation with cytokines, bacterial endotoxin or the antigens of infectious agents [2]. A role for antiparasitic effects of NO and RNI *in vivo* and *in vitro* has been demonstrated against a number of parasites, including *Plasmodium* spp, *Leishmania* spp, *Toxoplasma gondii, Schistosoma* spp and *Trypanosoma brucei* [3-6]. NO is thought

Corresponding author:
Hossein Nahrevanian
E-mail: mobcghn@yahoo.co.uk
Fax: +98 21 6465132

to be an important mediator [7], or critical signalling molecule, of malaria immunopathology and a target for novel drug therapy [8]. NO in the serum of malaria-tolerant people is generated by macrophages and appears to be responsible for the malaria tolerance [3]. NO also is reported to inhibit both the liver and blood forms of malaria parasites [9]. It is suggested a cascade of reactions leading to NO production is involved in the killing of infected hepatocytes [10]. However, there are some contradictory reports about the role of NO and related molecules in malaria [11]. Data reported by some researchers do not support a potent role for NO during malaria [12-13].

Despite the importance of NO as a biological mediator, few methods have been described for its assay. The Griess reaction measures nitrite, which, if applied after the catalytic reduction of nitrates in samples, measures both free nitrite and the larger nitrate pool. These values are commonly used as a measure of RNI [14-15]. The Griess assay is cheap and can be applied to the micromolar concentrations of relatively stable NO metabolites that accumulate both locally and away from the site of NO production. For such reasons as these, the Griess assay has been central in determining the involvement of NO in malaria [15].

The levels of NO metabolites are measured in plasma as the surrounding medium for PRBC. However, understanding the qualitative and quantitative level of NO production and its metabolites inside the PRBC seems to be important to elucidating the mechanism of parasite penetration and settlement inside the RBC, and its pathogenesis during immune response.

Blood flow may play a role in transfering NO and its metabolites from sources of production to other parts, in order to eliminate these toxic materials by their presentation to scavenger sites. Blood circulation appears to have a positive role as a fast distributor of high levels of NO or its metabolites throughout the whole body and among the host tissues [14].

In PRBC, nitrite reacts with hemoglobin (Hb) to form either nitrosothiol-Hb or *met*-Hb and also hydrogen peroxide, which are all toxic to plasmodia [5]. NO may be the precursor of chemical species that are even more toxic, such as peroxinitrite or hydroxyl radicals [5]. The objective of this study is to measure the RNI level inside the PRBC and compare this amount with that extracellularly. Another reason for this study is to measure the RNI inside both PRBC and URBC to assess the levels, and to compare between them. It is suggested that NO or RNI are critical for intracellular parasites such as plasmodia. In this study, RNI production is assayed in the plasma of *P. berghei*-inoculated MF1 mice (malarial group) during infection, to compare these values with those in URBC inoculated mice (control group). This may clarify the actual pattern of NO production during this murine malaria infection.

Materials and methods

Animals

Mice used in this study were MF1 supplied by the Biological Services Unit, University of Manchester. Animals were housed in plastic cages (length 38 cm × width 22 cm × height 11 cm) at room temperature 19-22˚C, on a 12 h light (08:00-20:00) and 12 h dark (20:00-08:00) cycle, with unlimited access to food (CRM feeding pellets, SDS) and tap water. Experiments were licensed under the Animals (UK Scientific Procedures) Act, 1986. In compliance with the conditions of this license, infected animals were humanely killed at the onset of the terminal phase of malaria. Malarial animals were inoculated iv with 2×10^7 PRBC from a donor *P. berghei*-infected mouse and the control group received an equivalent volume (0.2 ml/mouse) of diluted URBC.

Experiments and groups

Animals used in this experiment were 10 male MF1 mice. Body weight was 20 ± 0.7 g on day zero. Animals were divided into two groups; malarial animals were inoculated iv with a donor *P. berghei*-infected mouse with 45% parasitemia. The control group received an equivalent volume (0.2 ml/mouse, iv) of diluted URBC.

Hemolysed RBC and plasma samples were collected for RNI measurement using GMA.

Malaria parasites

P. berghei N/13/1A/4/203, originally obtained from the School of Tropical Medicine, University of Liverpool, UK, was used in this study. Parasites were maintained by blood passage in MF1 mice when active parasites were required; otherwise they were restored at -70˚C in Alserver's solution (2.33% glucose, 0.525% NaCl and 1% sodium citrate in deionized water) and glycerol (9:1 parts by volume).

Inoculation of malaria parasites

Mice were inoculated (0.2 ml, iv) into a tail vein with blood from a donor *P. berghei*- infected mouse diluted with 0.85% saline, to contain 2×10^7 PRBC.

Parasitemia

In all animals, parasitemia was determined using blood smears stained with Leishman's reagent (Sigma Chemical Co, USA) 2 mg/ml methanol. PRBC were counted in five different fields, each of approximately 200 cells. Results are expressed as the mean percentage (%) of erythrocytes containing Leishman-positive bodies.

Griess microassay (GMA)

Mice were terminally anesthetized by inhalation of diethyl ether (BDH, England) and blood taken by cardiac puncture into a 1 ml syringe containing 50 iu heparin (Monoparin, CP Pharmaceuticals Ltd, Wareham, UK). Plasma was prepared by centrifuging blood at 1,500 relative centrifugal force (RCF) (MSE Centaur 2, UK) for 10 min and stored at -70˚C until assayed. After blood collection, animals were humanely sacrificed by cervical dislocation. RNI were measured in samples using the Griess reaction after first converting nitrates to nitrites with nitrate reductase treatment. The Griess reaction was adapted, with modifications, from the methods of [12] and [16] to assay nitrite. RNI was determined indirectly by the GMA, as the nitrite produced from nitrate when incubated with nitrate reductase. 60 µl samples were treated with 10 µl nitrate reductase (NAD[P]H *Aspergillus* species 5 U/ml, Sigma Chemical Co, UK) and 30 µl NADPH β-nicotinamide adenine dinucleotide phosphate (1.25 mg/ml, Sigma Diagnostics, St Louis, USA). 200 µl Griess reagent (5% phosphoric acid, 1% sulfanilic acid and 0.1% N (1-naphthyl-1)-ethylendiamine dihydrochloride (NED), all from Sigma Chemical Co, UK, dissolved in 100 ml deionized water) was then added and proteins subsequently precipitated by 200 µl trichloroacetic acid 10%, (BDH, England). Tube contents were vortex mixed then centrifuged at 13,400 RCF (Model 1-13 Microcentrifuge, Sigma, UK). Duplicate 200 µl samples of supernatants were transferred to a 96-well flat-bottomed microplate (Costar, USA) and absorbances read at 520 nm using a microplate reader (Dynatech, MRX, USA).

Lysis of red blood cells by hypotonic shock

Blood was taken by cardiac puncture from all animals under terminal general anesthesia and a RBC pellet from each sample was prepared by centrifugation. This method was adapted from Rockett *et al* (1994), with the modification of using direct lysis in hypotonic distilled water. The method is based on osmosis and the effect of hypotonic solutions on cells, which leads to water flow through cell membrane from hypotonic to hypertonic medium to reach to an equilibrium (isotonic). The final result in this process is swelling and bursting of RBC with hemolysis. The protocol was applied according to the following procedure: blood samples were centrifuged at 600 RCF (2,500 rpm) (Centrifuge, MSE, Centaur 2, Co, UK) for 10 min and plasma with buffy coat removed as supernatant. Pellets were washed 3 times with PBS and after each wash, samples were centrifuged at 600 RCF for 10 min. Sedimented RBC were transferred to another tube. Deionized H_2O (dH_2O) in equal volume to RBC samples was added to tubes and the contents were vortex mixed thoroughly for 30 sec. Tube contents were incubated for complete hemolysis at room temperature (19-22˚C) for 30 min and finally centrifuged at 600 RCF at 15 min. Supernatants were stored at -20˚C

until subjected to GMA. The amounts of total nitrite at the end of calculation were multiplied by two, because of the 1:1 dilution of RBC in dH_2O used during cell lysis.

Statistical analysis

Values for RNI concentration are presented as the mean ± SEM for groups of n = 5 mice. The significance of difference ($P < 0.05$) was determined by Student's *t*-test using GraphPad Prism Software (GraphPad, San Diego, California, USA).

Results and discussion

The amounts of RNI inside the PRBC and URBC were too low (approximately 1.87 μM), which is the baseline level of total nitrite measured by the GMA in this experiment. Although, no difference was observed in the RNI levels between PRBC and URBC hemolysates, the RNI levels in the plasma of the malarial and control groups showed a significant difference (malaria 47.7 ± 9.1 μM, control 26.8 ± 2.1 μM, n = 5, $P < 0.05$) (Fig 1).

Low levels of total nitrite indicate that Hb is a powerful NO scavenger and may have prevented nitrite formation. On the other hand, NO is known to react rapidly with oxyhemoglobin (*Oxy*-Hb) to give nitrate and *met*-Hb. The GMA did not show NO_2^- accumulation inside the RBC suspensions [17]. Even if NO is cytotoxic for some species of plasmodia, some would argue it is an unlikely effector molecule *in vivo* against erythrocytic parasites, which are constantly surrounded by Hb, a very potent scavenger for this radical [18]. The scavenging of NO by Hb in blood vessels should represent a significant sink for this molecule [19].

$$\textbf{Hb-O}_2 + \textbf{NO} \rightarrow \textit{met}\textbf{-Hb} + \textbf{NO}_3^-$$

Nitrite reacts soon after production with *Oxy*-Hb and this could explain why it is present in such small amounts in samples [20]. Nitrite accumulation could not be detected in RBC, perhaps because of high levels of Hb, a powerful

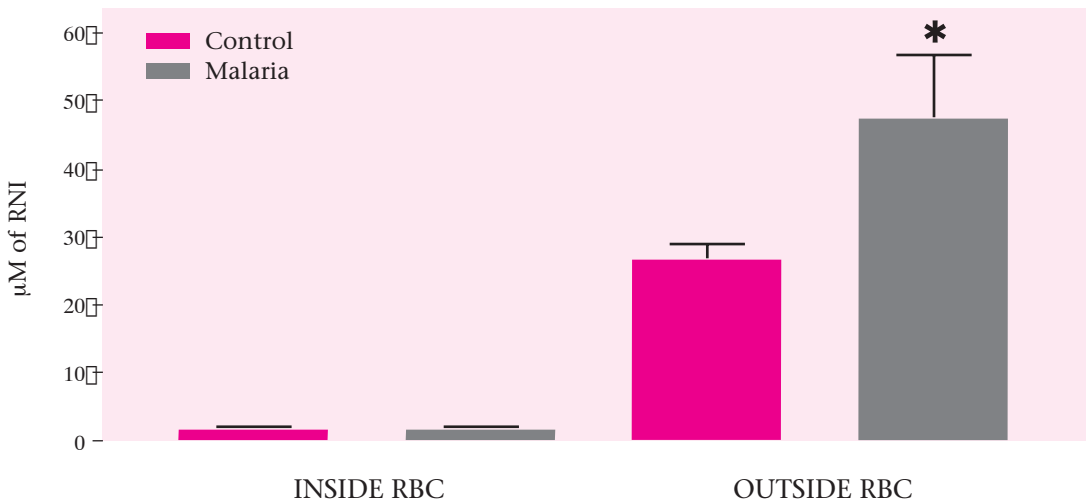

Fig 1 RNI levels inside and outside the RBC of control and *P. berghei*-infected host. Values of RNI from RBC hemolysed suspensions and plasma samples are expressed as mean ± SEM, n = 5. Malarial animals were inoculated with PRBC from a donor *P. berghei*- infected mouse. Control mice received an equivalent volume (0.2 ml/mouse) of diluted URBC. Statistical analysis were determined by Student's *t*-test with a significant difference of $P < 0.05$ using GraphPad Prism.

NO scavenger [17]. Thus, Hb within the RBC acts as a sink and prevents NO reaching the plasmodia [18]. *Oxy*-Hb in RBC is a target for RNI, and nitrite reacts with this molecule to form either nitrosothiol-Hb or *met*-Hb [5]. Both reactive oxygen intermediates (ROI) and Hb seem to oppose NO effects. An important function of NO in the blood is bound to thiols in Hb, forming S-nitroshemoglobin (*SNO*-Hb), which releases the NO group on deoxygeneration in the microcirculation [21]. When NO diffuses into the RBC, it reacts rapidly with *Oxy*-Hb to form *met*-Hb and nitrite. In the presence of oxygen and *met*-Hb reductase, these are rapidly converted back to Hb and nitrate [1]. NO is a freely diffusable molecule. Therefore, the increased permeability and changes in the lipid composition of the red cell membrane may have only a marginal effect on the entry of NO or its donor molecule into infected erythrocytes [22]. The possible anti-parasitic function of NO and RNI against malaria is less well accepted. This is due, at least in part, to the belief that Hb universally scavenges NO and thus RBC acts as a permanent sink for this molecule [23].

It now appears that this theory is incomplete. Under certain conditions *in vivo*, when RBC is saturated with oxygen, Hb binds to NO. However, with low oxygen tension, Hb readily releases NO [23]. Also, the sensitivity of malaria parasites to NO increases with decreasing oxygen tension. This is because, at lower oxygen tension, Hb releases NO rather than binds the molecule, making it available to exert antiparasitic effect [24]. Hb may serve a dual role, as a scavenger, but also as a donor for NO. It is thought that an equilibrium between the binding of NO to Hb (NO scavenging) and unbinding (NO releasing) is reached at a given, variable oxygen tension [23].

There is also some evidence indicating that human PRBC infected with *P. falciparum* can synthesize NO. The synthesis of NO appeared to be very high in comparison with human endothelial cells [17]. The NOS activity was also very high in the lysate of PRBC, while not measurable in that of URBC, suggesting a new pathogenic mode of parasite-host interaction. In addition, *P. falciparum* itself may generate NO and produce some soluble factors, able to evoke NO synthesis in host tissues. The data suggests that increased NO synthesis in malaria can be directly managed by soluble factor(s) released by the blood stages of the parasite, without requiring host cytokines [17].

Perhaps an inhibitory mechanism by hemozoin leads to a reduction of RNI and ROI production, to decrease the overproduction of these mediators causing malaria pathology. *P. vinckei* hemozoin decreases the production of ROI and NO in murine peritoneal macrophages [25]. NO could react with intra-parasitic molecules and participate in the assembly of free heme into hemozoin or the formation of S-nitrosothiols. It is well known that hemozoin accumulation is indispensable for parasite survival, as soluble heme can damage biological membranes and inhibit a variety of enzymes [17].

The increase in RNI during *P. berghei* malaria was found to be related to the stage of disease and degree of parasitemia, which suggests that NO/RNI have a role during *P. berghei* infection [14]. The parasite may release exotoxins to inhibit NO or RNI production. It is suggested a negative feedback by a high level of NO can reduce the amount of NO production as well, which was supported earlier by Taylor *et al* [8]. This feedback starts when NO concentration is saturated in the circulation *in vivo*; it can stop or may reverse the process of L-arginine oxidation to citrulline. This novel mechanism was identified whereby NO down-regulated iNOS gene expression, possibly to limit overproduction during the septic response [26].

Although, the protective immune responses against malaria parasite is multifactorial and the final effector molecules that mediate parasite death are not known, NOS, NO and RNI have been significantly implicated [14]. It is suggested the reason why the RNI level is low inside PRBC, but NOS protein expression is high, because Hb can only bind to NO and downstream metabolites eg RNI, but not to NOS as an upstream factor. Application of molecular assay including RT-PCR could be a more sensitive

method to detect the iNOS mRNA expression in target organs and even PRBC for intraerythrocytic activity of NO [27].

RNI levels revealed that NO had been involved during infection with *P. berghei*. However, failure of the host defence to eliminate parasites led to death. The contribution of NO in this study might be pathological rather than preventive in the *P. berghei* strain of murine malaria. The pathological consequences of lethal malaria, including hepato-splenomegaly, anemia, weight loss, hypothermia and a reduction of locomotor activity, may be related at least in part to the over-production of NO and RNI. Data from the *in vitro* study on the asexual stages of human parastic *P. falciparum* indicated that while NO itself may not be inhibitory to parasite development, its downstream products do have some anti-plasmodial activity [18]. However, the exact mechanism(s) by which NO acts during malaria infection is not fully understood and requires more investigation. Perhaps, NO comes from several cellular sources, which can contribute towards the protective immune responses against intracellular plasmodia. Further investigation in defining these sources will be important for the understanding of cell-mediated defense mechanism(s) in malaria.

Acknowledgements

The authors would like to thank Professor N Rothwell, Dr R Basir, Dr G Luheshi, Dr D Bristow, Mr WJ Manning for providing some materials, technical assistance and advice on methods. Special thanks to Professor M Assmar from the Department of Parasitology, Pasteur Institute of Iran, for his excellent support.

References

1. Ellis G, Adatia I, Yazdanpanah M, Makela SK. Nitrite and nitrate analyses: a clinical biochemistry perspective. *Clin Biochem* 1998;31:195-220.
2. Oliveira DM, Silva-Teixeira DN, Carmo SA, Goes AM. Role of nitric oxide on human schistosomiasis mansoni: upregulation of *in vitro* granuloma formation by N-omega-nitro-L-arginine methyl ester. *Nitric Oxide* 1998;2:57-65.
3. Clark IA, al-Yaman FM, Cowden WB, Rockett KA. Does malarial tolerance, through nitric oxide, explain the low incidence of autoimmune disease in tropical Africa? *Lancet* 1996;348:1492-4.
4. Motard A, Landau I, Nussler A, Grau G, Baccam D, Mazier D, *et al*. The role of reactive nitrogen intermediates in modulation of gametocyte infectivity of rodent malaria parasites. *Parasite Immunol* 1993;15:21-6.
5. Rockett KA, Awburn MM, Cowden WB, Clark IA. Killing of *Plasmodium falciparum in vitro* by nitric oxide derivatives. *Infect Immun* 1991;59:3280-3.
6. Roitt IM, Brostoff J, Male D. Immunology. 5th ed. London: Mosby Publication; 1998.
7. Bogdan C, Rollinghoff M, Diefenbach A. The role of nitric oxide in innate immunity. *Immunol Rev* 2000;173:17-26.
8. Taylor BS, Kim YM, Wang Q, Shapiro RA, Billiar TR, Geller DA. Nitric oxide down-regulates hepatocyte-inducible nitric oxide synthase gene expression. *Arch Surg* 1997; 132:1177-83.
9. Rockett KA, Cowden WB, Awburn MM, Clark IA. Arginine utilization and induction of nitric oxide synthase: cytokine-induced release of nitric oxide *in vivo* and its implications for the pathogenesis of cerebral malaria. In: Moncada S, Martella MA, Hibbs JrJB, Higgs EA, editors. The biology of nitric oxide: 2 Enzymology, Biochemistry and Immunology. London: Portland Press; 1992. p.135-7.
10. Hommel M. Immunology of malaria. In: WHO, Health co-operation papers, Quaderni di cooperazion sanitaria. Geneva: World Health Organisation; 1996. p. 53-70.
11. Chiwakata CB, Hemmer CJ, Dietrich M. High levels of inducible nitric oxide synthase mRNA are associated with increased monocytes count in blood and have a beneficial role in *Plasmodium falciparum* malaria. *Infect Immun* 2000;68:394-9.
12. Amante FH, Good MF. Prolonged Th1-like

response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice. *Parasite Immunol* 1997;19:111-26.
13. Favre N, Ryffel B, Rudin W. The development of murine cerebral malaria does not require nitric oxide production. *Parasitology* 1999;118:135-8.
14. Nahrevanian H, Dascombe MJ. Nitric oxide and reactive nitrogen intermediates in lethal and nonlethal strains of murine malaria. *Parasite Immunol* 2001;23:491-501.
15. White NJ. Malaria pathophysiology. In: Sherman IW. editor. Malaria, Parasite Biology, Pathogenesis and Protection. Washington DC: ASM Press; 1998. p. 371-85.
16. Rockett KA, Awburn MM, Rockett EJ, Cowden WB, Clark IA. Possible role of nitric oxide in malarial immunosuppression. *Parasite Immunol* 1994;16:243-9.
17. Ghigo D, Todde R, Ginsburg H, Costamagna C, Gautret P, Bussolino F, *et al*. Erythrocyte stages of *Plasmodium falciparum* exhibit a high nitric oxide synthase (NOS) activity and release an NOS-inducing soluble factor. *J Exp Med* 1995;182:677-88.
18. Jones IW, Thomsen LL, Knowles R, Gutteridge WE, Butcher GA, Sinden RE. Nitric oxide synthase activity in malaria-infected mice. *Parasite Immunol* 1996;18:535-8.
19. Lancaster JRJ. A tutorial on the diffusibility and reactivity of free nitric oxide. *Nitric Oxide* 1997;1:18-30.
20. Granger DL, Hibbs JB. High-output nitric oxide: weapon against infection? *Trends Microbiol* 1996;4:46-7.
21. Pawloski JR, Swaminathan RV, Stamler JS. Cell-free and erythrocytic S-nitrosohemoglobin inhibits human platelet aggregation. *Circulation* 1998;97:263-7.
22. Balmer P, Phillips HM, Maestre AE, McMonagle FA, Phillips RS. The effect of nitric oxide on the growth of *Plasmodium falciparum*, *P. chabaudi* and *P. berghei in vitro*. *Parasite Immunol* 2000;22:97-106.
23. Taylor-Robinson AW. Nitric oxide can be released as well as scavenged by haemoglobin: relevance to its antimalarial activity. *Parasite Immunol* 1998;20:49-50.
24. Taylor-Robinson AW, Looker M. Sensitivity of malaria parasites to nitric oxide at low oxygen tensions. *Lancet* 1998;351:1630.
25. Prada J, Malinowski J, Muller S, Bienzle U, Kremsner PG. Effects of *Plasmodium vinckei* hemozoin on the production of oxygen radicals and nitrogen oxides in murine macrophages. *Am J Trop Med Hyg* 1996;54:620-4.
26. Stevenson MM, Tam MF, Wolf SF, Sher A. IL-12-induced protection against blood-stage *Plasmodium chabaudi AS* requires IFN-gamma and TNF-alpha and occurs *via* a nitric oxide-dependent mechanism. *J Immunol* 1995;155:2545-56.
27. Nahrevanian H, Dascombe MJ. Expression of inducible nitric oxide synthase (iNOS) mRNA in target organs of lethal and non-lethal strains of murine malaria. *Parasite Immunol* 2002;24:471-8.