Phagocyte-Derived Reactive Oxygen Species Do Not Influence the Progression of Murine Blood-Stage Malaria Infections

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Phagocyte-derived reactive oxygen species have been implicated in the clearance of malaria infections. We investigated the progression of five different strains of murine malaria in gp91phox−/− mice, which lack a functional NADPH oxidase and thus the ability to produce phagocyte-derived reactive oxygen species. We found that the absence of functional NADPH oxidase in the gene knockout mice had no effect on the parasitemia or total parasite burden in mice infected with either resolving (Plasmodium yoelii and Plasmodium chabaudi K562) or fatal (Plasmodium berghei ANKA, Plasmodium berghei K173 and Plasmodium vinckeii vinckei) strains of malaria. This lack of effect was apparent in both primary and secondary infections with P. yoelii and P. chabaudi. There was also no difference in the presentation of clinical or pathological signs between the gp91phox−/− or wild-type strains of mice infected with malaria. Progression of P. berghei ANKA and P. berghei K173 infections was unchanged in glutathione peroxidase-1 gene knockout mice compared to their wild-type counterparts. The rates of parasitemia progression in gp91phox−/− mice and wild-type mice were not significantly different when they were treated with l-α′,β′-methylarginine, an inhibitor of nitric oxide synthase. These results suggest that phagocyte-derived reactive oxygen species are not crucial for the clearance of malaria parasites, at least in murine models.

Malaria is an enormous health, social, and economic burden for over 40% of the world’s population. More than half a billion people are infected with malaria each year, and many of these infections result in chronic parasitemia and anemia. It is believed that phagocytic cells such as monocytes are important in the clearance of the blood-stage parasite (42, 53). However, the mechanism by which monocytes and other phagocytic cells effect killing of the parasite is as yet unknown.

Phagocytic leukocytes are able to engulf bacteria, fungi, or antigens within a phagocytic vacuole (phagosome). The formation of the vacuole leads to the activation of the enzyme NADPH oxidase, present in a dormant state in all phagocytic leukocytes (39). NADPH oxidase is involved in the generation of the superoxide anion (·O2−) by the addition of an electron to oxygen. Superoxide and its derivatives such as hydrogen peroxide (H2O2) and ·OH, which collectively are referred to as reactive oxygen species (ROS), are released into the phagosome and are essential in the killing of the ingested pathogen (39, 70). Electron transport in NADPH oxidase is mediated by a membrane-bound flavocytochrome b (b558), a heterodimer. An essential component of the flavocytochrome is the β subunit, termed gp91phox. The gp91phox−/− mouse (54) does not express the flavocytochrome b558 complex and thus lacks NADPH oxidase activity. The phagocytic leukocytes of these mice therefore are unable to generate ROS and to kill various infectious agents.

It has long been appreciated that intraerythrocytic forms of malaria parasites are exposed to oxidative stress (reviewed in references 6, 32, and 36) and that increasing oxidative stress is inimical to their growth and survival (36, 69). For example, it has been proposed that increased generation of ROS in abnormal erythrocytes from people with genetically determined hemoglobinopathies inhibits the growth of malaria parasites and may be responsible for the preservation in human populations of these otherwise apparently deleterious traits (1, 47, 56). Furthermore, malaria parasites grow poorly in vitamin E-deficient mice (28). ROS-generating systems kill murine malaria parasites in vitro (10, 26, 27) and in vivo (14, 15, 16) and kill Plasmodium falciparum in vitro (7, 13, 45, 72).

The possibility that phagocyte-derived ROS are involved in the host antimalarial immune response has been investigated only indirectly in past studies. Leukocytes from mice (9, 25, 43, 44, 62, 71) and humans (2, 12, 23, 49, 58) infected with malaria show an increased ability to generate ROS. Leukocytes stimulated with malaria parasites in vitro generate ROS, and in some cases this has been linked to damage to the parasites in human (33, 40, 50) and murine (51) systems. However, ROS do not account for all the antimalarial activity of human leukocytes in such in vitro systems (41, 50). As discussed elsewhere (36), these correlative studies need to be interpreted cautiously: in an analogous situation, although earlier studies with free radical scavengers suggested a role for ROS in the pathogen-
Oxidative stress can result from increased generation of ROS or from compromised antioxidant defenses. Glutathione peroxidase (Gpx) is an important antioxidant enzyme activity present in erythrocytes. There are several isoforms of Gpx (8). Since the glutathione redox cycle is important in malaria parasite antioxidant defense (4, 60), we evaluated its possible role in influencing malaria parasite growth and/or survival in vivo using Gpx1 gene knockout mice (21), since this is the predominant form of the enzyme in mice, particularly in the relevant erythrocytic compartment.

Nitric oxide (NO) is another free radical species generated by phagocytes that has antimicrobial activity. NO derivatives (17, 57) kill P. falciparum in vitro. The importance of NO in the immune response to hepatic stages of malarial infection has been reviewed (17). A role for NO in the killing of blood-stage malarial parasites in mice is less clear (20, 29, 38, 67). We therefore considered the possibility that NO and ROS might work synergistically in parasite clearance, as has been shown to occur during the host response to infection with the intracellular bacterium Rhodococcus equi (19).

The first aim of this study was to definitively test the role played by phagocyte-derived ROS in parasite clearance, in a number of different malarial infections, through the use of gp91phox−/− mice. Second, using Gpx1−/− mice, we examined whether host Gpx influences parasite progression. Finally, we tested the effect on parasite survival of the inhibition of both ROS and reactive nitrogen intermediates by the treatment of gp91phox−/− mice with 1-NAME, an inhibitor of nitric oxide synthase and therefore of NO and reactive nitrogen intermediate production.

Materials and Methods

Mice. All experiments were carried out by procedures authorized by the Animal Ethics Committee of the University of Sydney. Female gp91phox−/− and gp91phox−/− mice (54) and Gpx1−/− and Gpx1+/+ mice (21) between 6 and 8 weeks of age were used. All mice had been backcrossed 11 times to a C57BL/6 background. Animals were housed under specific-pathogen-free conditions, with ad libitum access to food and water (except when they were undergoing L-NMA treatment; see below). The inability of phagocytes isolated from the gp91phox−/− mice to generate ROS in response to phorbol myristate acetate was verified (68), as was the absence of Gpx1 activity in erythrocytes.

Parasite maintenance and passage. The malaria strains used were Plasmodium berghei ANKA (courtesy of G. Grau, Marseilles, France), P. berghei K173, Plasmodium vinckeii vinckeii, Plasmodium chabaudi K562 (all courtesy of I. Clark, Australian National University, Canberra, Australia), and Plasmodium yoelii 17X (courtesy of H. Shear, New York, N.Y.). Parasitized red blood cells were cryopreserved in liquid nitrogen until they were required for experimental use. Parasite passaging was performed by taking a quantity of blood from the axillary artery of an infected mouse while it was under anesthesia induced by isoflurane (Rhodia Australia Pty. Ltd., Notting Hill, Australia). The blood was diluted with phosphate-buffered saline to a concentration of 10⁶ parasitized red blood cells per 100 μl and then warmed to 37°C. The inoculum was administered to the mice in 100-μl doses via intraperitoneal injection. Animals infected with the resolving strains of malaria, P. chabaudi K562 and P. yoelii, were rechallenged with a second dose of the same malaria strain no less than 4 weeks after full recovery from the primary infection.

In the case of the terminal malaria infections (P. berghei ANKA, P. berghei K173, and P. vinckeii vinckeii), mice were sacrificed via CO₂ asphyxiation when they appeared moribund. Certain tissues, for example, brain and lung tissues, were examined macroscopically and microscopically to ensure that the disease progression was normal (48). Some mice that were not used for secondary progression were examined macroscopically and microscopically to ensure that the disease progression was normal (48). Some mice that were not used for secondary progression were examined macroscopically and microscopically to ensure that the disease progression was normal (48).
infections with the resolving strains of malaria (P. chabaudi and P. yoelii) were also sacrificed for gross pathological analysis upon resolution of the infection.

Treatment with L-NMA. Mice were treated with L-NMA prepared and administered by the method previously described (18). In brief, the mice were housed individually and, starting 24 h prior to infection, were given drinking water containing 15 mmol/liter L-NMA throughout the course of infection. The drinking water without the addition of L-NMA was meted out to the untreated animals in an identical manner. Uninfected, L-NMA-treated control mice were given water containing the inhibitor for 14 consecutive days prior to sacrifice.

Hematological analysis of mice. Parasitemia was determined microscopically in tail vein blood smears by counting a minimum of 200 red blood cells. Hematocrit readings also were taken from infected mice. Approximately 5 µl of blood was obtained directly from the tail vein by capillary action into 75-mm-long, heparinized capillary tubes (Chase Instruments, Rockwood, TN) with an internal diameter of 0.5 to 0.6 mm. These tubes were then spun for 5 min on a microhematocrit centrifuge (Clements, Sydney, Australia). Because of the very small volume of blood, readings were taken using a microscope fitted with a graded 10× eyepiece (Olympus, Hamburg, Germany) to achieve maximum accuracy. Since anemia is seen in mice late in the course of malaria infection, the parasitemia does not reflect the total parasite burden of the mouse at later stages of infection. The hematocrit therefore was measured throughout the course of P. chabaudi infection, and the parasite burden calculated. The patterns of change in parasite burden seen in the wild-type and gp91phox−/− mice were similar throughout the course of infection with P. chabaudi (Fig. 1). The results did not support a major role for phagocyte-derived ROS in the host response to a primary P. chabaudi infection.

After surviving a primary infection with P. chabaudi, mice are resistant to a secondary infection. C57BL/6 and gp91phox−/− mice that had survived a primary infection with P. chabaudi were reinoculated with an equivalent number of parasites. For comparison of data from two groups, the Mann-Whitney rank test was applied.

RESULTS

Parasitemia was followed throughout the course of disease with P. chabaudi, a resolving infection, in C57BL/6 wild-type and gp91phox−/− mice (Fig. 1). No significant differences were observed. Because there is a loss of circulating erythrocytes in malaria infection, the parasitemia does not reflect the total parasite burden of the mouse at later stages of infection. The hematocrit therefore was measured throughout the course of P. chabaudi infection, and the parasite burden calculated. The patterns of change in parasite burden seen in the wild-type and gp91phox−/− mice were similar throughout the course of infection with P. chabaudi (Fig. 1). The results did not support a major role for phagocyte-derived ROS in the host response to a primary P. chabaudi infection.

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asitized red blood cells (PRBC). There was no significant parasitemia or change in hematocrit in either group of mice during the 25 days after the second inoculation (data not shown). Thus, resistance to a secondary *P. chabaudi* infection does not appear to depend on phagocyte-derived ROS.

Primary (Fig. 2) and secondary (data not shown) infections with another resolving parasite, *P. yoelii*, were studied in wild-type and *gp91phox*−/− mice. No significant differences in parasite burden throughout the course of infection were seen in primary (Fig. 2) or secondary (data not shown) infections. Parasitemia, hematocrit, and parasite burden were evaluated in wild-type and *gp91phox*−/− mice exposed to primary infections with the fulminating parasites *P. berghei* K173, *P. berghei* ANKA, and *P. vinckei vinckei*, but only parasite burden data are shown (Fig. 2). There were no significant differences in parasite burden between mice during the course of infection.

To evaluate whether host Gpx1 could influence malaria parasite growth and/or survival, wild-type and Gpx1−/− mice were inoculated with *P. berghei ANKA* or *P. berghei* K173 (Fig. 3). During the course of infection, the parasite burdens of the gene knockout and wild-type mice did not differ significantly.

The superoxide anion is a primary product of activated phagocyte NADPH oxidase. This radical can react with NO to yield peroxynitrite, which can react with many biological molecules. Furthermore, NO itself can kill intraerythrocytic malaria parasites in vitro, although this may not be relevant in vivo in mice (30, 67). We considered the possibility that, in *gp91phox*−/− mice, increased NO production from inducible nitric oxide synthase might compensate for the lack of phagocyte-derived ROS. Wild-type and *gp91phox*−/− mice therefore were treated with an inhibitor of nitric oxide synthase, l-NMA, and inoculated with *P. berghei ANKA* and *P. berghei* K173 (Fig. 4). The patterns of change in parasite burden throughout the course of infection were similar in all treatment groups.

**DISCUSSION**

Malaria immunity is complex and still incompletely understood. In primary infections, a cell-mediated immune response...
involving mononuclear phagocytes is believed to play a role (42, 53). There is a considerable body of evidence consistent with the concept that phagocyte-derived ROS are involved in host immunity against malaria infection (36). However, much of this evidence is indirect and correlative. In this study we have examined the course of several malaria infections in mice with an inactive NADPH oxidase. We found that there was no difference in the progression of primary infections with five different strains of malaria parasite, some of which were resolving and some fulminating types. Furthermore, in two resolving infections there also was no apparent involvement of phagocyte-derived ROS in secondary immune responses. Harada and colleagues have shown that there was no difference between the pattern of parasitemia in P. berghei ANKA infection in gp91phox−/− mice and that in control mice but that at one stage the infectivity of gametocytes in gp91phox−/− mice was greater than that in control mice (34). These results substantiate previous conclusions derived from indirect approaches (11). We also evaluated a possible role of NO as an immune system antimalarial effector molecule, operating in the absence of phagocyte ROS, by administering to gp91phox−/− mice an inhibitor of nitric oxide synthase and found this treatment to have no apparent effect.

Although phagocyte-derived ROS do not appear to play a role in immunity to malaria parasites in mice, at least, the exquisite sensitivity of the parasite to oxidative stress may still be a critical factor in its survival. Other potential sources of oxidative stress must be considered, the most obvious being the parasite itself. There is substantial evidence that malaria parasites inside erythrocytes exert an oxidative stress within the PRBC (32, 36). It has been suggested that the parasite generates ROS through one or more of the following pathways: an electron transport chain (24), degradation of hemoglobin and cytosolic proteins (3), or redox reactions of hemin (35). The parasite is protected against this oxidative stress by a number of host- or parasite-encoded enzymes (6, 36), by vitamin C (37), by vitamin E (28), and by glutathione (4, 5). Parasite antioxidant defense is believed to include export of oxidized glutathione to the erythrocyte cytosol (4). However, we evaluated the role of host Gpx1 in intraerythrocytic parasite progression by employing Gpx1−/− mice and found no significant difference from results for wild-type mice. Although we did not evaluate all four Gpx isoforms, Gpx1 is the most abundant one in erythrocytes (8). Furthermore, our results are consistent with previous studies using mice fed a selenium-depleted diet, which completely abrogates host Gpx activity (31). P. vinckei vinckei, P. berghei, and P. falciparum all contain a parasite-encoded, selenium-independent Gpx (31, 65), which may explain the apparent lack of importance of the host enzyme in parasite progression. Although we did not study the effect of eliminating other antioxidant enzymes, the activity of most of them, for example, catalase, decreases in PRBC (36, 61).

Although oxidative stress arising from the host immune response does not now appear to be important in influencing parasite progression, the redox balance of the PRBC remains clearly established as an important determinant of parasite survival. Indeed, several different antimalarial drug development strategies are based on the premise that tipping this balance towards oxidation will be inimical to the intraerythrocytic parasite (6, 22, 60).

The mechanisms that operate in malaria immunity are complex. In P. chabaudi infection, gamma interferon (64) and phagocytosis of PRBC by macrophages (63) are important in parasite clearance. Macrophages are known to be capable of phagocytosing and destroying PRBC in human and murine malaria infections (46, 52, 55). Any such killing of parasites is unlikely to be oxidative, since our current results suggest that phagocyte-derived ROS are not an important component of primary or secondary immune responses against malaria infection in mice. Nor do they play an important part in the pathogenesis of experimental cerebral malaria (59). Such roles cannot be ruled out in humans, although this is difficult to evaluate stringently.

Indirect associations previously had suggested that phagocyte-derived ROS might be involved in mammalian antimalarial responses (as reviewed in reference 36), but detailed evaluation with mice now has shown that this mechanism is not important in that species, throwing doubt on its relevance overall to malaria immunity.

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REFERENCES

blood-stage infection on macrophages in liver, spleen, and blood. J. Exp. Med. 163:54–74.
68. van Reyk, D. M., N. J. King, M. C. Dinauer, and N. H. Hunt. 2001. The