Are Reactive Oxygen Species Involved in the Pathogenesis of Murine Cerebral Malaria?

Latifu A. Sanni, Shanlin Fu, Roger T. Dean, Garry Bloomfield, Roland Stocker, Geeta Chaudhri, Mary C. Dinauer, and Nicholas H. Hunt

To investigate the involvement of oxidative tissue damage in the pathogenesis of murine cerebral malaria (CM), brain levels of protein carbonyls, 3,4-dihydroxyphenylalanine (DOPA), o-tyrosine, and dityrosine were measured during Plasmodium berghei ANKA (PbA) and P. berghei K173 (PbK) infections. During PbA infection in a CM model, brain levels of the substances were similar to those in uninfected mice. The role of phagocyte-derived reactive oxygen species in the pathogenesis of CM was examined in gp91phox gene knockout mice. The course of CM in these mice was the same as in their wild type counterparts. To examine whether superoxide production in the central nervous system could have occurred via increased xanthine oxidase activity, brain concentrations of urate were measured in CM mice and in mice infected with PbK (which does not cause CM). Brain urate concentration increased significantly in both groups of mice, suggesting that purine breakdown is not specific to CM. These results indicate that reactive oxygen species probably do not contribute to the pathogenesis of murine CM.

Although cerebral malaria (CM) is a major life-threatening complication of Plasmodium falciparum infection [1], its pathogenesis is largely unsolved [2]. The incidence of this condition is on the rise [3], and there is no specific treatment apart from antimalarials. A greater understanding of the pathogenesis of the disease is needed to develop a more specific and effective method of therapy. Studies on postmortem human material have the shortcoming of revealing mainly the outcome of the disease rather than the processes leading to its development. Experimental animal models have proved useful in this regard. In a murine CM model, CBA or C57 mice are inoculated intraperitoneally (ip) with Plasmodium berghei ANKA (PbA)–infected red blood cells [4–6]. These mice die on about post-inoculation (pi) day 7, exhibiting neurologic signs and histopathologic changes reminiscent of human CM, with some differences [4, 5, 7]. In this murine model, T lymphocytes and interferon (IFN)–γ are prerequisites for CM to develop [8–10] and tumor necrosis factor (TNF)–α is also thought to play a crucial role in the pathogenesis [7, 11].

IFN-γ synergizes with lipopolysacharride and TNF-α to enhance the superoxide anion radical production by phagocytes [12, 13] that accompanies phagocytosis of microorganisms. This superoxide production requires a protein complex known as NADPH oxidase, which contains the heterodimeric cytochrome b245/558, composed of an α-subunit (gp22phox) and a β-subunit (gp91phox) [14]. Following activation of NADPH oxidase, large quantities of superoxide are produced, which are rapidly converted into hydrogen peroxide and other reactive oxygen species (ROS) that are essential for effective phagocyte microbicidal activity. However, excessive or inappropriate release of ROS may contribute to tissue injury [15], such as endothelial cell damage [16]. Thus, activated phagocytes that attach to brain endothelial cells in murine CM [4] might cause oxidative damage to the endothelium and compromise the blood-brain barrier. Paraquat, a radical generator, causes cerebral lesions similar to those seen in CM [17]. Administration of butylated hydroxyanisole, an antioxidant, can prevent the development of CM in PbA-infected mice [18]. Thus, ROS may be involved in the pathogenesis of CM, especially since the brain is particularly vulnerable to oxidative damage [19].

To investigate this concept, we studied the effect of genetargeted disruption of the gp91phox β-subunit of cytochrome b245/558 in PbA infection. We also measured brain concentrations of protein carbonyls, protein-bound oxidized amino acids, such as 3,4-dihydroxyphenylalanine (DOPA), o-tyrosine, and dityrosine, and brain-glutamine synthetase (GS) activity as an indirect index of general protein oxidation. Brain uric acid concentration was also determined as an index of superoxide production from xanthine oxidase activity.

Materials and Methods

Inoculation of mice. We used 6- to 8-week-old female CBA/T6 mice weighing 20–25 g from Blackburn Animal House (University...
of Sydney). The gp91\(^{phox}\) gene knockout (GKO) (−/−) and gp91\(^{phox}\) (+/+ ) wild type (gp91\(^{phox}\) wt) mice, backcrossed to C57/Bl mice, were developed in the laboratory of Mary C. Dinauer [20] and bred at Blackburn Animal House. The malaria parasites used were either P. berghei ANKA (G. Grau, Geneva) or P. berghei K173 (PbK) (I. Clark, Australian National University, Canberra). Mice were inoculated by ip injection of 10\(^{9}\) parasitized erythrocytes obtained from tail blood of infected animals and suspended in 200 \(\mu\)L of PBS, pH 7.4. Controls were uninfected CBA mice or CBA mice injected ip with 200 \(\mu\)L of PBS. As expected, between pi days 5 and 6, CBA and gp91\(^{phox}\) wt mice inoculated with PbA showed evidence of cerebral involvement, such as convulsions, paralysis, ataxia, and stupor, progressing to coma and death [5, 6]. They died within 24 h of developing the symptoms, with low parasitemias of \(~15\%\). To ascertain that the changes observed during CM are not general to all malaria infections, we also studied mice infected with PbK.

Mice inoculated with PbK did not exhibit any of the cerebral signs seen in PbA-infected mice, although they became sick and died with severe anemia, as expected, between pi days 15 and 22 [4].

Mice were sacrificed when they became sick with CM (pi day 6 for CBA mice infected with PbA) or anemia (pi day 15 for PbK-infected mice). Sacrifice was by decapitation in a manner that caused the heads to fall straight into liquid nitrogen. After decapitation, blood samples were taken from the neck stumps into heparinized 1-mL syringes and used for determination of parasitemia and hematocrit levels.

Sample preparation. Whole brain samples used for the measurement of protein carbonyls, glutamine synthetase activity, and protein-bound oxidized amino acid concentrations (DOPA, o-tirosine, and dityrosine) were chiseled out of the frozen head, quickly weighed in 3 mL of cold (4\(^\circ\)C) homogenization buffer and then homogenized in the same buffer (50 mM phosphate buffer, pH 7.4, containing 0.1% digitonin, 1 mM EDTA, and a cocktail of protease inhibitors [40 \(\mu\)g/mL phenylmethylsulfonyl fluoride, 5 \(\mu\)g/mL leupeptin, 7 \(\mu\)g/mL pepstatin, and 5 \(\mu\)g/mL aprotinin]; all from Sigma, St. Louis) using a motor-driven Teflon pestle and glass mortar homogenizer (Wheaton, Millville, NJ). The brain homogenates produced were treated as described by Reznick and Packer [21].

For determination of oxidized amino acids on proteins from the brain homogenates, a previously established method of delipidation, removal of free amino acids, and protein hydrolysis was used [22, 23]. In brief, the brain homogenates were mixed with sodium deoxycholate and trichloroacetic acid. After centrifugation, the pellets were resuspended and washed twice with cold acetone and once with diethyl ether. As judged by the recoveries of added modified amino acids (DOPA and o-tirosine), free amino acids were removed completely by this method. The protein samples thus obtained were subsequently hydrolyzed, and the homogenates were analyzed for protein-bound oxidized amino acids by high-performance liquid chromatography (HPLC).

For urate analysis, mice were perfused intracardially with normal saline, before the whole brain was removed, weighed, and homogenized in 2 vol of 5% (wt/vol) metaphosphoric acid using the same homogenizer set up as described above. Supernates were recovered by centrifugation at 10,000 \(\times\) g for 5 min at 4\(^\circ\)C and extracted with an equal volume of ethyl acetate. After centrifugation, 20 \(\mu\)L of the aqueous phase was filtered (Acrodisk LC 13, 0.2 \(\mu\)m; Gelman, Sydney) and subjected to HPLC analysis.

HPLC analyses. Protein-bound oxidized amino acids (DOPA, o-tirosine, and dityrosine) present in protein hydrolysate were analyzed by HPLC on an octadecylsilanized silica column (Zorbax; Hewlett-Packard, Palo Alto, CA) with a Pelligrini guard column as described previously [24]. Elution positions were defined on the basis of standards.

Uric acid was determined by HPLC with electrochemical detection (LC-4C; Bioanalytical Systems, West Lafayette, IN) on a 250-\(\times\)0.46-cm C18 column with a 2.5-cm guard column (Supelco, Bellefonte, PA). The mobile phase was run at 0.9 mL/min and consisted of 24 mM sodium acetate, 0.54 mM EDTA, 7.5% (vol/vol) methanol, and 1.5 mM dodecyltrimethylammonium phosphate (Q12), pH adjusted to 4.75 with glacial acetic acid. The potential was set at 500 mV versus an Ag-AgCl reference electrode. Uric acid was quantified by area comparison with uric acid standards (Sigma).

Biochemical analysis. Protein carbonyls in brain homogenate supernatants were determined spectrophotometrically by the 2,4-dinitrophenylhydrazine (DNPH) procedure as described [21]. GS activity was determined in brain homogenate supernatant by the \(\gamma\)-glutamyl transfer assay method of Miller et al. [25].

Statistical analysis. Results were analyzed by the Mann-Whitney test. \(P \leq .05\) was considered significant.

Results

Concentration of protein carbonyls. Oxidative modification of proteins by ROS is accompanied by the generation of protein carbonyls that can be detected upon reaction with DNPH. The protein carbonyl content of soluble fractions of crude brain extracts from CBA mice infected with PbA (CM mice) was higher than in PbK-infected mice (non-CM [NCM] mice), although comparable to that in control, uninfected mice (table 1).

<table>
<thead>
<tr>
<th>Table 1. Brain concentration of protein carbonyls and glutamine synthetase activity in mice.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control uninfected mice</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Protein carbonyls</td>
</tr>
<tr>
<td>Glutamine synthetase activity</td>
</tr>
</tbody>
</table>

NOTE. Protein carbonyl concentration (nmol/mg protein) and glutamine synthetase activity (nmol/mg protein/min) in brain of control (uninfected) mice and CBA mice infected with P. berghei ANKA (CM mice) or with P. berghei K173 (non-CM mice). Data are mean ± SE; no. of mice is in parentheses.

\(^{a}\) Significantly different from controls on postinoculation days 6–7 (\(P < .05\)).

\(^{b}\) Significantly different from CM mice on postinoculation days 6–7 (\(P < .05\)).
Brain GS activity: As previous reports suggest that GS is sensitive to ROS, and a decrease in brain GS activity was used by Oliver et al. [26] as an index of oxidation in the brain, we assessed GS activity in brain preparations from age-matched female mice. Brain GS activity decreased slightly, although significantly and specifically in murine CM (table 1). In contrast to protein carbonyl measurements, this observation is consistent with a role for ROS and protein oxidation in murine CM. These apparently contradictory results, and the fact that protein oxidation can render proteins more susceptible for proteolysis, led us to seek other “footprints” of ROS in the brains of CM mice and to examine the progress and outcome of infection with PbA in mice with a defective NADPH oxidase.

Brain concentrations of protein-bound DOPA, o-tyrosine, and dityrosine. The brain levels of the protein-bound oxidized amino acids (DOPA, o-tyrosine, and dityrosine) were similar in

Table 2. Outcome of P. berghei ANKA infection in wild type and gp91phox gene knockout mice.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. that died on pi days 6–8</th>
<th>Cerebral symptoms on pi days 6–8</th>
<th>Brain histology score for cerebral malaria</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57 gp91phox +/+</td>
<td>0/4</td>
<td>0/4</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>C57 gp91phox −/−</td>
<td>0/4</td>
<td>0/4</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>C57 gp91phox +/+ infected with PbA</td>
<td>8/8</td>
<td>8/8</td>
<td>10, 8, 8, 8, 8, 8, 8, 11, 9</td>
</tr>
<tr>
<td>C57 gp91phox −/− infected with PbA</td>
<td>9/9</td>
<td>9/9</td>
<td>9, 9, 9, 10, 10, 9, 10</td>
</tr>
</tbody>
</table>

NOTE. Gp91phox gene knockout C57/B1 mice developed cerebral malaria and died, like wild type C57/B1 mice, 6–8 days after inoculation (pi) with P. berghei ANKA. Cerebral symptoms included convulsions (tonic/clonic), paralysis, ataxia, and stupor progressing to coma. Brain histology score signifies degree of cerebral edema, hemorrhage, and monocyte margination on vascular endothelial cells, observed under light microscopy; maximum score was 11.
control uninfected mice and CM mice (figure 1). In NCM mice, the brain concentrations of DOPA, o-tyrosine, and dityrosine decreased, consistent with decreased brain protein carbonyl concentrations (table 1).

**Outcome of P. berghei infection in gp91-phox GKO mice.** Gp91-phox GKO mice infected with P. berghei developed CM and died similarly to their wild type counterparts (table 2). However, in the absence of the gp91-phox gene, the parasitemia increased faster (table 3), consistent with a protective role for phagocyte-derived ROS in the host response against the blood stages of P. berghei.

Because there is evidence that xanthine oxidase is an important source of ROS [27, 28], we assessed a possible link between increased superoxide production from xanthine-oxidase activity and CM by determining the brain uric acid concentration. This was significantly increased in both CM and NCM mice (P = .01 and .03, respectively) compared with control, uninfected mice (figure 2). Thus, increased brain urate was not specifically associated with the pathogenesis of CM.

**Table 3.** Effect of gp91-phox gene knockout on parasitemia in mice.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Parasitemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp91-phox +/-</td>
<td>5.9 ± 0.5 (8)</td>
</tr>
<tr>
<td>gp91-phox -/-</td>
<td>10.8 ± 1.3 (9)*</td>
</tr>
</tbody>
</table>

NOTE. Parasitemia (% parasitized red blood cells) in gp91-phox gene knockout (-/-) C57 mice and wild type (gp91-phox +/-) counterparts. Values are mean ± SE; no. of mice in each group is in parentheses.

* Significantly different from gp91-phox +/- mice infected with P. berghei ANKA (PbA) on postinoculation (pi) day 5 (P < .05).

b Significantly different from gp91-phox +/- mice infected with PbA on pi day 7 (P < .05).

Discussion

We found similar levels of protein carbonyls, protein-bound DOPA, o-tyrosine, and dityrosine (footprints of ROS) in the brains of mice infected with CM and in uninfected controls. These results suggest that overproduction of ROS, at least in the brain parenchyma, does not play a major role in the pathogenesis of murine CM. However, because oxidative modification of proteins predisposes them to proteolysis, an increased production of protein carbonyls might not be detected if rapid proteolysis were to occur. Furthermore, if increased ROS production occurs only in discrete brain regions, this may not be evident as changes in total brain protein carbonyl, protein-bound DOPA, o-tyrosine, and dityrosine content. These considerations led us to use gp91-phox -/- mice as a tool to further study the possible role of phagocyte-derived ROS in murine CM.

When infected with PbA, gp91-phox -/- mice became sick about pi day 6 and died of CM, like the wild type gp91-phox gene +/- mice (table 2). This is consistent with the results obtained for protein carbonyls and oxidized amino acids and strongly suggests that ROS overproduction from phagocytes does not contribute to the pathogenesis of murine CM. Gene-targeted disruption of the gp91-phox gene had no effect on the brain histologic changes seen in CM (table 2); similar to wild type mice suffering from CM, brain petechial hemorrhages, edema, and monocyte margination to endothelial cells were observed in gp91-phox -/- mice infected with PbA. The development of petechial hemorrhages and vasogenic cerebral edema in the gp91-phox -/- mice is an indication that the increased vascular permeability and blood-brain barrier breakdown seen in CM are not mediated by ROS produced by monocytes adhering to endothelial cells in the brain.

We previously showed that administration of butylated hydroxyanisole, a radical scavenger, protected mice against CM [18]. However, butylated hydroxyanisole has other effects apart from being an antioxidant, such as being able to inhibit T lymphocyte proliferation [29]. T lymphocytes and their cytokine product IFN-γ are necessary for CM development in mice [8–10, 30].

It has been suggested [31] that nitric oxide (NO) produced locally in the central nervous system is important in the pathogenesis of human CM. Since NO can react with the superoxide anion radical to yield the highly reactive species peroxynitrite [32], interactions between ROS and NO might theoretically play a role in the pathogenesis of murine CM. However, there is increasing evidence that NO plays a protective role in murine CM [33, 34]. Since the gp91-phox -/- mice, which are incapable of producing superoxide anion from NADPH oxidase, were not protected, it seems unlikely that peroxynitrite is an important factor in the pathogenesis of murine CM.

In the NCM mice on pi day 15, when anemia was evident, the levels of protein carbonyls, protein-bound DOPA, o-tyr-
sine, and dityrosine in the brain decreased. This implies that a dynamic equilibrium exists between the rates of production of these substances and their rates of catabolism and that a perturbation of the balance can occur. The cause of the decrease remains to be determined, but brain hypoxia incident on the hemolytic anemia present in these mice may provide an explanation. Molecular oxygen is the precursor of superoxide and other ROS from which oxidized proteins and amino acids are formed.

Brain GS activity decreased significantly in murine CM. The exact reason for this is unknown, but the enzyme is sensitive to oxidation. Although a decrease in its activity may be due to increased ROS production in the brain as suggested by Oliver et al. [26] during ischemia-reperfusion-induced injury to gerbil brain, there is no direct evidence in this or other in vivo situations that oxidation is responsible for the altered GS activity. Other factors, such as altered astrocyte function, may be responsible because, in the brain, GS is exclusively found in astrocytes [35], and perturbation of central nervous system astrocyte morphology occurs in murine CM [36].

Another significant finding in this study was that the percentage of parasitized red blood cells in the circulation on pi days 5 and 7 was higher in the gp91

10phox

1/− mice than in the gp91

phox

1+/

+ mice (table 3). This suggests that phagocyte-derived ROS are involved in curbing the rate of parasite growth during PbA infection. Macrophages from malaria-infected animals can release ROS when incubated with parasitized red blood cells and T cells [37]. Furthermore, ROS are toxic to malaria parasites [38]. Thus, ROS might be involved in the ability of the host to curtail malaria infection. The antimalarial drug qinghaosu is thought to work through a free radical mechanism [39]. Levander et al. [40] reported that dietary-induced oxidative stress has antimalarial actions and also protects against murine CM. This is consistent with our results. Administration of prooxidant diets and qinghaosu might be expected to exacerbate the symptoms of CM if ROS were involved in its pathogenesis.

We also observed that brain uric acid was significantly increased in both CM and NCM mice compared with control, uninfected mice. This indicates that the increased rate of purine catabolism and production of uric acid is a general phenomenon during malaria infection and unlikely to contribute to the pathogenesis of murine CM. In fact, the increased brain uric acid concentration may be due to increased xanthine dehydrogenase rather than to xanthine oxidase activity. Xanthine oxidase is formed in tissues from xanthine dehydrogenase, which converts hypoxanthine to uric acid without the formation of ROS.

Together our results suggest that phagocyte-derived ROS are not involved in the pathogenesis of murine CM. The cause of death in murine CM is probably not due to excessive production of ROS in the brain parenchyma leading to protein oxidation, lipid peroxidation, or DNA damage.

References


