CEREBROSPINAL FLUID STUDIES IN CHILDREN WITH CEREBRAL MALARIA: AN EXCITOTOXIC MECHANISM?

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Abstract. The pathogenesis of cerebral malaria is poorly understood. One hypothesis is that activation of microglia and astrocytes in the brain might cause the cerebral symptoms by excitotoxic mechanisms. Cerebrospinal fluid was sampled in 97 Kenyan children with cerebral malaria, 85% within 48 hr of admission. When compared with an age-matched reference range, there were large increases in concentrations of the excitotoxin quinolinic acid (geometric mean ratio cerebral malaria/reference population [95% confidence limits] = 14.1 [9.8–20.4], \( P < 0.001 \)) and total neopterin (10.9 [9.1–13.0], \( P < 0.001 \)) and lesser increases in tetra-hydrobiopterin, di-hydrobiopterin, and 5-hydroxyindoleacetic acid. There was no change in tryptophan concentration. In contrast, nitrate plus nitrite concentrations were decreased (geometric mean ratio = 0.45 [0.35–0.59], \( P < 0.001 \)). There was a graded increment in quinolinic acid concentration across outcome groups of increasing severity. The increased concentration of quinolinic acid suggests that excitotoxic mechanisms may contribute to the pathogenesis of cerebral malaria.

Cerebral malaria is the most serious complication of infection with *Plasmodium falciparum*, which causes the death of more than 1 million children in sub-Saharan Africa each year. The pathogenesis of cerebral malaria is not well understood. A central occurrence in the production of the clinical syndrome is adherence of parasitized erythrocytes to endothelial cells of the cerebral vasculature. This initiates a cascade of events that cause intense endothelial activation on the abluminal side of the blood-brain barrier. These events include cytoadherence itself, and the production of inflammatory cytokines, malarial toxins, and nitric oxide. Much less is known about events occurring on the abluminal side of the blood-brain barrier in cerebral malaria and the cause of cerebral symptoms.

The clinical syndrome of childhood cerebral malaria is characterized by the rapid onset and recovery from a diffuse encephalopathy. The coma is complicated by raised intracranial pressure and seizures occur in more than half the patients. Between 5% and 15% of survivors of cerebral malaria have neurologic sequelae suggesting focal brain damage. These clinical findings could be explained by an excitotoxic mechanism.

Experimental stimulation of cells of the macrophage/monocyte lineage by various cytokines causes the parallel induction of indoleamine-pyrrole 2,3-dioxygenase (EC 1.13.11.42, indoleamine 2,3-dioxygenase), GTP cyclohydrolase I (EC 3.5.4.16), and nitric oxide synthase (EC 1.14.13.39). These enzymes catalyze the first step of the serotonin biosynthetic pathway (Figure 1), in which dihydroneopterin triphosphate is an intermediate. Human macrophage/monocyte lineage cells are relatively deficient in the enzyme that catalyzes the formation of 6-pyruvoyltetrahydropterin from dihydronopterin triphosphate, and when activated, cause the accumulation of the breakdown product neopterin. Neopterin concentration in biological fluids is widely used as a marker of immune activation. Tetrahydrobiopterin is also required as a cofactor for tryptophan monooxygenase, which is the first and rate-limiting step of the serotonin biosynthetic pathway. 5-Hydroxyindoleacetic acid is an acidic metabolite of serotonin whose concentration in cerebrospinal fluid (CSF) reflects serotonin turnover.

We hypothesized that the intense cerebrovascular endothelial activation in cerebral malaria causes activation of microglia and/or astrocytes in the brain. The activated cells then produce the excitotoxin quinolinic acid, which may be one cause of the cerebral symptoms. To address this hypothesis, we have measured CSF concentrations of quinolinic acid, its precursor tryptophan, 5-hydroxyindoleacetic acid, individual pterin species, and nitrate plus nitrite (stable breakdown products of nitric oxide) in CSF sampled from Kenyan children recovering from cerebral malaria.

**PATIENTS AND METHODS**

Ninety-seven children with cerebral malaria were studied. Their median (95th percentile confidence interval) age was 2.2 (0.7–6.9) years, and nearly 70% were less than three years old. All were admitted to the hospital in Kilifi, a coastal town in Kenya where malaria is endemic. Before devel-
opining cerebral malaria, each child was well and had no features suggestive of encephalitis due to human immunodeficiency virus.

Two different syndromes were defined depending upon the degree of loss of consciousness.35 Strict cerebral malaria was defined as a febrile encephalopathy with *P. falciparum* parasitemia in which children >8 months old were unable to localize pain and children ≤8 months old extended their limbs with pain. Children with *P. falciparum* parasitemia and a febrile encephalopathy causing a lesser disturbance in consciousness (Blantyre coma scale <4) were classified as having malaria with impaired consciousness. All children were treated in a standardized manner with either intravenous quinine (a 15 mg/kg loading dose, then 10 mg/kg every 12 hr) or intramuscular artemether (a 3.2 mg/kg loading dose, then 1.6 mg/kg every 24 hr) and treatment was completed with a single dose of oral or intramuscular sulfadoxine (500 mg)/pyrimethamine (25 mg). The outcome for each child with cerebral malaria was determined on discharge from hospital: death, survival with neurologic sequelae, and survival with no neurologic sequelae.

Cerebrospinal fluid was obtained from each child once it was clinically judged to be safe to do so; usually once the conscious level had started to increase. It was also taken post-mortem within 15 min of death from 11 children. The CSF was taken in aliquots: the first 1 ml was used for diagnostic purposes, and the second 1 ml and third 1 ml was used for this study. The third 1 ml was collected into a bottle containing 1 mg each of dithioerythritol (DTE) and diethylenetriaminepentaacetic acid (DETA-PAC). The second 1 ml and third 1 ml were frozen at the bedside on dry ice and stored at −70°C until analysis. All CSF analyzed had a normal cell count.

These studies were approved by the Ethical Committee of the Kenya Medical Research Institute. Lumbar puncture was performed as a routine clinical practice. Verbal consent was obtained from the patients for the storage and subsequent analysis of redundant CSF for research purposes.

The concentrations of the following compounds were measured in CSF: quinolinic acid, tryptophan, 5-hydroxyindoleacetic acid, total neopterin, tetrahydrobiopterin, dihydrobiopterin, and nitrate plus nitrite. Quinolinic acid was measured by gas chromatography electron impact mass spectrometry after tert-butyldimethylsilyl derivatization.36 Tryptophan was measured by high-performance liquid chromatography (HPLC) with fluorometric detection after precolumn derivatization with o-phthalaldehyde-2-mercaptoethanol.37 5-Hydroxyindoleacetic acid was measured by HPLC with electrochemical detection.38 Total neopterin (neopterin plus dihydroneopterin, breakdown products of dihydroneopterin triphosphate), tetrahydrobiopterin, and dihydrobiopterin were measured by HPLC with dual electrochemical and fluorometric detection.38 Nitrate plus nitrite were measured by spectrophotometry.39 Reference ranges for the metabolite concentrations were constructed from children and young adults living in the United Kingdom with a variety of neurologic or metabolic diseases in whom no disturbance of the biochemical pathways was expected; CSF was sampled after at least a 4-hr fast.36,38,39 With the exception of total neopterin, each metabolite shows an age-related decrement in CSF concentration and the reference ranges (n = 24–78 depending upon the metabolite assayed) were exactly age-matched to the children with cerebral malaria. We were also able to collect an age-matched local reference group of 9 children who had seizures or staging for Burkitt’s lymphoma for comparison of nitrate plus nitrite concentrations; they were not suitable as a reference group for quinolinic acid or the pterin species because the majority were investigated in the context of a fever.

The results were analyzed after logarithmic transformation.
to equalize the variances. Mean metabolite concentrations were compared across the diagnostic and treatment groups using Student’s t-test. In children with cerebral malaria, mean metabolite concentrations were compared across the outcome groups using analysis of variance with post hoc significance testing using Duncan’s new multiple range test. P values <0.05 were considered statistically significant.

RESULTS

Eighty-two children (85%) had CSF taken within 48 hr of admission. There were no differences in any metabolite concentration in CSF fluid taken within 48 hr of admission (n = 82) compared with that taken after 48 hr (n = 15). There were also no significant differences in metabolite concentrations between children treated with quinine (n = 79) and those treated with artesunate (n = 18). The ratios of the geometric means (quinine/artesunate) (95th percentile confidence limits for the ratio) for each metabolite were quinolinic acid = 0.9 (0.6–1.5), neopterin = 0.8 (0.6–1.2), tetrahydrobiopterin = 1.0 (0.7–1.3), dihydrobiopterin = 0.8 (0.6–1.1), 5-hydroxyindoleacetic acid = 1.0 (0.7–1.3), tryptophan = 0.7 (0.5–1.1), and nitrate plus nitrite = 1.0 (0.7–1.3). There were no significant differences in metabolite concentrations between the diagnostic groups. Geometric mean ratios (strict cerebral malaria/malaria with impaired consciousness) (95th percentile confidence limits) for each metabolite were quinolinic acid = 1.1 (0.7–1.9), neopterin = 0.9 (0.6–1.2), tetrahydrobiopterin = 0.8 (0.6–1.1), dihydrobiopterin = 1.1 (0.8–1.5), 5-hydroxyindoleacetic acid = 1.0 (0.8–1.4), tryptophan = 1.2 (0.8–1.8), and nitrate plus nitrite = 1.2 (0.9–1.5). Therefore, for the rest of the analysis, children with strict cerebral malaria (n = 79) and malaria with impaired consciousness (n = 18) were grouped together and referred to as cerebral malaria for simplicity (Table 1).

In comparison with the reference range, there were highly significant and very large increases in the CSF concentrations of quinolinic acid (ratio of the geometric means) (cerebral malaria/reference range [95th percentile confidence interval for the ratio] = 14.1 [9.8–20.4], P < 0.001) and neopterin (geometric mean ratio = 10.9 [9.1–13.0], P < 0.001) in children with cerebral malaria. There was also a highly significant but moderate increase in concentrations of tetrahydrobiopterin (geometric mean ratio = 3.5 [3.0–4.2], P < 0.001) and dihydrobiopterin (geometric mean ratio = 3.3 [2.6–4.5], P < 0.001). There was a significant, but small increase in 5-hydroxyindoleacetic acid (geometric mean ratio = 1.2 [1.0–1.4], P = 0.02). There was no change in tryptophan concentration (geometric mean ratio = 0.93 [0.77–1.1], P = 0.48). In contrast, CSF nitrate plus nitrite concentrations were highly significantly and moderately reduced in cerebral malaria compared with both the United Kingdom reference range (geometric mean ratio = 0.45 [0.35–0.59], P < 0.001) and the local reference range (geometric mean ratio = 0.55 [0.45–0.68], P < 0.001). There was no difference between nitrate plus nitrite concentrations in the local range compared with the United Kingdom reference range (geometric mean ratio = 0.82 [0.60–1.11], P = 0.19).

In children with cerebral malaria, concentrations of CSF quinolinic acid, dihydrobiopterin, and 5-hydroxyindoleacetic acid were significantly different between the 3 outcome groups (Table 2). The CSF quinolinic acid was significantly increased in survivors with abnormal neurologic signs (n = 11) and those who died (n = 12) compared with survivors with no neurologic sequelae (n = 74). The CSF dihydrobiopterin and 5-hydroxyindoleacetic acid concentrations were significantly increased in the children who died compared with survivors with no neurologic sequelae and those with a neurologic deficit. There was no significant difference in age across the outcome groups (F2.40 = 0.55, P = 0.58).

DISCUSSION

Our results show that Kenyan children with cerebral malaria have a characteristic neurochemical profile with increased CSF quinolinic acid and neopterin concentrations and a reduced nitrate plus nitrite concentration. Central nervous system quinolinic acid and neopterin have previously been shown to be increased in a variety of diseases and experimental models where there is immune activation,46 including cerebral malaria.45,46 Experimentally, this has been shown to be caused by induction of indoleamine 2,3-dioxoxygenase and GTP cyclohydrolase I, the first enzymes in the pathways leading to quinolinic acid and neopterin synthesis, respectively.14,31,32

There is some experimental support for our finding of increased quinolinic acid concentrations in children recovering from cerebral malaria. Mice infected with P. berghei ANKA, who develop fatal malaria with cerebral involvement, showed a 3-fold increase in brain quinolinic acid concentrations caused by induction of indoleamine 2,3-dioxoxygenase.40 They also had normal brain tryptophan concentrations.40 Whether quinolinic acid could be chronically elevated in Kenyan children who have a greater exposure to infectious disease than our reference population might also be questioned. We think this is unlikely because lumbar CSF concentrations of quinolinic acid in American children with septicemia but without central nervous system infection are increased only 1.3–3.5 times those of non-septic controls.29 In comparison, much higher values (10–20 times) were found here in patients with cerebral malaria. Importantly, we also

### Table 1

Cerebrospinal fluid metabolite concentrations in cerebral malaria

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Reference range</th>
<th>Cerebral malaria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinolinic acid (nmol/L)</td>
<td>16.2 (12.3–21.5)</td>
<td>229* (188–278)</td>
</tr>
<tr>
<td>Nitrate plus nitrite (μmol/L)</td>
<td>12.6 (10.5–15.1)</td>
<td>5.7* (5.1–6.5)</td>
</tr>
<tr>
<td>Total neopterin (nmol/L)</td>
<td>17.0 (15.2–19.1)</td>
<td>186* (162–213)</td>
</tr>
<tr>
<td>Tetrahydrobiopterin (nmol/L)</td>
<td>33.9 (30.2–38.0)</td>
<td>119* (106–134)</td>
</tr>
<tr>
<td>Dihydrobiopterin (nmol/L)</td>
<td>3.8 (2.9–4.9)</td>
<td>12.7* (11.2–14.4)</td>
</tr>
<tr>
<td>5-Hydroxyindoleacetic acid (nmol/L)</td>
<td>194 (172–218)</td>
<td>233* (210–259)</td>
</tr>
<tr>
<td>Tryptophan (μmol/L)</td>
<td>2.7 (2.4–3.0)</td>
<td>2.5 (2.1–3.0)</td>
</tr>
</tbody>
</table>

* Significantly increased (Student’s t-test).
† Significantly reduced (Student’s t-test).

* Significantly increased (Student’s t-test).
† Significantly reduced (Student’s t-test).
Nitrate and nitrite are stable breakdown products of nitric oxide and the peroxynitrite anion. The breakdown of nitric oxide and the peroxynitrite anion is the major source of nitrate and nitrite in biological fluids of fasted humans. In rodents, brain nitrate plus nitrite concentrations were found to correlate accurately with brain nitric oxide synthase activity. Cisternal CSF concentrations were found to parallel those of the brain and there was no effect of plasma concentrations upon brain concentrations. In humans, evidence for a rostro-caudal gradient of nitrate plus nitrite concentrations suggests that nitrate and nitrite measured in lumbar CSF are generated higher up the neuraxis. The evidence therefore suggests that concentrations of nitrate plus nitrite in lumbar CSF reflect nitric oxide synthase activity in the brain that is independent of systemic nitric oxide metabolism.

There is also evidence of a species difference in the ability of cells to produce parallel induction of indoleamine 2,3-dioxygenase, GTP cyclohydrolase I, and nitric oxide synthase. While parallel induction of nitric oxide synthase, GTP cyclohydrolase I, and indoleamine 2,3-dioxygenase can be induced in rodent microglia by cytokines, this is not the case for human microglia. In human cells, cytokine stimulation does induce indoleamine 2,3-dioxygenase, but not nitric oxide synthase to any great degree. However, there is good evidence that nitric oxide synthase can be induced in human astrocytes by cytokine stimulation, although these cells cannot produce quinolinic acid. While species differences might explain why there is no parallel induction of nitric oxide synthase with the induction of GTP cyclohydrolase I and indoleamine 2,3-dioxygenase, it does not explain the reduction in nitrate plus nitrite concentrations. The reduction can only occur by reduced activity of constitutive nitric oxide synthase or increased scavenging of nitric oxide. We can only speculate about the mechanisms whereby activity of constitutive nitric oxide synthase is reduced. The activity of both constitutive isoforms of nitric oxide synthase in the brain is both calcium-dependent and requires tetrahy-
drobioprotein as a cofactor. We have shown here that tetrahydrobioprotein concentrations are increased in cerebral malaria and cofactor deficiency does not seem likely as a cause; although the increased concentrations of dihydrobioprotein might inhibit nitric oxide synthase. It is possible that free intracellular calcium is reduced in the brain in cerebral malaria and this question needs to be addressed. Concentrations of the substrate arginine may also be limiting to nitric oxide synthase activity in the brain and this too needs to be addressed. Alternatively, other inhibitors of nitric oxide synthase or scavengers of nitric oxide might be produced.

The combination of increased quinolinic acid and reduced nitric oxide concentrations in the brain may cause an excitotoxic mechanism for the production of symptoms in cerebral malaria. When quinolinic acid is synthesized in excess it readily enters the extracellular compartment. There is neither extracellular metabolism nor active transport of quinolinic acid in cerebral tissue; therefore, toxic quantities of quinolinic acid cannot be removed from the synaptic cleft. Nitric oxide has been shown to protect against NMDA-mediated neurotoxicity by reducing conductance of calcium through the receptor pore. A reduction in nitric oxide production could therefore enhance quinolinic acid neurotoxicity.

The mechanisms by which NMDA-toxicity is mediated can be separated into 2 components distinguishable on the basis of differences in time course and ionic dependence. First, an influx of sodium causes reversible neuronal swelling while delayed neuronal disintegration is caused by an influx of calcium. Excitotoxic mechanisms can thus explain the neurologic symptoms of childhood cerebral malaria: coma, cerebral edema, and seizures that may be reversible, and permanent neuronal damage. That such a mechanism occurs in cerebral malaria is supported by the graded increase in quinolinic acid found across the 3 outcome groups. It is possible that the increased concentration of quinolinic acid found in the children who died is caused by post-mortem artefact, but we think that this is unlikely because intracellular accumulation of quinolinic acid does not occur in the central nervous system, and the cerebrospinal fluid was sampled within 15 min of death.

In conclusion, we have found biochemical evidence for an excitotoxic mechanism causing the cerebral symptoms in cerebral malaria. This is of potential importance because selective NMDA-receptor blockers are becoming more widely used in medicine and would provide a novel approach to the treatment of cerebral malaria.

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