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Dramatic Changes in Oxidative Tryptophan Metabolism along the Kynurenine Pathway in Experimental Cerebral and Noncerebral Malaria

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The pathogenesis of human cerebral malaria (CM) remains unresolved. In the most widely used murine model of CM, the presence of T lymphocytes and/or interferon (IFN)-γ is a prerequisite. IFN-γ is the key inducer of indoleamine 2,3-dioxygenase (IDO), which is the catalyst of the first, and rate-limiting, step in the metabolism of tryptophan (Trp) along the kynurenine (Kyn) pathway. Quinolinic acid (QA), a product of this pathway, is a neuro-excitotoxin, like glutamic acid (Glu) and aspartic acid (Asp). Kynurenic acid (KA), also produced from the Kyn pathway, antagonizes the neuro-excitotoxic effects of QA, Glu, and Asp. We therefore examined the possible roles of IDO, metabolites of the Kyn pathway, Glu, and Asp in the pathogenesis of fatal murine CM. *Plasmodium berghei* ANKA infection was studied on days 6 and 7 post-inoculation (p.i.), at which time the mice exhibited cerebral symptoms such as convulsions, ataxia, coma, and a positive Wooly/White sign and died within 24 hours. A model for noncerebral malaria (NCM), *P. berghei* K173 infection, was also studied on days 6 and 7 and 13 to 17 p.i. to examine whether any changes were a general response to malaria infection. Biochemical analyses were done by high-pressure liquid chromatography and gas chromatography/mass spectrometry/mass spectrometry (GC/MS/MS). IDO activity was low or absent in the brains of uninfected mice and NCM mice (days 6 and 7 p.i.) and was induced strongly in the brains of fatal murine CM mice (days 6 and 7 p.i.) and NCM animals (days 13 to 17 p.i.). This induction was inhibited greatly by administration of dexamethasone, a treatment that also prevented CM symptoms and death. Furthermore, IDO induction was absent in IFN-γ gene knockout mice, which were also resistant to CM. Brain concentrations of Kyn, 3-hydroxykynurenine, and the neuro-excitotoxin QA were significantly increased in both CM mice on days 6 and 7 p.i. and NCM mice on days 13 to 17 p.i., whereas an increase in the ratio of brain QA to KA occurred only in the CM mice at the time they were exhibiting cerebral symptoms. Brain concentrations of Glu and Asp were significantly decreased in CM and NCM mice (days 13 to 17 p.i.). The results imply that neuro-excitation induced by QA may contribute to the convulsions and neuro-excitatory signs observed in CM. *(Am J Pathol 1998, 152:611–619)*

Cerebral malaria (CM), the most common fatal complication of *Plasmodium falciparum* infection, causes well over one million deaths annually.[1] Its incidence is increasing[2] and there is no specific cure apart from anti-malarial treatment. The nature of its pathogenesis is controversial, although ischemic[3] and immunological[4] mechanisms, working independently or together,[5] may be responsible. Additional studies are needed to better characterize the pathogenesis of CM with a view to designing an effective therapy to reduce the associated mortality. To help elucidate mechanisms occurring early in the development of CM, before the symptoms are manifest, murine experimental models of the disease have been developed.[4,6–8] In one such model, CBA mice are inoculated intraperitoneally with 10^6 *Plasmodium berghei* ANKA (PbA)-infected red blood cells. These mice die between days 6 and 8 post-inoculation (p.i.), exhibiting cerebral symptoms such as convulsions, hemiplegia, and stupor progressing to irreversible coma.[4,6–8] Petechial hemorrhages and cerebral edema, which also occur in human CM,[9] are notable features of CM in this animal model.[4,6–8] However, although monocytes are the predominant cells that adhere to endothelial cells and plug the central nervous system blood vessels in these mice,[10] parasitized red blood cells attach to endothelial cells and plug the brain vessels in the majority of fatal human CM cases.[11] Nevertheless, similar endothelial cell changes, notably increased expression of ICAM-1, may mediate the adherence of these different cell types to vascular endothelial

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cells in murine[12,13] and human CM.[11] Moreover, scanty inflammatory infiltrates in the meninges are occasionally seen in severe human CM[14] and mononuclear cell sequestration in CNS microvessels has been reported there also.[15] Despite the differences between human and murine CM, a study of the pathogenesis of this mouse model may provide useful insights into the development of CM in humans.

T lymphocytes are thought to play a pivotal role in the pathogenesis of murine CM, as athymic nude mice do not develop this complication when inoculated with PbA.[16] Depletion of $CD4^+$ T cells by treatment with specific antibody also prevents the development of CM.[4] Monoclonal anti-interferon (IFN)-γ antibody administered not later than day 4 p.i. protects mice from developing CM.[17] Hence, IFN-γ could conceivably mediate the actions of T lymphocytes in murine CM.

IFN-γ is the key inducer of indoleamine 2,3-dioxygenase (IDO),[18,19] the enzyme that converts tryptophan (Trp) into N-formylkynurenine, the first and rate-limiting step in the pathway leading to the production of the neuro-excitotoxin quinolinic acid (QA; 2,3-pyridinedicarboxylic acid)[20,21] (Figure 1). Kynurenic acid (KA; 4-hydroxy-2-quinolinecarboxylic acid), which protects neurons from the neuro-excitotoxicity of QA,[22,23] is also produced from this pathway. The production of a potentially dangerous receptor agonist and a protective antagonist from the same pathway suggests that it plays an important role in physiological body metabolism. A balanced production of QA and KA would protect the brain from the toxic effects of QA. On the other hand, an imbalance favoring QA overproduction would predispose to convulsions and neurodegeneration, which are common features of CM. In general support of this, QA and the endogenous excitatory amino acid neurotransmitters (glutamic acid (Glu), 1-aminopropane-1,3-dicarboxylic acid; and aspartic acid (Asp) 2-aminobutane-dioic acid) have been increasingly implicated in the pathogenesis of neuronal injury following some central nervous system insults (eg, AIDS dementia,[24] inflammatory neurological diseases,[25] and experimental bacterial meningitis[26]).

As murine CM is an IFN-γ-dependent inflammatory neurological disease, with neuro-excitatory signs such as convulsions, we have investigated a possible role for IDO and QA in its pathogenesis, by measuring in wild-type and IFN-γ gene knockout mice the brain IDO activity, the concentrations of kynurenine (Kyn) pathway metabolites, and concentrations of Glu and Asp. Clear-cut changes in the kynurenine pathway of tryptophan metabolism were observed in malaria infection, and certain of these were specific to CM.

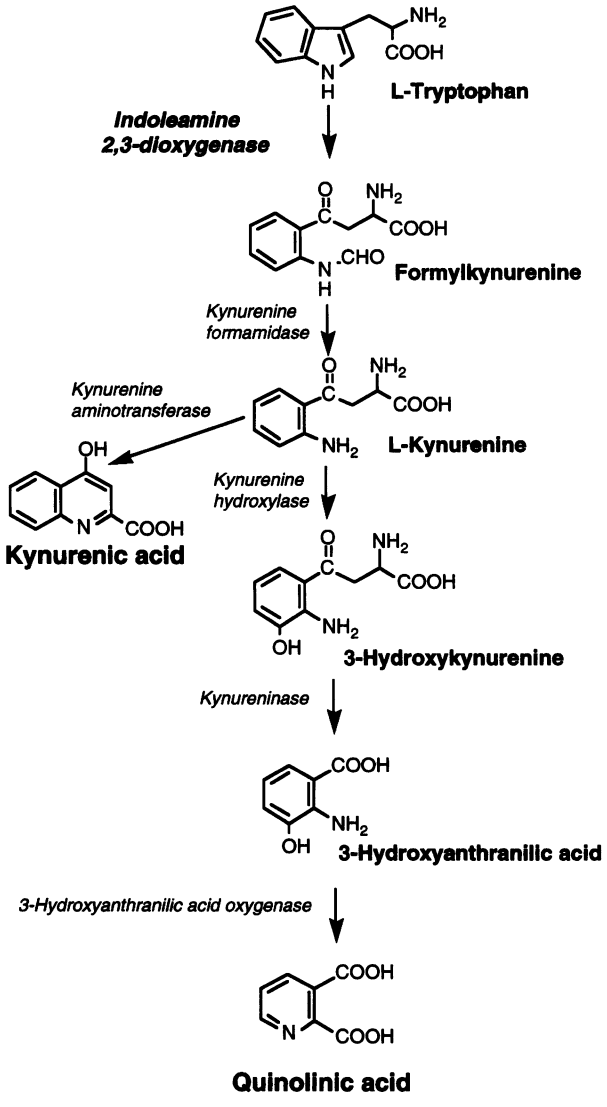

Figure 1. Oxidative tryptophan metabolism along the kynurenine pathway.

Materials and Methods

Inoculation of Mice

Six- to eight-week-old female CBA/T6 and C57 black mice weighing 20 to 25 *g* were from Blackburn Animal House, University of Sydney. The IFN-γ gene knockout mice were from Genentech (South San Francisco, CA).[27]

The malaria parasites used were either PbA (from Dr. G. Grau, Geneva, Switzerland), or *Plasmodium berghei* K173 (PbK) (from Dr. Ian Clark, Australian National University, Canberra, Australia). Mice were inoculated by intraperitoneal injection of 10^6 parasitized erythrocytes obtained from the blood of infected animals and suspended in 200 μl of phosphate-buffered saline (PBS), pH 7.4. Controls were uninfected CBA mice or CBA mice injected intraperitoneally with 200 μl of PBS. As expected, between days 5 and 6 p.i., CBA and C57 mice inoculated with PbA showed evident signs of cerebral involvement such as convulsions, paralysis, ataxia, and coma.[7,8] They died within 24 hours of developing these symptoms, with parasitemias of approximately 15%. IFN-γ gene knockout mice inoculated with PbA did not show any of the above cerebral signs (Table 1). They became sick and died between days 20 and 28 p.i. with severe anemia and parasitemias of approximately 60 to 70%.

Mice inoculated with PbK did not exhibit any of the cerebral signs seen in the PbA-infected mice, although

Table 1. Outcome of *P. berghei* ANKA Infection in Wild-Type and IFN-γ Gene Knockout Mice

Experimental group	Died on days 6 to 8 p.i.	Cerebral symptoms on days 6 to 8 p.i.
C57 wild type not infected	0/7	0/7
C57 wild type infected	8/8	28/29
C57 IFN-γ GKO not infected	0/4	0/4
C57 IFN-γ GKO infected	0/8	0/23

IFN-γ gene knockout (IFN-γ GKO) C57/Bl mice did not develop cerebral malaria after inoculation with *P. berghei* ANKA. Cerebral malaria signs include convulsions, ataxia, positive Wooly/White sign, and loss of consciousness. The numbers of animals in columns 2 and 3 are different because some were killed for other investigations.

they became sick and died between days 15 and 22 p.i. with severe anemia, as expected.[28]

At a dose of 80 mg/kg body weight, dexamethasone (DXM; Intervet International, Boxmeer, The Netherlands), an immunosuppressant and anti-inflammatory agent, was administered subcutaneously at the back of the neck to some of the mice inoculated with PbA on days 0 and 1 p.i. This treatment, as reported previously,[28,29] completely protected the mice from developing CM.

Mice were sacrificed when they became sick or on the designated day, as indicated in Results, by exsanguination under ether (Ajax Chemicals, Australia) anesthesia, except when stated otherwise. Before sacrifice, blood samples were taken from the axillary artery for determination of the percentage of parasitemia and hematocrit.

Sample Preparation

A total of six parameters related to the kynurenine pathway were assessed in brain (Tables 2 to 4). It was not possible to measure all six in each mouse because of the volume of tissue required for each assay.

Whole-brain and spleen samples used for the measurements of IDO activity and L-Trp and L-Kyn concentrations were homogenized in 2 vol of cold (4°C) 140 mmol/L KCl/20 mmol/L KPO_4 buffer (pH 7.0) containing 1 mmol/L EDTA and the protease inhibitors leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), phenylmethylsulfonyl fluoride (40 μg/ml), and aprotinin (0.5 μg/ml; all from Sigma, St. Louis, MO). The homogenates were centrifuged at 30,000 rpm (30,000 × *g*) for 30 minutes at 4°C using a Beckman TL-100 ultracentrifuge. The supernatants were removed and stored at −80°C until analyzed. For IDO activity determinations, 100-μl aliquots of the supernatants were analyzed by the ascorbate/methylene blue assay.[30]

The concentrations of 3-hydroxykynurenine (3-OHKYN) and 3-hydroxyanthranilic acid (3-OHAA) were determined in whole-brain samples from mice perfused intracardially with normal saline. The brain tissues were homogenized in 2 vol of 5% (w/v) metaphosphoric acid. Supernatants were recovered by centrifugation at 10,000 × *g* for 5 minutes at 4°C and were then extracted with an equal volume of ethyl acetate. After centrifugation (2000 × *g* for 10 minutes at 4°C), the ethyl acetate layer was removed and evaporated. The dried material was then dissolved in 5% metaphosphoric acid, and 20 μl was subjected to high-pressure liquid chromatography (HPLC) for determination of endogenous 3-OHAA.[31] For 3-OHKYN, 20 μl of the aqueous phase was analyzed by HPLC after filtration (Acro LC 13, 0.2 μm).

For the measurement of QA, brain specimens from malaria-infected and control mice, perfused intracardially with normal saline, were homogenized in 1 ml of nanopure water pretreated with Chelex-100 (Bio Rad, Richmond, CA) and containing 50 ng of [^{18}O]QA as internal standard. [^{18}O]QA was prepared as previously described.[32] The homogenates were centrifuged at 12,000 × g for 20 minutes at 0°C. Supernatants were retrieved and stored at −80°C until assayed.

For the determination of KA, Glu, and Asp, brain tissues obtained as described above were homogenized in 2 vol of 0.1 mol/L perchloric acid. Supernatants from the homogenates were retrieved by centrifugation at 8000 × *g* for 5 minutes at 0°C. Aliquots of the homogenate supernatants used for Glu and Asp determinations were neutralized with 1 mol/L KOH, and the unneutralized remnant was used for KA analysis. All samples were stored at −80°C until analyzed.

HPLC Analyses

Trp and Kyn were analyzed as described previously.[33] 3-OHKYN was determined by HPLC with electrochemical detection using a BAS LC-4C HPLC (Bioanalytical Systems, West Lafayette, IN). The column was a 25 × 0.46 cm C18 column with a 2.5-cm guard column

Table 2. IDO Activity in Control and Malaria-Infected Mice

	IDO activity (pmol/mg protein/minute)		IDO activity in plasma (pmol/ml/minute)	Plasma protein concentration (mg/ml)
	Brain	Spleen		
Control	0.2 ± 0.1 (17)	1.7 ± 0.2 (11)	6.3 ± 2.6 (12)	60.7 ± 1.9 (9)
CM d6–7	8.2 ± 1.1*** (18)	4.7 ± 0.5*** (9)	36.1 ± 9.6** (5)	58.5 ± 0.9 (6)
CM d6–7 plus DXM	1.5 ± 0.2***††† (9)	1.9 ± 0.3†† (5)	14.6 ± 5.3 (5)	61.2 ± 3.6 (5)
NCM d6–7	1.7 ± 0.1***††† (8)	2.1 ± 0.2†† (6)	15.5 ± 7.2 (6)	55.3 ± 3.1 (6)
NCM d14–15	210.6 ± 28.8***†††‡‡‡ (10)	2.6 ± 0.5† (7)	171.4 ± 32.1***†††‡‡‡ (9)	70.9 ± 4.2*†‡ (8)

IDO activity in plasma (pmol/ml/min) and homogenates of brain and spleen (pmol/mg protein/min) of control uninfected mice, *Plasmodium berghei* ANKA-infected (cerebral malaria, CM) mice with and without dexamethasone (DXM) treatment, and *P. berghei* K173-infected (noncerebral malaria, NCM) mice. Plasma protein concentration (mg/ml) is also shown. Values are mean ± SEM, with the number of mice in each group given in parentheses. The composite data are from 2–4 separate experiments. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$, significantly different from control; †$P < 0.05$, ††$P < 0.005$, †††$P < 0.0001$, significantly different from CM d6–7; ‡$P < 0.05$, ‡‡$P < 0.005$, ‡‡‡$P < 0.0001$, significantly different from NCM d6–7.

Table 3. Trp and Kyn Concentrations in Control and Malaria-Infected Mice

	Concentration in brain (nmol/mg protein)		Concentration in spleen (pmol/mg protein)		Brain concentration (nmol/ml)	
	Trp	Kyn	Trp	Kyn	Trp	Kyn
Control	0.7 ± 0.1 (16)	9.4 ± 2.2 (17)	0.5 ± 0.1 (12)	11.7 ± 4.4 (12)	84.2 ± 10.5 (12)	2.3 ± 0.3 (12)
CM d6–7	0.7 ± 0.1 (17)	149.4 ± 14.9*** (17)	1.4 ± 0.2*** (9)	45.6 ± 2.4** (9)	60.4 ± 4.8 (8)	5.6 ± 0.2*** (8)
CM d6–7 plus DXM	0.8 ± 0.1 (9)	23.3 ± 3.3*††† (9)	1.1 ± 0.1** (5)	24.0 ± 4.0†† (5)	78.2 ± 11.3 (5)	3.9 ± 0.4*† (5)
NCM d6–7	0.8 ± 0.1 (9)	20.0 ± 3.7*††† (9)	1.1 ± 0.1*** (6)	18.3 ± 3.1†† (6)	91.7 ± 8.5 (6)	3.9 ± 0.3**†† (6)
NCM d14–15	0.3 ± 0.04*** (10)	1410.0 ± 121.5***†††‡‡‡ (10)	1.5 ± 0.1***‡ (9)	80.0 ± 12.0***†‡‡ (9)	64.5 ± 4.4 (9)	14.9 ± 2.2***†††‡‡‡ (9)

Endogenous Trp and Kyn concentrations in homogenates of brain and spleen, expressed in nmol/mg protein and pmol/mg protein, respectively, and in plasma (nmol/ml) of control uninfected mice, *P. berghei* ANKA-infected (cerebral malaria, CM) mice with and without dexamethasone (DXM) treatment, and *P. berghei* K173-infected (noncerebral malaria, NCM) mice. Values are mean ± SEM, with the number of mice in each group given in parentheses. The composite data are from two to four separate experiments.

*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$, significantly different from control.

†$P < 0.05$, ††$P < 0.005$, †††$P < 0.0001$, significantly different from CM d6–7.

‡$P < 0.05$, ‡‡$P < 0.005$, ‡‡‡$P < 0.0001$, significantly different from NCM d6–7.

(Supelco, Bellefonte, PA). The mobile phase was used at 0.9 ml/minute and consisted of 24 mmol/L sodium acetate, 0.54 mmol/L EDTA, 7.5% (v/v) methanol, and 1.5 mmol/L dodecyl triethylammonium phosphate (Q12), with the pH adjusted to 4.75 with glacial acetic acid. The potential was set at +500 mV *versus* a Ag/AgCl reference electrode. 3-OHKYN was quantified by area comparison with that of a freshly prepared standard, dissolved in 5% metaphosphoric acid. 3-OHAA was determined as described for 3-OHKYN, except that a 25 × 0.46 cm C18 DB column with a 2.5-cm guard column (Supelco) and a mobile phase consisting of 100 mmol/L sodium acetate (pH 5) and acetonitrile (4:1, v:v) were used.[31] KA was quantified as described by Shibata,[34] using Spectroflow model 400 pumps and an LC-18 (7.5 × 4.6 mm) column with a 25-cm guard column (Supelco) eluted with 50 mmol/L sodium acetate plus 4.5% acetonitrile, pH 6.2 (adjusted with glacial acetic acid) at 1.0 ml/minute. Zinc acetate at 0.5 mol/L was delivered post-column at a flow rate of 1.0 ml/minute. A high-pressure mixer was used, and before detection an additional mixing loop with a volume of 0.2 μl was installed. A Perkin Elmer (Norwalk, CT) LC 240 fluorescence detector (7-μl flow cell), set at an excitation wavelength of 344 nm and an emission wavelength of 398 nm, was used for detection. Glu and Asp were determined as described previously.[35]

Gas Chromatography/Mass Spectrometry/Mass Spectrometry (GC/MS/MS) Quantification of QA

Brain homogenate supernatants were placed in 13 × 100 mm screw-capped glass tubes containing 2 ml of ice-cold chloroform, and the tubes were shaken for 5 minutes. The tubes were then centrifuged at 2000 × *g* for 5 minutes. The top aqueous layers were retrieved and used for the derivatization of endogenous QA and [^{18}O]QA to their pentafluorobenzyl esters using tetrabutylammonium hydrogen sulfate as catalyst.[36] The samples were analyzed on a Finnigan/MAT TSQ 46 GC/MS/MS system (San Jose, CA) in negative ion chemical ionization mode, linked to a Hewlett Packard (Palto Alto, CA) 7673 A autosampler. The SE30 bonded phase capillary column (30 m × 0.25 mm I.D., 0.25-μm film thickness, Alltech N.S.W, Australia) was inserted directly into the ion source of the mass spectrometer. Helium was used as the carrier gas at 2 ml/minute and methane (UHP) as the chemical ionization reagent gas at a total ion source pressure of 0.9 torr (120 Pa). The optimal ion source temperature for most abundant daughter ion formation was determined to be 140°C. The optimal collision pressure and collision energy were determined to be 2.0 milliTorr (0.26 Pa) and 10.0 ev, respectively. Argon was used for the collision experiment. After injection of 1-μl aliquots the GC was

Table 4. Brain Concentration of 3-OHKYN, KA, and QA and Ratio of QA to KA

	Concentration (fmol/mg wet weight)			
	3-OHKYN	QA	KA	QA:KA ratio
Control	45.3 ± 0.3 (4)	139.0 ± 20.5 (14)	5.7 ± 0.7 (13)	17.2 ± 1.8 (8)
CM d6–7	192.1 ± 15.9** (8)	377.0 ± 19.2*** (15)	9.2 ± 0.8** (15)	38.5 ± 3.2*** (11)
NCM d6–7	ND	124.6 ± 7.5††† (11)	5.9 ± 0.9† (11)	18.2 ± 1.6††† (11)
NCM d13–17	1115.1 ± 46.2*†† (7)	493.7 ± 48.7***†‡‡ (10)	22.9 ± 4.0***†††‡‡ (11)	22.5 ± 1.8†† (6)

Endogenous 3-OHKYN, KA, and QA concentrations in the brains of control uninfected mice, *P. berghei* ANKA-infected (CM) mice, and *P. berghei* K173-infected (NCM) mice expressed in fmol/mg wet weight. Values are mean ± SEM, with the number of mice in each group given in parentheses. ND, not determined.

*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$, significantly different from control.

†$P < 0.05$, ††$P < 0.005$, †††$P < 0.0001$, significantly different from CM d6–7.

‡$P < 0.05$, ‡‡$P < 0.0005$, ‡‡‡$P < 0.0001$, significantly different from NCM d6–7.

programmed to 300°C at 25°C/minute. Details of the validation of the GC/MS/MS assay would be described elsewhere (Tattam BN, Awad V, Moore DE, Sanni LA, Hunt NH; manuscript in preparation).

Statistical Analysis

Results were analyzed by the Mann-Whitney test. Values of $P \leq 0.05$ were considered significant.

Results

IDO Activity

The activity of IDO in plasma and homogenates of brain and spleen was assessed at certain critical times during the course of infection in CBA mice inoculated with PbA (CM mice) and PbK (NCM mice). In uninfected controls, IDO activity in the brain homogenate was very low (0.2 ± 0.1 pmol/mg protein/min) (Table 2). When compared with controls, very large and highly significant increases ($P < 0.0001$) in brain IDO activity were observed in CM mice and in NCM mice on days 6 and 7 and 14 to 17 p.i., respectively, which were the times when the mice died in these models. A significant increase ($P < 0.0001$) in IDO activity was also seen in the spleen of CM mice, whereas in NCM mice, even at 14 to 17 days p.i., the splenic activity was similar to that in control animals. Plasma IDO activity was significantly increased ($P < 0.001$) over the controls in CM (days 6 and 7 p.i.) and NCM mice (days 14 to 17 p.i.; Table 2). The results indicate that induction of IDO was a feature of malaria infection and that this was much more pronounced in CM than NCM on days 6 and 7 p.i., a time at which CM mice exhibit cerebral symptoms and start to die. It should be noted that we did not make measurements throughout the course of the infections but concentrated on those critical periods when the mice were approaching death from CM or noncerebral complications.

Previous studies[19] indicate that *in vivo* induction of IDO can essentially be ascribed to the action of the proinflammatory cytokine IFN-γ. To assess a possible link between IDO induction and CM, we tested the effect of administration of the anti-inflammatory agent DXM on IDO induction in CM. As can be seen in Table 2, DXM effectively reduced the extent of IDO induction in brain, spleen, and plasma of CM mice to levels indistinguishable from those in NCM at the same time p.i. In agreement with previous reports,[28,29] DXM also prevented the development of CM even though it did not affect parasitemia (not shown). Induction of IDO was totally prevented in IFN-γ gene knockout mice infected with PbA, even though infection of mice of the corresponding background strain, C57, caused both CM (Table 1) and IDO induction in the brain (Figure 2) to an extent similar to that seen in CBA mice (Table 2). Importantly, IFN-γ knockout mice were also totally refractory to CM (Table 1), consistent with a link between IDO induction in brain, or its consequences, and CM.

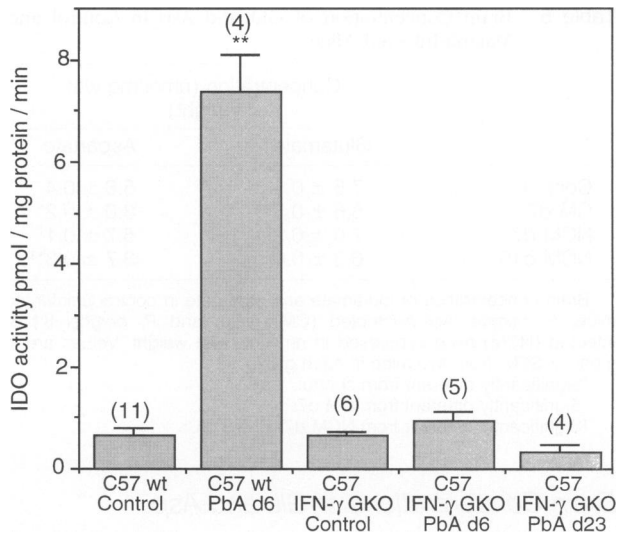

Figure 2. Brain IDO activity (pmol/mg protein/min) in control (uninfected) C57 wild-type mice (wt), IFN-γ gene knockout (IFN-γ GKO) mice and their PbA-infected counterparts. d6, day 6 after inoculation (p.i.); d23, day 23 p.i. Values are in mean ± SEM, with the number of mice in each group given in parentheses. $^{**}P < 0.005$ when compared with the uninfected controls.

Endogenous Concentration of Trp and Its Metabolites

We next characterized the effects of IDO induction on the concentrations of its substrate, Trp, and its products. Brain Trp concentrations in controls, CM, and NCM (days 6 and 7) mice were similar (Table 3). However, there was a significant decrease ($P < 0.0001$) in Trp concentration in the brain of the NCM mice on days 14 to 17 p.i., when IDO activity was highest. There were small, though significant, increases in splenic Trp concentration above that in control mice in CM mice and NCM mice on all days studied ($P < 0.0001$ in all cases), but plasma Trp concentration was similar in all groups (Table 3).

Kyn concentrations increased significantly in the brain and spleen of CM mice and NCM mice on all days studied when compared with controls (Table 3). The brain concentration of 3-OHKYN significantly increased in CM and NCM mice (days 13 to 17 p.i.; $P < 0.01$ in both cases; Table 4). 3-OHAA was not detectable in the brains of the control or malaria-infected mice. The limit of detection of our procedure was 1.3 pmol.

Brain concentrations of QA increased in CM and NCM mice (days 13 to 17 p.i.) when compared with controls ($P < 0.0001$ and $P = 0.0003$, respectively; Table 4). However, the value for NCM mice on days 6 and 7 p.i. was similar to that in controls. In addition, there were significant increases ($P = 0.01$ and 0.0002, respectively) in the level of brain KA in CM and NCM mice (days 13 to 17 p.i.; Table 4). Importantly, there was a specific and significant ($P = 0.02$) increase, by a factor of >2, in the brain ratio of QA to KA in CM mice on days 6 and 7 p.i. (Table 4), whereas this ratio did not change significantly in NCM mice even on days 13 to 17 p.i.

Table 5. Brain Concentration of Glu and Asp in Control and Malaria-Infected Mice

	Concentration (nmol/mg wet weight)	
	Glutamate	Aspartate
Control	7.5 ± 0.3	5.6 ± 0.4
CM d7	5.6 ± 0.3*	3.0 ± 0.2*
NCM d7	7.6 ± 0.2†	5.7 ± 0.1†
NCM d19	6.3 ± 0.4‡	3.7 ± 0.3*‡

Brain concentration of glutamate and aspartate in control uninfected mice, *P. berghei* ANKA-infected (CM) mice, and *P. berghei* K173-infected (NCM) mice expressed in nmol/mg wet weight. Values are in mean ± SEM, from five mice in each group.
*Significantly different from control.
†Significantly different from CM d7.
‡Significantly different from NCM d7; $P < 0.05$.

Brain Concentrations of Glu and Asp

Brain concentration of Glu was decreased significantly ($P = 0.02$) in CM mice, when compared with controls (Table 5). No change occurred in NCM. Brain Asp concentration decreased significantly in CM (days 6 and 7 p.i.) and NCM mice (day 13 p.i.; $P = 0.008$ in both cases). However, Asp was normal in NCM mice brains on days 6 and 7 p.i.

Discussion

A striking induction of brain IDO activity was observed in CM mice on days 6 and 7 p.i., and this was significantly greater than that in NCM mice at the same time p.i. However, when NCM mice became moribund and severely anemic (by days 14 to 17 p.i.), brain IDO activity increased dramatically to levels above that seen in CM mice. As NCM mice, even at the late stages of infection, do not show any cerebral symptoms, we conclude that IDO induction in brain *per se* is neither specific to CM nor solely responsible for the symptoms. However, as discussed below, other changes in this pathway of Trp metabolism, notably the increased QA to KA ratio (Table 5), did correlate with the development of CM, suggesting that neuro-excitotoxicity mediated by QA could contribute to the pathogenesis. We believe that this is the first demonstration of derangements in the kynurenine pathway of tryptophan metabolism in malaria or, indeed, any parasitic disease (see Table 6 for summary). Intervention with DXM treatment, or through the use of IFN-γ gene knockout mice, blocked the increase in brain IDO activity

Table 6. Summary of Brain IDO Activity and Levels of Trp Metabolites

	CM d6–7	NCM d6–7	NCM d13–17
Brain IDO	↑ ↑	↑	↑ ↑ ↑ ↑
Kyn	↑ ↑	↑	↑ ↑ ↑ ↑
3-OHkyn	↑ ↑	ND	↑ ↑ ↑ ↑
QA	↑ ↑	→	↑ ↑
KA	↑	→	↑ ↑
QA:KA Ratio	↑	→	→

ND, not determined.

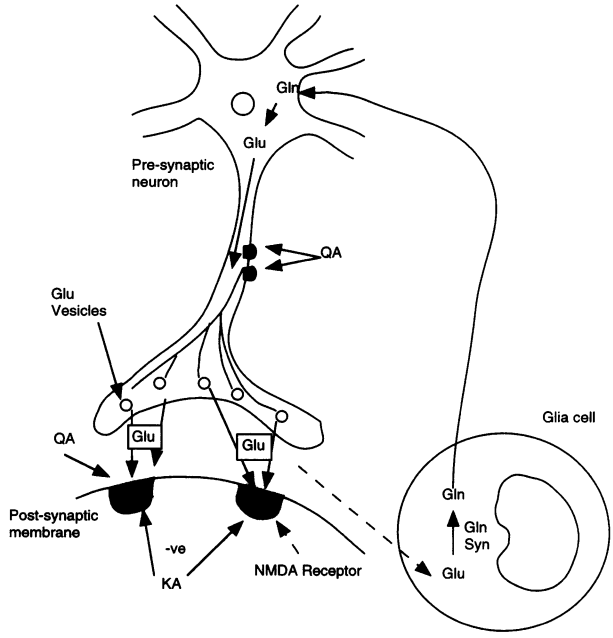

Figure 3. Proposed mechanism of decrease in Glu and Asp concentrations in murine CM and NCM.

in mice infected with PbA and prevented their death from CM.

Despite the increased and very high concentrations of QA in the brain of the CM and NCM mice on days 13 to 17 p.i., only the former exhibited signs of neuro-excitation, notably convulsions. KA is an antagonist of the neuro-excitotoxic actions of QA,[23] and in CM the brain KA concentration remained low, whereas in NCM it increased proportionally to QA. This led to a CM-specific increase in the QA to KA ratio and may explain why the NCM mice, even on days 13 to 17 p.i., were resistant to convulsions. If indeed the imbalance in QA to KA ratio is responsible for the convulsions in CM, strategies to reduce QA concentration in brain or inhibit its excitotoxic effect may prove protective in murine CM. This could be achieved in a number of ways: 1) employing IDO gene knockout mice, which have not yet been described, 2) using drugs that inhibit IDO activity, 3) using drugs that inhibit the activity of kynurenine hydroxylase, thereby increasing KA production at the expense of QA (see Figure 1), or 4) employing NMDA receptor antagonists (see Figure 3 for rationale). Appropriate drugs have been reported to be under development, and we intend to explore their application to this system.

The mechanisms responsible for induction of IDO in the brain during the malaria infections are unknown. However, inflammatory and immune processes may play a role, at least in the CM mice, because the anti-inflammatory and immunosuppressant agent DXM significantly inhibited the brain IDO induction seen on days 6 and 7 p.i. and also totally abolished the development of cerebral complications and symptoms. This is consistent with the findings of Bianchi and colleagues,[38] who demonstrated the inhibitory effect of DXM on IDO induction in the lung *in vivo* caused by lipopolysaccharide administration.

IFN-γ is the only agent known to directly induce IDO activity *in vitro* or *in vivo*.[18,19] Consistent with this, induction of IDO in the brain in murine CM is absolutely dependent on this cytokine, because it was abolished in IFN-γ gene knockout mice inoculated with PbA (Figure 2). These IFN-γ-deficient mice did not develop cerebral symptoms or die from CM (Table 1). However, despite these correlations, a direct causative effect of IDO induction on CM cannot be deduced as the absence of IFN-γ may have prevented other key steps in the immunopathology of CM. Similarly, DXM may act at several stages in the pathogenesis of CM.[28]

Malaria-induced increases in brain IDO activity were reflected in plasma IDO activity, the source of which remains to be elucidated but could conceivably be brain cells or other tissues releasing the enzyme into the extracellular space. The specific cell type(s) in the central nervous system in which IDO is induced is not known because there is as yet no anti-mouse IDO antibody available for immunohistochemistry. As IDO activity is induced by IFN-γ in cells of the macrophage lineage,[37] monocytes and microglia are obvious candidates. Monocytes adhere to the microvascular endothelium during the later stages of murine CM but never do so in PbK infection. Although a small amount of blood might have remained in the central nervous system microcirculation after saline perfusion, measurement of blood IDO activity (data not shown) demonstrated that this could not account for any significant part of the increase in brain IDO activity in murine malaria. Microglia become activated during the course of murine CM[38] and so remain potential sites of IDO induction. Whether microglia are activated in NCM is not known. IFN-γ has been shown to induce IDO activity in a murine microglial cell line.[18]

The pathophysiological significance, if any, of the massive IDO induction in NCM mice is not clear. Activation of this pathway has been reported in a number of systemic infectious diseases, such as septicemia,[39] human,[24] and simian AIDS.[40] It is conceivable that it is related to the sorts of changes in central nervous system activity, for example, drowsiness, loss of appetite, or changes in body temperature regulation, that are common to a number of infectious diseases, although this idea is purely speculative at present.

Although the spleen is rich in macrophages and malaria infection is hematogenous in nature, splenic IDO activity was unchanged in NCM mice and only minimally elevated in CM mice. This apparent resistance of the spleen to IDO induction may be attributable to the upregulation of inducible nitric oxide synthase (iNOS). Spleen iNOS is increased in some malaria infections,[41] and it is induced by IFN-γ.[42] The product of its action, nitric oxide (NO), inhibits IDO activity in macrophages.[43] This possible relationship between IDO and iNOS in malaria is being tested in our laboratories.

The increases in brain IDO activity led to a corresponding rise in the brain concentrations of its product, Kyn. However, only in the NCM mice on days 14 and 15 p.i., where brain IDO activity was increased massively, was the brain concentration of the enzyme substrate Trp decreased. This reduction appeared to be localized to the brain as plasma and splenic concentrations of Trp remained steady (Table 3). As NCM mice do not show cerebral involvement, IDO induction does not appear to affect brain functions, grossly at least, through depletion of Trp, which is a precursor of the transmitter substance 5-hydroxytryptamine. However, significant changes at the level of individual neurons cannot be ruled out.

An important observation in the present study was that the brain concentrations of QA and 3-OHKYN did not reflect the IDO activities, indicating that determination of IDO alone is not sufficient to assess the significance of potential changes in the levels of the different Trp metabolites along the Kyn pathway and that enzymes in addition to IDO appear to be under regulatory control, in an as yet undefined manner. QA is a neurotoxin,[20,21] and QA phosphoribosyl transferase is responsible for its conversion to nicotinic acid ribonucleotide. A reduction in the activity of this enzyme could contribute to the observed accumulation of QA in the brain of CM mice. Differences in the activity of this enzyme in CM and NCM mice could explain why the brain QA concentrations in CM (days 6 and 7 p.i.) and NCM mice (days 13 to 17 p.i.) were only slightly different despite much greater differences in their brain IDO activity and 3-OHKYN levels. This explanation, however, remains to be tested.

One source of elevated QA detected in the brain of malaria-infected mice could be extracerebral. Dendritic cells and phagocytes in the spleen, and adherent monocytes in the vasculature of the brain, could be the source of QA, as was reported for retroviral infection in mice.[44] However, the normal blood-brain barrier (BBB) is impermeable to QA.[45] A compromised BBB induced by malaria infection during the late stages of the CM and NCM could allow influx of QA into the brain from the plasma. Against this possibility must be set the clear evidence for IDO induction and increased levels of other kynurenine pathway metabolites seen in malaria infection (Table 6).

In murine CM, brain concentrations of Glu and Asp decreased to a much greater extent than that observed in NCM (day 19 p.i.). QA can cause the release of Glu and Asp from cerebral cortex *in vivo*,[46] and this could predispose the brain to excitotoxic damage. We propose that the brain attempts to protect itself from such excitotoxic damage by rapid uptake of the extracellular Glu and Asp by nerve and glial cells.[47] Inside glial cells, Glu is amidated to glutamine (Gln) by glutamine synthetase, possibly leading to the observed decrease in total brain concentration of Glu (Figure 3). As Glu is also the precursor of Asp, the brain level of the latter would be expected to also decrease. Intracranial hypertension from vasogenic cerebral edema, a prominent feature of murine CM, is known to increase extracellular Glu concentration.[48] This, then, may explain the more pronounced decrease of Glu and Asp levels in murine CM than in NCM, where cerebral edema is not seen.

As preliminary studies indicate increased IDO immunoreactivity in the brains of patients who died from CM

(Sanni LA, Ogunbiyi JO, Osifo BOA, and Hunt NH, unpublished observation), this pathway could also contribute to the pathogenesis of CM in humans. A number of new pharmaceutical approaches to the treatment of neurological deficits associated with AIDS dementia and inflammatory neurological diseases have been proposed,[24,25] and these could be of potential benefit to human patients with CM although additional work needs to be done in the experimental model to justify this approach.

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