Malaria is a major cause of morbidity and mortality with an annual death toll exceeding one million. Severe malaria is a complex multisystem disorder, including one or more of the following complications: cerebral malaria, anemia, acidosis, jaundice, respiratory distress, renal insufficiency, coagulation anomalies, and hyperparasitemia. Using a combined in vivo/in vitro metabolic-based approach, we investigated the putative pathogenic effects of Plasmodium berghei ANKA on brain, in a mouse strain developing malaria but resistant to cerebral malaria. The purpose was to determine whether the infection could cause a brain dysfunction distinct from the classic cerebral syndrome. Mice resistant to cerebral malaria were infected with P. berghei ANKA and explored during both the symptomless and the severe stage of the disease by using in vivo brain magnetic resonance imaging and spectroscopy. The infected mice did not present the lesional and metabolic hallmarks of cerebral malaria. However, brain dysfunction caused by anemia, parasite burden, and hepatic damage was evidenced. We report an increase in cerebral blood flow, a process allowing temporary maintenance of oxygen supply to brain despite anemia. Besides, we document metabolic anomalies affecting choline-derived compounds, myo-inositol, glutamine, glycine, and alanine. The choline decrease appears related to parasite proliferation. Glutamine, myo-inositol, glycine, and alanine variations together indicate a hepatic encephalopathy, a finding in agreement with hepatic encephalopathy associated with fitting and loss of consciousness (1). Together with CM, malarial anemia is a leading cause of mortality (3). Its pathogenesis is complex and undoubtedly involves multiple processes related to both the destruction and the reduced production of red blood cells (6).

P. berghei ANKA (PbA) is a widely recognized experimental model of malaria. Mice are infected with parasitized red blood cells to skip the hepatocyte stage of the parasite cycle. They directly undergo the red blood cell stage, which is the symptomatic stage. Infected mice develop a different syndrome.

Malaria is one of the major tropical diseases. This parasitic infection results in 200 to 300 million clinical cases and 1 to 2 million deaths annually, mostly children under 5 and pregnant women in sub Saharan Africa (1, 2). Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, and Plasmodium falciparum are the four species infecting humans. The latter one causes the most virulent and deadly infections (3). The Plasmodium life cycle includes firstly a non-pathogenic symptomless hepatocyte stage followed by a pathogenic red blood cell stage. Transmission via the bite of an infected female Anopheles species mosquito induces the hepatocyte stage; sporozoites invade hepatocytes, replicate, and produce merozoites, and these later initiate the red blood cell stage with invasion of red blood cells. During the red blood cell stage, clinical malaria may develop (4). The classic clinical presentation consists of fever accompanied by other symptoms such as headache, malaise, nausea, muscular pains, and mild diarrhea (5). A minority of cases progress toward a life-threatening condition due to complications, including severe anemia, acidosis, respiratory distress, jaundice, renal failure, and cerebral malaria (CM) (6) (3). The clinical course of CM, the most lethal complication of P. falciparum infection, is characterized by diffuse, potentially reversible encephalopathy associated with fitting and loss of consciousness (7).
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depending on their genetic background (8). Susceptible mice (CBA/J strain) develop an experimental cerebral syndrome and die approximately 1 week after the infection. On the contrary, resistant mice (BALB/c strain) do not present any apparent neurological signs and die of severe anemia during the third and fourth weeks (5). The molecular mechanisms underlying this difference in susceptibility to CM remain to be elucidated (9, 10). Although CM occurs only with certain combinations of parasites and inbred mouse strains, anemia is a pathological feature of all rodent malarial infections (3, 11, 12).

We previously performed the first characterization of the experimental cerebral syndrome using in vivo magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) techniques (13). We found that blood-brain barrier (BBB) rupture, major edema, and reduced brain perfusion were the main pathological features. Our findings demonstrated the coexistence of inflammatory and ischemic lesions and proved the preponderant role of edema in the fatal outcome of experimental CM (13).

Here, we were interested in examining the cerebral effects of PbA infection in a mouse strain (BALB/c) resistant to experimental CM. The purpose was to test the hypothesis that PbA infection could elicit brain dysfunction, despite the absence of experimental CM, and by mechanisms different from those involved in CM. We examined the brain of CM-resistant mice at day 8 post-infection, a time when susceptible mice develop the cerebral syndrome with the same parasitemia. In our previous study on CBA/J mice, we identified structural, functional, and metabolic markers characteristic of experimental CM. Here, we aimed at screening these pathological indicators in BALB/c mice to delineate the main differences in the cerebral response to the infection between these two strains. The mice explored at day 8 post-infection were referred to as “non-cerebral malaria” (NCM). Because BALB/c mice progressively develop severe anemia, we explored the impact of this complication on brain, by imaging mice at day 15 post-infection, a stage when hyperparasitized (HP) mice present anemia. We applied multimodal magnetic resonance techniques that provide non-invasive characterization of anatomic, structural, functional, and metabolic brain features. The in vivo metabolic study was completed by high resolution MRS analysis of brain extracts, a technique that gives access to a wider range of metabolites. Finally, taking into account that, during the blood stage of experimental malaria, although the parasite cannot invade hepatocytes as in humans, the infection induces liver injury (14), we investigated hepatic damages and their possible incidence on brain metabolism and function.

**EXPERIMENTAL PROCEDURES**

**Mice**

Thirty female BALB/c mice from Charles River Laboratories (l’Arbresle, France) (8–10 week old, 20–25 g body weight) were maintained at 23–25 °C with a 12-h light/12-h dark cycle and had free access to food and water. Animal studies were in agreement with the French guidelines for animal care and approved by the local Committee on Ethics.

**Parasite, Infection, and Disease Assessment**

Twenty mice were infected with PbA by intraperitoneal injection of 10⁶ parasitized red blood cells. Parasitemia was determined on Giemsa-stained blood smears and was expressed as the percentage of parasitized red blood cells. Total red blood cell counts and hemoglobin levels were determined from heparinized blood. Hemoglobin levels were assessed in blood collected from healthy BALB/c and infected mice at 8 or 15 days post-infection. Blood was diluted in Drabkin’s solution, and the optical density at 580 nm was measured. Hemoglobin level in blood was calculated using standard curves (Randox Kit, Mauguio, France).

**Hepatic Function**

**Biochemistry**—Blood samples from control and infected mice were collected from retro-orbital plexus of isoflurane-anesthetized mice with capillaries containing heparin. Blood samples were centrifuged, and plasma was collected. Among indicators of hepatocellular integrity, the most commonly measured in clinical toxicology studies are the enzymatic activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GT), and alkaline phosphatase (ALP), as well as the levels of ammonia and bilirubin. Analysis of AST, ALT, γ-GT, and ALP activities and measurement of total bilirubin and ammonia concentrations in serum were performed using the Beckman Synchrone LX (Beckman Coulter, Villepinte, France). Enzymatic activities of AST, ALT, γ-GT, and ALP were obtained by colorimetric methods and were expressed as international units per liter. Serum concentrations of total bilirubin and ammonia were derived from a colorimetric method and expressed in micromolar.

**Histology**—Livers from control and infected mice at 8 or 15 days post-infection were collected and fixed with 4% paraformaldehyde and subsequently embedded in paraffin. Paraffin-embedded sections (5 μm) were processed for hematoxylin-eosin staining. Slides were pictured using an Eclipse 800 microscope (Nikon, Champigny-sur-Marne, France) and a digital camera.

**In Vivo MR Protocol**

The infected mice were explored at days 8 and 15 after infection, on a horizontal Bruker 47/30 AVANCE Biospec magnetic resonance system operating at 4.7 T (Bruker, Ettlingen, Germany) following a procedure previously described (13). Animals were anesthetized by an intraperitoneal injection of ketamine (50–100 mg/kg body weight) and xylazine (10–20 mg/kg body weight). The mice were placed in prone position in a cradle equipped with a stereotaxic holder and an integrated heating system that allowed the body temperature to be maintained at 36 ± 1 °C.

**MRI**—T₁, T₂, and T₂*-weighted images were obtained with the following parameters: 15 contiguous slices; slice thickness, 0.7 mm; matrix, 256 × 256; and field of view, 25 × 25 mm². Multislice axial T₁-weighted images were acquired using a spin echo sequence (time of echo (TE), 15 ms; time of repetition (TR), 0.6 s; and 2 averages), before and after the injection of gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA, 1 ml/kg body weight, Schering, Levallois-Perret, France) in the caudal vein. Injection of Gd-DTPA was performed at the end of
the MR protocol. Multislice axial and sagittal T₂*-weighted images were acquired with a spin echo sequence (TE, 40 ms; TR, 2.5 s; rapid acquisition with relaxation enhancement factor 4; and 2 averages). Multislice axial T₂*-weighted images were acquired with a gradient-echo sequence (TE, 17 ms; TR, 1 s; flip angle, 20°; and 3 averages).

Multislice diffusion-weighted spin-echo segmented echoplanar imaging was used to map the mean apparent diffusion coefficient (ADC, TE, 55 ms; TR, 3 s; 11 contiguous slices, slice thickness, 3 mm; matrix, 128 × 128; field of view, 25 × 25 mm²; and 2 averages for each direction (x, y, and z)). Half-sine-shaped diffusion gradients were applied separately along x-, y-, and z-axis directions, with five increasing values of gradient magnitude (ADC, TE, 55 ms; TR, 3 s; flip angle, 20°; and 3 averages).

Quantitative cerebral blood flow (CBF) maps were obtained with a Look-Locker arterial spin labeling technique based on flow-alternating inversion recovery (15). A series of 50 gradient echoes was acquired after each inversion pulse. The in-plane spatial resolution was 156 × 312 μm²; TE, 1.56 ms; slice thickness, 3 mm; matrix, 128 × 64; field of view, 25 × 25 mm²; and flip angle, 10°.

Magnetic resonance angiography was performed on a vertical Bruker AVANCE 500WB wide-bore MR system operating at 11.75 T using a transmitter/receiver with a 2.5-mm diameter resonator. Angiograms were acquired with a three-dimensional gradient echo time of flight sequence (TR, 30 ms; TE, 3.6 ms; flip angle, 50°; matrix, 256 × 192 × 64; field of view, 18 × 12 × 9 mm³; and 2 averages).

MRS—Localized 1H MR spectra of brain were obtained at 4.7 T by using the point resolved spatially localized spectroscopy sequence (TE, 135 ms or TE, 16 ms; TR, 1.5 s; 256 averages; and 1,024 points). Water suppression was achieved using a variable power radiofrequency pulses with optimized relaxation delays sequence. The volume of interest (3.5 mm³) was positioned centrally on both hemispheres mainly in striatum and thalamus. At short TE (16 ms) the number of metabolite signals that can be detected is greater than at long TE (135 ms) due to a "T₂ effect." Indeed, brain metabolites are characterized by diverse longitudinal and transverse relaxation times (T₁ and T₂) that influence the intensity of the MR signal. During the TE, the transverse relaxation is responsible for an exponential decay of the MR signal with a time constant T₂. Consequently, this signal will be "T₂-weighted" according to the T₂ values of the molecules. Because of these differences in the T₂ relaxation time, certain metabolite signals are eliminated or too low to be resolved at long TE. Thus, spectra acquired at long TE present less signals when compared with those obtained at short TE. Although the T₂ effect may be a source of discrepancies between results obtained at short and long TE, the acquisition of spectra at long TE is motivated by two important reasons: 1) the resulting simplification of the spectrum may facilitate the assignment of metabolites in spectral regions with overlapping signals and 2) long TE allow the unambiguous detection of lactate when present owing to its characteristic negative signal at 1.31 ppm.

31P MRS data processing was performed as follows: after zero-filling (2048 points), the free induction decays were apodized with a line broadening of 15 Hz and Fourier-transformed. Zero-order phase correction was applied. The chemical shifts were referenced to phosphocreatine (PCr, −2.45 ppm). The signal amplitudes corresponding to PCr, inorganic phosphate (Pᵢ), phosphomonooesters, and α-, β-, and γ-ATP were calculated using the AMARES time-domain-fitting procedure. The chemical shift (δ) between Pᵢ and PCr was used to calculate brain pH using: pH = 6.8 + log[(δ - 0.77)/(3.39 - δ)]. The metabolites were expressed as ratios of metabolites or as the ratio of each metabolite to the sum of all metabolites (Pᵢtot).

1H MRS of Whole Brain Extracts

At the end of the in vivo exploration, while still anesthetized, mice were sacrificed and the brain was freeze-clamped. Tissues frozen in liquid nitrogen were pulverized and extracted with perchloric acid 6%. Whole brain extracts were homogenized and allowed to settle at 4 °C during 20 min. The samples were then centrifuged and neutralized with a solution of K₂CO₃ (2 M). The salts were removed by centrifugation, and the extracts were treated by a Chelex resin and subsequently lyophilized. Lyophilized samples were reconstituted in D₂O 99.9% and 3-(trimethylsilyl)propionic-2,3,3-d₄ acid was used as an

In Vivo MR Data Processing

All MR data were processed under an interactive data language (IDL) environment (Interactive Data Language Research System, Boulder, CO) as previously described (13, 16).

MRI Data—Brain, cerebellum, and ventricle (lateral and third ventricles) volumes were calculated from T₁-weighted images. Cerebellar and ventricular fractions were calculated as the ratio between the volume of a given structure to the volume of brain (cerebrum and cerebellum). These ratios were expressed as percentages. Regional mean ADC values were evaluated as an average of pixel values in regions of interest in the cortex and the striatum.

Perfusion post processing and CBF maps were done using a customized program under the IDL environment (15), assuming a blood/tissue partition coefficient for water λ of 0.9 ml/g. T₁ of blood was measured for each mouse in a large cerebral vessel. Blood T₁ values are different in HP mice probably owing to the modification of blood properties due to the parasite. CBF values >8 ml/g/min were masked in the CBF map to exclude large vessels. Regional CBF was evaluated as an average of pixel values in regions of interest in the cortex and the striatum. Angiograms were produced by generating maximum intensity projections after interpolating raw data to obtain an isotropic resolution (588 μm³/pixel³).

MRS Data—1H MRS data processing was performed as already described with an exponential filtering set to 4 Hz (17). Results were expressed as ratios of the relative area of each metabolite signal to the sum of all metabolite signal areas (S) detectable at each TE: S = N-acetylaspartate (NAA) + total creatine + choline-containing compounds (for spectra recorded with TE = 135 ms) and S = NAA + total creatine + choline-containing compounds + (glutamine + glutamate) (Glx) + myoinositol + taurine (for spectra recorded with TE = 16 ms).

31P MRS data processing was as follows: after zero-filling (2048 points), the free induction decays were apodized with a line broadening of 15 Hz and Fourier-transformed. Zero-order phase correction was applied. The chemical shifts were referenced to phosphocreatine (PCr, −2.45 ppm). The signal amplitudes corresponding to PCr, inorganic phosphate (Pᵢ), phosphomonooesters, and α-, β-, and γ-ATP were calculated using the AMARES time-domain-fitting procedure. The chemical shift (δ) between Pᵢ and PCr was used to calculate brain pH using: pH = 6.8 + log[(δ - 0.77)/(3.39 - δ)]. The metabolites were expressed as ratios of metabolites or as the ratio of each metabolite to the sum of all metabolites (Pᵢtot).
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RESULTS

NCM Mice Do Not Show Cerebral Lesions—As expected, BALB/c mice did not develop any of the neurological signs associated with cerebral malaria. Mice were explored at day 8 post-infection with a parasitemia of 5.8 ± 1.9% (similar to that seen in susceptible mice developing CM). T$_2$-weighted images did not reveal any cerebral lesions in the cerebral parenchyma, in the corpus callosum, or in the periphery of the caudate putamen, a result in sharp contrast with the lesion profile in experimental CM (Fig. 1A).

To assess BBB integrity in infected BALB/c mice, T$_1$-weighted images were acquired before and after the injection of a contrast agent (Gd-DTPA). No signal enhancement attributable to BBB rupture was observed in the group of mice studied at day 8 post-infection (Fig. 1, B and C). In addition, T$_2$*-weighted images did not reveal any hemorrhage or abnormal signal in the brain of NCM mice, whereas petechial hemorrhages are a feature of experimental CM (Fig. 1D).

NCM Mice Present No Sign of Brain Edema—To assess a possible brain swelling in relation to a vasogenic edema, which is characteristic of experimental CM, we performed a volumetric study of different cerebral structures (brain, ventricle, and cerebellum). NCM mice did not present any change in brain volume (control mice: 340 ± 7 mm$^3$; NCM mice: 344 ± 10 mm$^3$, n = 9), ventricle fraction (control mice: 1.6 ± 0.4%; NCM mice: 1.4 ± 0.2%, n = 9), or cerebellar fraction (control mice: 13.3 ± 0.9%; NCM mice: 13.2 ± 1%, n = 9). Cellular edema is a feature of experimental CM, which is associated with a significant and uniform decline in ADC in the whole brain (18). We acquired diffusion-weighted images to determine ADC values (Fig. 1E). ADC values were not different between control and NCM mice in the cortex (control mice: 0.76 ± 0.06 × 10$^{-3}$ mm$^2$/s; NCM mice: 0.71 ± 0.04 × 10$^{-3}$ mm$^2$/s, n = 7) and in the striatum (control mice: 0.75 ± 0.07 × 10$^{-3}$ mm$^2$/s; NCM mice: 0.70 ± 0.03 × 10$^{-3}$ mm$^2$/s, n = 7).

NCM Mice Do Not Have a Cerebral Ischemic Profile—We acquired perfusion-weighted images to measure CBF values (Fig. 2 A). There was no difference in CBF values between con-
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control and NCM mice in the cortex (control mice: 1.2 ± 0.3 ml/g/min; NCM mice: 1.0 ± 0.3 ml/g/min, n = 10 and n = 7, respectively) and in striatum (control mice: 1.3 ± 0.3 ml/g/min; NCM mice: 1.2 ± 0.4 ml/g/min, n = 10 and n = 7, respectively). Moreover, angiograms did not evidence any blood void or compression of the arteries (Fig. 2B), another difference with experimental CM.

Because metabolic disturbances may precede the onset of lesions at MRI, we performed both in vivo 1H and 31P MRS. On the contrary to previous observations made at the onset of the cerebral syndrome in CBA/J mice, the 1H MRS spectra of NCM mice were normal with no lactate, no reduced NAA, and no increase in Glx (Fig. 3 and Tables 1 and 2). High resolution 1H MRS analysis of brain extracts confirmed the in vivo metabolic results obtained for NCM mice with no significant anomaly (data not shown). In addition, in vivo 31P spectra did not reveal any energetic failure. Cerebral pH values were unchanged (control mice: 7.11 ± 0.14; NCM: 7.16 ± 0.07, n = 7).

HP Mice Suffer from Severe Anemia—Parasites keep on proliferating until host death, which is caused by anemia and hyperparasitemia. At day 8, parasitemia was only 5.8 ± 1.9% but reached 66 ± 2% of parasitized red blood cells at day 15 post-infection. We evaluated anemia by total red blood cell count and hemoglobin concentration. The red blood cell number per microliter of blood dropped with parasitemia (control mice: 7.957 × 10^6 ± 2.053 × 10^6; NCM: 6.765 × 10^6 ± 1.231 × 10^6; HP mice: 2.605 × 10^6 ± 8.424 × 10^5; n = 10; Scheffé test: control mice versus HP, p < 0.001; NCM versus HP, p < 0.001) as did the hemoglobin concentration in grams/dl of blood (control mice: 13.87 ± 0.61; NCM: 13.07 ± 0.96; HP mice: 5.12 ± 0.48; n = 10; Scheffé test: control mice versus HP, p < 0.001; NCM versus HP, p < 0.001).

HP Mice Show Moderate Cerebral Swelling—HP mice investigated with T2*, T1*, and T2*-weighted MRI did not show parenchymal lesions, BBB breakdown, or hemorrhages. However, we detected a significant increase in the cerebral volume in HP mice (Fig. 1), but cerebellar and ventricular fractions were normal (brain volume: control mice: 339.7 ± 7.3 mm³; HP mice: 363.0 ± 8.7 mm³ (p < 0.0001), n = 9; ventricle fractions: control mouse: 1.6 ± 0.4%; HP mice: 1.5 ± 0.4%, n = 9; cerebellar fractions: control mouse: 13.3 ± 0.9%; HP mice: 13.4 ± 0.4%, n = 9).

HP Mice Show an Increase in CBF—we measured a significant increase in the CBF values of HP mice in the striatum (control mice: 1.3 ± 0.3 ml/g/min; HP mice: 1.7 ± 0.4 ml/g/min (p < 0.005), n = 10) as well as in the cortex (control mice: 1.2 ±
Brain Metabolism Is Altered in HP Mice—To evaluate metabolic disturbances caused by the presence of high levels of parasite in blood, we performed in vivo cerebral \(^1\)H and \(^31\)P MRS. There was no detectable sign of hypoxia. However, \(^1\)H spectra obtained from a voxel comprising thalamus and striatum at a TR of 16 ms showed a significant decrease of Cho/S, whereas myo-inositol/S was reduced, although this result did not reach statistical significance (Fig. 3 and Table 1). To better understand the variations observed in vivo, we completed the analysis by an in vitro approach. To this aim, we compared whole brain extracts of control and HP mice. High resolution \(^1\)H MRS analysis of brain extracts allowed precise quantification of important metabolites for cerebral function, osmoregulation, and neurotransmission. We observed a significant increase in glutamine, glycine, and alanine concentrations, whereas the rise in lactate did not reach significance. Acetate and aspartate were reduced (Fig. 4 and Table 3) as glutamate, although this latter result was not significant. A Spearman rank correlation test was applied to check whether there was a link between acetate and NAA level or between acetate and aspartate level. The Spearman rank correlation test failed to support any correlation between these metabolites.

All these anomalies were not detectable in vivo due to a lower resolution compared with in vitro analysis. Indeed, glutamine and glutamate signals cannot be distinguished in vivo on our \(^1\)H spectra recorded at 4.7 T. An additional source of variation between in vivo and in vitro results may stem from the fact that in vivo MRS data were obtained from a limited region of the brain essentially containing gray matter structures (mainly striatum and thalamus), whereas in vitro data represented the analysis of whole brain extracts.

We also measured a significant reduction in myo-inositol concentration confirming the in vivo finding. Another important result was the statistically significant decrease in glycerophosphocholine (GPC) concentration, in line with the reduction of choline-containing compounds observed in vivo. Regarding in vivo \(^31\)P MRS results, inorganic phosphate (Pi) appeared to decrease in HP mice, although this difference was not statistically significant (Table 2). Apparently, energetic metabolism was not impaired by the infection, and normal levels of \(\beta\text{-ATP}/P\text{tot}\) and \(\text{PCr}/P\text{tot}\) were found. In addi-
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Hepatic Function Is Significantly Altered in HP Mice but Moderately Affected in NCM Mice—There was an increase in AST and ALT activity in HP mice (Fig. 5A), in line with a significant increase in serum ammonia levels. AST, ALT, and ammonia remained within the normal range in NCM mice (Fig. 5B). Whereas we could not detect γ-GT activity in control mice, we could measure this activity in the sera of HP mice (5.86 ± 1.56 IU/liter). Regarding the level of total bilirubin in the serum, there was a slight but significant increase in NCM mice and a more pronounced increase in HP mice (Fig. 5C). Histological examinations of livers confirmed the presence of hepatic damage in HP mice (Fig. 6). The liver of HP mice was characterized by a strong portal and lobular inflammation. Some eosinophil infiltrates were observed. Pericentrolobular necrotic foci and iron-containing hemosin pigment deposits were also detected (Fig. 6, E and F). NCM mice presented with an intermediate stage, without any malarial pigment deposition but with an inflammation essentially affecting lobules at the level of sinusoids and portal spaces (Fig. 6, C and D).

DISCUSSION

We previously reported the first characterization of cerebral malaria in susceptible mice by using in vivo brain MRI and MRS (13). Our findings herein concur with our previous work (13) in that BBB breakdown, major brain edema, reduced CBF, and dramatic arterial flow perturbations were the crucial events leading to the fatal outcome in experimental CM. Here, we are interested in examining brain response to malaria infection, in the absence of the classic cerebral syndrome.

As expected, NCM mice did not present any of the pathological features of CM previously identified by magnetic resonance techniques, despite parasite loads similar to those found in susceptible mice. Furthermore, their brain was apparently normal when compared with healthy subjects. Regarding HP mice, they showed important brain anomalies, although unrelated to CM pathophysiology. Thus, the MRI and MRS findings reported before in susceptible mice are characteristic of the cerebral syndrome per se and do not mirror any systemic effect resulting from the mere presence of the parasite in blood. However, our data on resistant mice provide the first evidence for significant brain dysfunction as a consequence of hyperparasitemia, anemia, and liver involvement and shed a new light on the pathological processes in malaria.

A major consequence of the elevated parasitemia in CM-resistant mice was anemia. Indeed, an important drop in hemoglobin concentration and in red blood cell count was observed in HP mice, which both point to severe anemia (19). In humans, the level of parasitemia is generally lower than in mice infected with PbaA. However, percentages of parasitized red blood cells in circulating blood greater than 10% (20), up to 45% in children (21), and to 80% in adults (22) have been reported. Therefore, HP mice may be seen as a model of the hyperparasitemia occurring in some cases of the human disease. We found that the important blood parasite load and the ensuing alteration of hematological parameters produced notable hemodynamic alteration in the brain of HP mice, characterized by an unexpected elevation of CBF. It is well known that vasoregulatory mechanisms modulate CBF to maintain adequate levels of oxygen and nutrients. An adaptation of these mechanisms has been reported in various types of anemia and blood pathologies. In animal models of acute normovolemic anemia, and hemodilutional anemia, CBF increased in proportion to the reduction of hemoglobin content, and nitric oxide synthase up-regulation, respectively (23, 24). Similarly, CBF was augmented in patients with uncomplicated sickle cell disease as a result from low hemato-crit and compensatory vasodilation (25). Thus, the increase in

**TABLE 3**

| Assignment | Metabolites   | CTL mice (n = 9) | HP mice (n = 9) |
|------------|--------------|-----------------|-----------------|
|            | mmol/g of fresh tissue (mean ± S.D.) |                |                |
| 1          | Lactate      | 2.82 ± 0.42     | 2.37 ± 0.99     |
| 2          | Alanine      | 0.25 ± 0.07     | 0.43 ± 0.17     |
| 3          | γ-Aminobutyric acid | 1.30 ± 0.21 | 1.27 ± 0.31     |
| 4          | Acetate      | 0.11 ± 0.04     | 0.06 ± 0.02     |
| 5          | NAA          | 3.37 ± 0.44     | 3.06 ± 0.56     |
| 6          | Glutamate    | 3.41 ± 0.64     | 3.15 ± 0.60     |
| 7          | Glutamine    | 1.13 ± 0.22     | 0.89 ± 1.73     |
| 8          | Pyruvate     | 0.03 ± 0.01     | 0.02 ± 0.01     |
| 9          | Succinate    | 0.19 ± 0.03     | 0.19 ± 0.05     |
| 10         | Aspartate    | 0.73 ± 0.11     | 0.53 ± 0.11     |
| 11         | Creatine     | 3.47 ± 0.47     | 3.70 ± 0.68     |
| 12         | Choline      | 0.07 ± 0.04     | 0.07 ± 0.04     |
| 13         | Phosphocholine | 0.79 ± 0.11 | 0.94 ± 0.22     |
| 14         | GPC          | 1.83 ± 0.28     | 1.46 ± 0.30     |
| 15         | Taurol       | 2.20 ± 0.69     | 2.66 ± 0.97     |
| 16         | myo-Inositol | 2.10 ± 0.35     | 1.28 ± 0.29     |
| 17         | scylo-Inositol| 0.05 ± 0.04     | 0.07 ± 0.13     |
| 18         | Glycine      | 0.34 ± 0.12     | 0.51 ± 0.23     |
| 19         | Betaine      | 0.25 ± 0.04     | 0.24 ± 0.08     |

*p < 0.05.

*p < 0.001.

*p < 0.0001.
CBF appears as a common regulatory process in various types of anemia. Surprisingly, we did not observe any ischemic lesion and/or metabolic profile in HP mice that one would expect in such cases given the alteration of red blood cells. This result indicates that the increase in CBF may be sufficient, temporarily at least, to avoid oxygen supply default. However, MR angiography was not able to detect vasodilation in larger brain arteries. Anatomical images showed a small increase in cerebral volume, apparently unrelated to any vasogenic edema, because the BBB was found intact. This brain swelling of unknown origin might partly result from capillary vasodilation as part of a regulatory mechanism to maintain sufficient oxygen delivery. This question needs further investigations.

Another complication detected in hyperparasitized mice was liver injury. Indeed, all our biochemical and histological data on liver function confirm this finding. The increases in serum ammonia in AST, ALT, and γ-GT activities in HP mice are indicative of hepatic failure. Along this line, the large increase in total bilirubin concentration detected in HP mice could originate not only from red blood cell destruction but also from hepatic dysfunction and inefficient elimination of blood bilirubin. Our histological findings confirmed liver involvement in HP mice with the presence of hemozoin deposits, inflammation, and necrosis. Actually, the infection induces liver injury (14) via the alteration in blood flow through the organ as parasitized red blood cells adhere to endothelial cells, blocking the sinusoids and obstructing the intrahepatic vessels (26).

The presence of hemozoin, the malaria pigment, in the liver of infected mice may have major adverse effects and is probably involved in local inflammation and necrosis. Extensive deposition of hemozoin and parasitized red blood cells in liver has been reported in murine models of malaria obtained with blood stage parasites (27, 28). Hemozoin is formed during the asexual intra-erythrocytic development of the parasite and is a crystalline by-product of hemoglobin degradation by the parasite (29). There is growing evidence suggesting that hemozoin modulates the host immune response to malaria. Indeed, after parasitized red blood cell rupture, hemozoin is engulfed by macrophages, endothelial cells, or platelets. Hemozoin impairs phorbol ester-stimulated oxidative burst, killing of ingested bacteria, fungi, or tumor cells, and response to interferon γ stimulation (30). Moreover, cytokine production is altered, and an increase in immunosuppressive molecules occurs (30). Indeed, hemozoin is a potent catalyst of lipid peroxidation that leads to the production of hydroxylated fatty acids such as 4-hydroxy-2-nonenal and 15-S-hydroxyeicosatetraenoic acid (31, 32). Both these compounds may significantly impair macrophage function. 4-Hydroxy-2-nonenal leads to major cellular anomalies, including damage to the cytoskeleton and mitochondria and inhibition of DNA, RNA, and protein synthesis. It also stimulates chemotaxis and modulates platelet aggregation. Likewise, 15-S-hydroxyeicosatetraenoic acid may induce altered permeability of endothelial cells, edema, and chemotaxis (32).

In murine models of malaria obtained with blood stage parasites, large numbers of infected red blood cells pass through the liver (14). It was found that hemozoin increased the level of a pro-inflammatory mediator termed as macrophage migration inhibitory factor in inflammatory cells within the lumen of liver vessels, hepatocytes, endothelium, and Kupffer cells, the macrophages residing in the sinusoids (27).

In addition to the local production of pro-inflammatory cytokines, other mechanisms may contribute to liver dysfunction and damage as the decrease in hepatic mono-oxygenase activity, including microsomal and mitochondrial cytochrome P-450 (33, 34). Interestingly, NCM mice, which showed moderate signs of liver damage and dysfunction and a slight but significant increase in bilirubin, did not present any cerebral metabolic alteration at MRS.

Our report on brain metabolic impairment in hyperparasitized mice resistant to cerebral malaria is a new finding that may have multiple causes. On the one hand, in CM-resistant mice, the parasite load becomes extremely important within days (66% of parasitized red blood cells at day 15 post-infection), which represents a burden for the host. This rapid proliferation requires an active production of new membranes, with phosphatidylcholine being the most abundant phospholipid in parasite membranes (35). To proliferate, *Plasmodium* takes up choline from the external medium for *de novo* synthesis of phosphatidylcholine (36). An important metabolic result of the current study is the finding of a decrease in cerebral choline-containing compounds, which are involved in membrane and myelin synthesis and degradation, in HP mice. This alteration was attributed to a reduction in GPC on the basis of the analysis of cerebral extracts. GPC can be degraded to free choline that is able to cross the BBB (37). Thus, the decrease in brain GPC could result from a compensatory mechanism to replenish blood choline used by the parasite to proliferate, because choline concentration in brain closely parallels choline fluctuations in plasma (38).

On the other hand, a striking feature of the cerebral metabolic pattern of HP mice was the concomitant increase in glu-
tamine and reduction in myo-inositol, characteristic of hepatic encephalopathy (39, 40). Hepatic encephalopathy is a neuropsychiatric syndrome, which is associated with acute liver failure or certain chronic liver diseases such as cirrhosis. In acute liver dysfunction, hepatic encephalopathy is characterized by astrocyte swelling, brain edema, and intracranial hypertension that may ultimately lead to coma and death. In chronic diseases, gray matter astrocytes show the morphological features of Alzheimer type II astrocytosis, with anomalies of the nucleus, chromatin, and cytoplasm and altered expression of glutamate transporters, peripheral-type benzodiazepine receptors, and glial acidic fibrillary protein. The neuropsychiatric features develop slowly and involve psychomotor dysfunction, concentration difficulties, or sensory anomalies but may progress toward stupor and coma (41, 42).

Liver failure is associated with a rise in blood ammonia that leads in turn to an increase in brain ammonia, a crucial event leading to the pathogenesis of hepatic encephalopathy (41, 42). Ammonia produced by the degradation of amino acids in liver, kidney, and gut is detoxified in the liver. Two ammonia-scavenging systems, involving urea and glutamine synthesis, have been identified in the liver. Urea synthesis is present in perivascular hepatocytes, whereas glutamine synthetase is found only in perivenous hepatocytes (43). The development of hyperammonemia in acute or chronic hepatic diseases has been ascribed to several mechanisms, including urea cycle deficits and destruction of perivenous hepatocytes containing glutamine synthetase (43).

In brain, which is devoid of a urea cycle, ammonia is detoxified by astrocytes, essentially through the formation of amino acids (42, 44). In particular, ammonia is eliminated via theamination of glutamate to form glutamine. The 3-fold increase in cerebral glutamine in HP mice is consistent with the high serum ammonia concentration found in these mice. An alternative detoxification pathway for ammonia in brain involves alanine synthesis via the transamination of pyruvate to alanine (45–47). This amino acid was found augmented in hyperammonemia, and fulminant hepatic failure, and its level correlated to the degree of neurological dysfunction (48, 49). As a matter of fact, we observed a 2-fold increase in alanine concentration in the brain of HP mice, a finding that further substantiates the mechanism of increased ammonia detoxification. The decrease of myo-inositol, an astrocytic osmolyte, is generally viewed as a compensatory mechanism against the increased intracellular osmolarity evoked by glutamine accumulation (39), which may adversely affect brain function by eliciting astrocyte swelling. Along this line, the decrease in cerebral choline observed in HP mice could also partly stem from an osmoregulatory mechanism. Indeed, reduced brain choline content has been reported in hepatic encephalopathy (50) and was accounted for by the osmolyte role of certain choline derivative compounds.

Besides glutamine and alanine, other amino acids are implicated in the pathogenesis of hepatic encephalopathy (51). For example, reduced amounts of cerebral excitatory amino acids (glutamate and aspartate) have been reported (52, 53). Moreover, the extracellular concentration of brain glycine was found increased in animal models of hepatic encephalopathy, particularly at the coma stage (51, 54). This increase in glycine was accompanied by an alteration of the expression of glycine transporter Glyt-1 (54). In HP mice, we also found a significant decrease in brain aspartate and an elevation in total cerebral glycine concentration accompanying liver damage.

The origin of the decrease in acetate in brain is not clear but was not linked to NAA metabolism. The decrease in acetate could presumably reflect liver dysfunction, because this compound is essentially produced within the liver.

The metabolic profile identified in HP mice is characteristic of hepatic encephalopathy, and undoubtedly confirms increased ammonia elimination. The resulting accumulation of certain amino acids could in turn be detrimental to brain function. The rise in extracellular glycine, which is a positive allosteric modulator of N-methyl-D-aspartate receptors, combined to a reduced expression of glycine transporter Glyt-1, could impair glutamatergic neurotransmission via excessive N-methyl-D-aspartate activation (54). On the other hand, high glutamine concentration may induce astrocytic swelling if not efficiently buffered by astrocytic release of myo-inositol. Moreover, it was recently shown that cerebral glutamine and myo-inositol concentrations were correlated to the neuropsychological deterioration in patients with cirrhosis (55).

In conclusion, the results of the current study indicate that hyperparasitemia is associated with impaired cerebral choline metabolism, a crucial compound for membrane and myelin synthesis in brain, whereas anemia induces an alteration of cerebral hemodynamics. Furthermore, our findings reveal that hepatic encephalopathy is an important feature of experimental malaria. We did not detect astrocytic cellular edema, which is typical of acute liver failure but not of chronic liver failure (46). This result suggests that the hepatic damage at the time of our investigations was not yet critical and, rather, point to a chronic liver disease. One could assume that liver manifestations would have been more acute provided the parasite life cycle had included a hepatocyte stage as in human malaria. Our finding of a hepatic encephalopathy is relevant to the understanding of the human disease, because hepatocellular dysfunction ranging from conjugated hyperbilirubinemia, with or without mild elevation in transaminases, to fulminant hepatic failure has been described in P. falciparum malaria (56, 57). Therefore, subjects with malaria are at risk to develop a hepatic encephalopathy. Our results highlight the vulnerability of brain to malaria even in the absence of the classic cerebral syndrome and clearly demonstrate that cerebral MRS is a powerful technique of great potential value to diagnose brain dysfunction in infectious diseases.

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