Short Communication

In the Eye of Experimental Cerebral Malaria

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Cerebral malaria is the most severe complication of *Plasmodium falciparum* infection, accounting for 1 million deaths per year. We characterized the murine disease using *in vivo* magnetic resonance imaging (MRI) at 4.7 T, proving that ischemic edema is responsible for fatality. The aim of the present study was to identify early markers of experimental cerebral malaria using very high field conventional MRI (11.75 T). CBA/J mice infected with *Plasmodium berghei* ANKA were observed at an early stage of the disease, before the onset of detectable brain swelling and at the most acute stage of cerebral malaria. Herein, we report the first detection of damage to the optic and trigeminal nerves on T₂-weighted MRI. The trigeminal nerves appeared hypointense, with significantly reduced diameter and cross-sectional area. The optic nerves were hypointense and often not visible. In addition, the internerve distance between the optic nerves was significantly and progressively reduced between the early and severest stages. Cranial nerve injury was the earliest anatomic hallmark of the disease, visible before brain edema became detectable. Thus, cranial nerve damage may manifest in neurologic signs, which may assist in the early recognition of cerebral malaria. (Am J Pathol 2011, 179:1104–1109; DOI: 10.1016/j.ajpath.2011.05.044)

Malaria is a major disease of the developing world, and cerebral malaria (CM) is the most lethal complication of infection with *Plasmodium falciparum*, with an estimated death toll of approximately 1 million in 2008.¹ In sub-Saharan Africa, children younger than 5 years account for more than 80% of malaria-related deaths, and 10% to 20% of those surviving an episode of CM develop long-term cognitive impairment.^{2,3} Magnetic resonance imaging (MRI) is a powerful tool for the diagnosis and follow-up of central nervous system disease but also for the

understanding of pathophysiologic processes. However, CM radiologic reports are sparse, and findings are inconsistent among patients. The small number of experimental CM (ECM) imaging studies^{4–7} conducted have identified hallmarks of the disease at its most acute stage and at an earlier stage already characterized by significant brain swelling.⁴ However, the field lacks a thorough and extensive account of early MRI-detectable markers of CM visible before the onset of cerebral edema. It is essential, therefore, that early sensitive indices of disease be identified. The use of MRI in a clinically relevant animal model is an approach that allows investigation of the acute effects of parasitic infection and host immune response on the brain. Previously, we performed the first *in vivo* MRI studies of ECM-susceptible or ECM-resistant mice infected with *Plasmodium berghei* ANKA (PbA) at 4.7 T.^{4,8} We showed that ECM is characterized by blood-brain barrier breakdown, hemorrhages, reduced brain perfusion, ischemia, hemodynamic dysfunction, and brain edema. We identified that a potential cause of death in CM was compression of the cerebral arteries by a swollen cerebrum, resulting in a severe reduction in cerebral blood flow. Herein, we present an anatomic imaging study of PbA-induced ECM lesions. We show that at higher field (11.75 T), using conventional MRI, it is possible to detect pathologic changes at the level of the cranial nerves, and we present a comprehensive MRI-visible characterization of the ECM model, identifying potentially clinically relevant markers of human CM.

Materials and Methods

Mice

Female CBA/J mice (8 to 10 weeks old) from Harlan (Venray, The Netherlands) were maintained at 23°C to 25°C with a 12-hour light/dark cycle with free access to food and water. All the animal experiments were in agreement with

Supported by the French National Center for Scientific Research and a Centre National de la Recherche Scientifique postdoctoral fellowship (R.S.).

Accepted for publication May 26, 2011.

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the French guidelines for animal care and were approved by the Marseille Medical School Committee for Animal Ethics.

Parasite Infection and Disease Monitoring

Twenty-one mice were used: 14 were infected with PbA by i.p. injection of 10^6 parasitized red blood cells⁹ and 7 were kept as controls (CTs). Parasitemia was assessed on days 4 and 7 after infection by Giemsa staining of blood smears. Animals were monitored daily for clinical signs of CM. Two stages of clinical manifestation were selected for this study depending on the time of examination after infection. The animals were explored at an early stage of the disease (day 4 to 5 after infection, referred as early CM; $n = 14$). Because mice within a cohort do not develop the disease exactly at the same pace (there is often a delay of several days in the time of death of the animals), we chose to group animals presenting the same noticeable early clinical signs of the cerebral syndrome in the CBA strain (ie, ruffled fur, closing eyes, first manifestation of lethargy, and rare seizures but no hypothermia, ataxia, hemiplegia or paraplegia, or coma). In the present cohort, this stage was reached at day 4 (2 mice) or at the end of day 5 (12 mice). Three of these mice were reexamined at the most acute stage (day 7 to 8 after infection, referred as severe CM). All animals infected with PbA died of the disease within 7 to 10 days of the injection of parasitized red blood cells. Early CM mice displayed ruffled fur, closed eyes, and lethargy, whereas severe CM mice exhibited, in addition, hypothermia, redness of the ears, paraparesis, ataxia, hemiplegia or paraplegia, seizures, prostration, and, ultimately, coma.

In Vivo MRI Protocol

Mice were explored using an 11.75-T vertical Bruker AVANCE 500 WB wide-bore MRI system (Bruker, Ettlingen, Germany) with a transmitting and receiving head resonator of 2.5 mm. Animals were anesthetized by i.p. injection of ketamine (50 to 100 mg/kg body weight) and xylazine (10 to 20 mg/kg body weight). Mice were positioned in a cradle equipped with a stereotaxic holder and a pressure probe to monitor the respiratory rate. Body temperature was maintained at 37°C using the magnet gradients. T_2 -weighted images were acquired in the axial and sagittal planes using a spin-echo sequence (echo time, 37.1 milliseconds; repetition time, 5000 milliseconds; rapid acquisition with relaxation-enhanced factor 8; 2 averages). Geometric parameters for T_2 -weighted images were as follows: 30 contiguous 0.5-mm-thick slices; matrix, 256×256 ; and field of view, 400 mm². T_1 -weighted images were acquired in the axial plane using a three-dimensional gradient echo with a first-order flow compensation sequence with strong T_2^* -weighting (echo time, 5 milliseconds; repetition time, 30 milliseconds; field of view, 8000 mm³; matrix, $256 \times 256 \times 64$).

In Vivo MRI Data Processing and Analysis

All MRI data were processed using ImageJ software (NIH).¹⁰ Brain volume was calculated from T_2 -weighted

images. The optic nerves were identified at the chiasma and the optic tracts at different postchiasma levels on axial T_2 -weighted images. The horizontal distance (internerve distance) between each optic nerve was measured in each animal at the level of the chiasma. Trigeminal nerve damage was assessed by measuring the vertical and horizontal axes and the cross-sectional area on selected axial T_2 -weighted images. T_1 -weighted images with strong T_2^* weighting were used to identify hemorrhages.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad Software Inc., San Diego, CA). Values are reported as mean $\pm$ SD. A nonparametric analysis was used because all the values tested were not from normal distributions (d'Agostino and Pearson omnibus normality test). Nerve diameters and cross sections were compared between the left and right hemispheres using the Wilcoxon matched pairs test. The internerve distance was compared between the CT and early CM groups using the Mann-Whitney *U*-test. Cerebral volume was compared among the three groups using the Kruskal-Wallis test followed by Dunn's post hoc test. Values of $P < 0.05$ were considered significant.

Results

Characterization of Cerebral Lesions in Early CM and Severe CM Mice

Conventional MRI was performed on CT animals ($n = 7$) and throughout the evolution of CM in early CM mice (day 4 to 5 after infection, $n = 14$) and severe CM mice (day 7 to 8 after infection, $n = 3$). As previously described,⁴ severe CM was associated with massive brain edema and focal parenchymal lesions that were detected as hyperintensities or hypointensities on T_2 -weighted images (Figure 1 A–D). Alteration of the external and internal capsules (Figure 1, A and D) and cerebral hemorrhages (Figure 1E) were detected in all cases of severe CM. Severe CM animals ($n = 3$) exhibited significant brain swelling compared with CT and early CM animals (mean $\pm$ SD: CT mice, 348.8 ± 12.64 mm³; early CM mice, 353 ± 24.14 mm³; and severe CM mice, 453.6 ± 6.044 mm³; Kruskal-Wallis test: $P = 0.0224$; Dunn's test: CT mice versus severe CM mice, $P < 0.05$; early CM mice versus severe CM mice, $P < 0.05$) (Figure 1C). This swelling was accompanied by protrusion of the brainstem into the foramen magnum, crushing the cerebellum and the lateral and third ventricles in all severe CM animals (Figure 1, A and B).

Hyperintense parenchymal lesions in the caudate putamen, corpus callosum, and external capsule were detected on sagittal and axial T_2 -weighted images in severe CM animals⁴ but not in early CM mice (Figure 1, A and B). In addition, hyperintensities in the piriform cortex and in the olfactory bulbs were observed in severe CM animals (Figure 1, A and B). These focal lesions were accompanied by whitening of the striatal border and hypointensity of the optic

Figure 1. Typical brain T₂-weighted images of CT and CM mice. **A:** Sagittal T₂-weighted images of CT, early CM, and severe CM brains. Note the brain swelling, crushing of the cerebellum, and hyperintensity of the corpus callosum, indicating edema and demyelination in the severe CM mouse. In addition, focal hyperintensities were detected in the cortex (cx), olfactory bulb (ob), and optic chiasma (ox). The thalamus (tha) exhibited loss of delineation, whereas the hippocampus showed a reduction in contrast. **Arrowheads** point to changes in T₂-contrast. bst, brainstem; Cb, cerebellum; cc, corpus callosum. **B:** Axial T₂-weighted images showing changes in contrast in the corpus callosum (cc), caudate putamen (CPU), external capsule (ec), internal capsule (ic), optic tracts (opt), piriform cortex, and trigeminal nerve (5n) in early CM and severe CM animals compared with the CT mouse. **Arrowheads** point to changes in T₂-contrast. ac, anterior commissure. **C:** Histogram showing mean cerebral volume of CT, early CM, and severe CM brains calculated from T₂-weighted images. Brain volume is significantly increased in severe CM animals compared with in CT and early CM mice (Kruskal-Wallis and Dunn's post hoc tests). Statistical significance for the post hoc test is indicated by **P* < 0.05. Error bars indicate SD. **D:** T₂-weighted features of early and severe CM. **E:** Typical brain T₁-weighted images of severe CM mice showing petechiae. Discrete hypointensities (**arrows**) correspond to focal hemorrhages. Note the extensive lesions in the striatum and cortex. Scale bar = 1 mm.

area on T₂-weighted images (Figure 1B). In addition, loss of delineation of the thalamus and hypothalamus was visible on sagittal T₂-weighted images in all severe CM mice but not in early CM mice (Figure 1A). However, on axial T₂-weighted images, the thalamus and the internal capsule appeared hypointense in early CM and severe CM mice, although more pronounced in the latter (Figure 1B). In addition, the optic tracts appeared more hypointense in severe CM mice (Figure 1B). Discrete focal hemorrhages were visible on T₁-weighted images throughout the cerebrum, which extended into the cerebellum and brainstem in severe CM mice but not in early CM mice (Figure 1E).

Cranial Nerve Damage Is the Earliest Anatomic Hallmark of ECM Observable Using Conventional MRI

At high field, damage to the visual system was easily detectable on T₂-weighted images in CM animals (Figure

2). The optic nerves and optic tracts were examined at the chiasmatic and postchiasmatic levels, respectively, in early CM and severe CM mice (Figure 2). Early CM and severe CM mice showed alteration of the optic nerves and tracts. Damage to the visual system was characterized by loss of visibility of the chiasma and hypointensity of the optic nerves and tracts on axial T₂-weighted images (Figure 2A). The change in contrast was such that the optic nerve could not be detected at the chiasma in most severe CM animals (Figure 2B). The optic tracts were more hypointense in all severe CM animals (Figure 1B) compared with in CTs. In addition, early CM animals exhibited a significant reduction in the internode distance at the chiasmatic level (*P* = 0.0043) (Figure 2E), but the optic tracts could be detected at the postchiasmatic level in all the animals.

In addition to the damage to the optic nerves, ECM induced early MRI-visible changes in the structure of the trigeminal nerves (Figure 3A). The trigeminal nerves ap-

peared crushed and hypointense in CM animals on T_2 -weighted images. The vertical and horizontal axes of the trigeminal nerve and the cross-sectional area were examined in CT, early CM, and severe CM animals at different anatomic levels on T_2 -weighted images (Figure 3, B–D). The vertical axis and surface area were significantly reduced in early CM mice compared with CTs; severe CM mice exhibited a further reduction in the vertical axis and area of the trigeminal nerve compared with early CM mice.

Discussion

Improved knowledge of brain lesion formation at the initial stage of CM is essential for the understanding of pathogenic mechanisms and for the early detection of subtle

Figure 2. Morphologic changes of the optic nerve in CM. **A:** Axial T_2 -weighted imaging of the optic nerves (2n) in CT, early CM, and severe CM animals. Note the loss of visibility of the chiasmatic region (ox) and the reduction of the internerve distance in early CM mice. Optic nerves at the chiasma level are undetectable in the severe CM mouse. Scale bar = 1 mm. **B:** Percentage of subjects in which the optic nerve is clearly visible (+), partly visible (+/-), or not visible (-) at the chiasma on T_2 -weighted images. **C:** Mean internerve distance between the two optic nerves in CT and early CM animals at the chiasma. Optic nerves are not visible in severe CM mice. The Mann-Whitney U -test showed significantly reduced internerve distance in early CM animals. Error bars indicate SD. Statistical significance is indicated by $*P < 0.01$.

Figure 3. Morphologic changes of the trigeminal nerve in CM. **A:** Axial T_2 -weighted imaging of the trigeminal nerves (5n) in CT, early CM, and severe CM animals. Note the hypointensity and the crushing of the trigeminal nerve in early CM and severe CM mice. Scale bar = 1 mm. **B:** Histogram representing the mean vertical diameter of the trigeminal nerve in CT and CM mice. Kruskal-Wallis followed by Dunn's post hoc tests showed a significantly reduced diameter in severe CM mice compared with CT animals in both hemispheres and a significant reduction in the right hemisphere in early CM mice. Error bars indicate SD. Statistical significance for the post hoc test is indicated by $*P < 0.05$, $**P < 0.01$. **C:** Histogram representing the mean horizontal diameter of the trigeminal nerve in CT and CM mice. Kruskal-Wallis tests did not reveal any differences in diameter among all the groups. Error bars indicate SD. **D:** Histogram representing the mean cross-sectional area of the trigeminal nerve. Kruskal-Wallis followed by Dunn's post hoc tests showed a significantly reduced surface area in severe CM compared with CT animals. Error bars indicate 1 SD. Statistical significance for the post hoc test is indicated by $**P < 0.01$.

neurologic signs that may help in disease diagnosis. In this study, we sought to identify early MRI-detectable lesions in ECM to produce a more complete characterization of lesion distribution and evolution. Clinical imaging findings in human CM are limited. Computed tomography scans have been shown to underestimate the extent of disease at pathologic examination,¹¹ with normal computed tomography scan findings being common.^{11–13} A paucity of MRI investigations in patients with CM have reported increased brain volume,^{14,15} diffuse cerebral edema,^{15,16} and herniation through the foramen magnum¹⁴ associated with either ventriculomegaly or crushing of the lateral ventricles.¹⁷ Parenchymal lesions assessed by T₂-weighted imaging consisted in focal hyperintensities in several white matter tracts, including the corpus callosum, indicative of vasogenic edema, gliosis, or cell death.^{16,18} T₁-weighted imaging allowed the detection of focal hemorrhages, a hallmark of the disease, and infarction in some patients^{14–16,19} at the cortical and subcortical levels, believed to be a consequence of parasitized erythrocytes obstructing capillaries.^{15,16} More recently, susceptibility-weighted imaging²⁰ has proved more sensitive in the detection of petechiae and seems to correlate better with postmortem findings of diffuse petechial hemorrhages.¹⁷

We previously demonstrated that MRI provides specific and reliable markers of ECM that could help decipher some of the pathogenic processes underlying this cerebral syndrome.⁴ In the present study, we confirmed the lesion pattern associated with massive brain edema in severe CM mice consisting of crushing of the brainstem and cerebellum, and focal lesions in the caudate putamen and the white matter (external capsule, internal capsule, and corpus callosum). We also showed that at 11.75 T, superior resolution and better localization of brain hemorrhages are obtained in mice than at 4.7 T,⁴ which is comparable with what has been obtained in the few human cases explored at a clinical magnetic field.¹⁷ In addition, we report on a variety of novel MRI-visible features of ECM. In the gray matter of the forebrain, T₂-weighted changes were detected in the olfactory system, cortex, thalamus, and hypothalamus. Focal T₂-weighted hyperintensities were visible in the olfactory bulbs and diffuse T₂-weighted hyperintensities were observed in the piriform cortex, whereas the thalamus showed a loss of delineation. To our knowledge, the MRI finding of thalamic abnormality is the first such description in the murine model. T₂-weighted hyperintensity of the thalami has previously been reported in two patients,^{18,21} although thalamic lesions were first detected on computed tomography.¹¹

For the first time in the murine model, we detected pathologic changes in the cranial nerves: optic nerve and trigeminal nerve. Damage to the cranial nerves seems to be the earliest anatomic hallmark of the disease and is visible before brain edema becomes detectable. The diagnostic importance of the visual system in patients with CM has recently been highlighted,²² and the optic nerve has been proposed as an important diagnostic marker for patient classification, risk assessment, and monitoring of CM.²³ White and colleagues²⁴ specifically showed that

although the detection of hemorrhages *per se* in the retina is not a clear predictor of outcome in patients with CM, the number and severity of hemorrhages correlate with morbidity. Ma and colleagues,²⁵ who were the first to examine the pathologic abnormalities of the optic nerves in murine CM, demonstrated loss of axonal viability in this disease in any central nervous system tissue, which corroborated with reports of demyelination in human CM. They showed that although patchy demyelination and scattered axonal degeneration were visible in the optic nerves initially, by day 7 after PbA infection, there was an increase in the number of demyelinated axons and degenerated axons. In the present study, these axonal changes associated with inflammation²⁵ and ischemia⁴ may be responsible for the complete loss of MRI visibility of the optic nerves in severe CM animals on day 7 after infection and the alteration of the optic tracts in severe CM animals. The optic nerves of early CM animals showed a clear reduction in the internerve distance. Recently, a study using ultrasound detected increased optic nerve sheath diameter in 100% of patients diagnosed as having CM.²³ We, therefore, propose that the internerve distance observed in early CM animals may be due to local edema at the level of the optic nerve. In addition to the optic nerve damage, other components of the visual system were affected in CM animals. The thalamus, an area that receives axons, including those from retinal ganglion cells transmitting visual information, showed a marked loss in delineation on T₂-weighted imaging in CM animals. Furthermore, white matter T₂-weighted changes were detected at the level of the optic nerve, optic chiasma, and optic tract. Clinically, it is well-known that lesions anterior to the optic chiasma, at the level of the chiasma, or posterior to the chiasma may cause visual defects. The MRI changes of the optic nerves do not necessarily translate as irreversible damage and loss of vision, but it is likely that the severe CM animals in whom the optic nerves and tracts were altered have impaired visual acuity. In a recent study examining sensorimotor function and behavior in ECM mice, loss of visual acuity was detected 36 hours before death.²⁶

The trigeminal nerve is a mixed cranial nerve with three sensory branches: the ophthalmic branch, which transmits sensory information from the retina; the maxillary branch, which carries sensation from the eyelids, cheeks, and lips; and the mandibular branch from the mandibula and lips. In this study, we measured a reduction in the diameter of the trigeminal nerve, which may be a consequence of axonal degeneration and/or demyelination, as has been shown in the optic nerves.²⁵ Brain edema that may lead to nerve compression possibly plays a role in nerve injury at the most acute stage of the disease, when brain swelling is significant. The MRI findings on the trigeminal nerve may correspond to behavioral changes in CM animals, such as half-open or closed eyelids, a common observation in CM mice; in humans, trigeminal neuralgia is known to induce intense facial pain. The present findings on the optic and trigeminal nerves suggest that assessment of cranial nerve damage should receive particular attention when applying clinical or behavioral tests to evaluate cerebral dysfunction in CM. In

addition, these changes in the visual system highlight another shared pathologic feature of the murine model and the human disease.²⁷

In conclusion, the present results demonstrate that the earliest MRI hallmark of ECM is cranial nerve damage, which may be noninvasively assessed using conventional MRI without the need for sophisticated contrast agents. It is possible that cranial nerve damage may translate to the manifestation of neurologic signs, which may assist in the early recognition of CM. These novel MRI findings may help improve our understanding of the pathogenesis of CM and provide additional markers for the evaluation of new therapeutic strategies.

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