

**Imaging Inflammation: Direct Visualization of Perivascular Cuffing in EAE by Magnetic Resonance Microscopy**

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**Purpose:** To determine if the architectural features revealed by magnetic resonance microscopy (MRM) allow one to detect microscopic abnormalities associated with neuroinflammation in fixed brain sections from animals with experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis (MS).

**Materials and Methods:** Imaging was performed at the Center for In Vivo Microscopy (CIVM) using a 9.4-Tesla, 89-mm bore, superconducting magnet with actively shielded gradients capable of 850 mT/m. A number of MR contrasts and spatial resolutions were explored.

**Results:** The assessment of EAE brain showed that it is possible to visualize perivascular cuffing in vitro by MRM on three-dimensional T1 proton stains.

**Conclusion:** Inflammatory cell infiltration is a prerequisite for the development of lesions in EAE and MS. Thus, the ability to directly detect individual perivascular cuffs of inflammation may provide a useful means of monitoring the time course of inflammatory events, as conventional histopathological scoring of perivascular cuffs is utilized, but in the absence of sectioning and staining.

**Key Words:** magnetic resonance imaging; multiple sclerosis; magnetic resonance microscopy; experimental allergic encephalomyelitis; perivascular cuffing; inflammation


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MULTIPLE SCLEROSIS (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) (1). The symptoms of MS vary widely depending on the pathological nature and the location of lesions within the CNS. Acute MS lesions display edema, demyelination, and inflammation consisting of perivascular lymphocyte and macrophage infiltration. Chronic plaques may be completely demyelinated with a variable degree of axonal loss, and may show variable degrees of remyelination (2). Experimental allergic encephalomyelitis (EAE) is a widely used animal model for studying the pathology of MS. EAE encompasses a group of CNS inflammatory/autoimmune diseases in various laboratory animals, including the mouse, rat, guinea pig, primate, and marmoset (3).

Magnetic resonance imaging (MRI) studies have had a major impact on the understanding of the natural history of MS. MRI is a predictive measure of the future development of MS, and an effective method of monitoring the progression of the disease. Conventional MRI easily detects focal white matter (WM) lesions in MS patients. Although not specific, there are characteristic MRI findings in MS. Patients usually exhibit multiple lesions, with high signal intensity (SI) on both proton density (PD)- and T2-weighted images, that are preferentially located in close proximity to the lateral ventricles (4). Lesion burden, defined as the total lesion volume measured from MR images covering the entire brain, is considered the most complete currently available MR-based quantitative measure of disease status (5).

Demyelination and axonal loss are said to be the key pathological substrates of functional impairment in MS. Several quantitative MRI methods have been proposed to measure these pathologic features, including magnetization transfer imaging (6–8), multiexponential T2 relaxation decay analysis (9,10), and MR spectroscopy (11).

Most evidence indicates that blood-brain barrier disruption (BBB), which is associated with marked inflammation, is the initial event in the development of the lesion in MS (12,13). Gadolinium (Gd) enhancement of T1-weighted images correlates well with inflammation in EAE and in MS (14,15). Gd-enhanced MRI is commonly used, in a qualitative or semi-quantitative fashion, to monitor disease activity in MS patients. There are actually few reports of quantitative MRI assess-
mments of inflammation. Morrissey et al (16) quantitatively assessed the integrity of the BBB using T1 measurements in a rodent EAE model and showed that inflammatory cell infiltration and edema could be distinguished in the early phase of the disease.

Currently, the most common method for assessing the extent of inflammation in EAE or the effectiveness of various therapeutic strategies that inhibit inflammation involves a histopathologic evaluation of multiple tissue sections. An early indication of inflammation can be seen as perivascular cuffs of infiltrating inflammatory cells (T cells and macrophages), primarily in WM regions of the brain tissue (17). Pathologic grading systems are frequently used to estimate the extent of this perivascular cuffing using a cellular stain, such as hematoxylin and eosin (H&E), typically scoring sections on a scale of 0 to 3, reflecting normal to severely inflamed tissue (18).

MR microscopy (MRM) theoretically provides the spatial resolution necessary to visualize microscopic pathologic features associated with EAE (19,20). In 1993, Johnson et al (21) coined the term “proton stain” to describe an ultra-high resolution MR image with high contrast- and signal-to-noise ratios (SNR) that showed microscopic detail comparable to conventional histological stains. This technique has the capacity to allow the direct visualization of features of inflammation in EAE, such as perivascular cuffing. Visualization of inflammation in EAE may provide exciting new information about events involved in the early stages of lesion development. The primary objective of this study was to determine if the architectural features revealed by MRM allow one to detect microscopic abnormalities in fixed brain sections from EAE guinea pigs.

MATERIALS AND METHODS

Animal Model

EAE was induced in female Hartley (Charles River) guinea pigs (200–250 g) by an intranuchal injection of whole CNS homogenate in complete Freund’s adjuvant with inactivated Mycobacterium tuberculosis. The clinical signs of EAE begin approximately nine days after immunization and are characterized by ascending paralysis due to inflammation and myelin damage in the brain and spinal cord. Animals were weighed and scored daily for the clinical features of EAE (Table 1). Scoring began the day after immunization. The acute phase of EAE (days nine to 14) is characterized primarily by inflammation without signs of alterations to myelin. During the chronic progressive (CP) phase (> day 30), there may be evidence of myelin damage and/or inflammation in the brain and spinal cord. In this study, we examined guinea pig brains (N = 5) removed during the acute phase of EAE (day 13) from animals that were assigned a clinical score of 2. Brains from age-matched control animals were also examined (N = 3). Animals were killed and exsanguinated, and the brains were removed and fixed in formalin.

Imaging

Before imaging, the brains were trimmed to fit into a 1-cm diameter plastic tube. The sample was immersed in Fomblin® (perfluoropolyether; Ausimont, Morris-town, NJ), an embedding medium that limits tissue dehydration and thereby limits susceptibility effects at the surfaces of the specimen. The sample was placed in a vacuum for several hours to remove air bubbles and then positioned within a custom-built, 19-mm diameter copper birdcage radiofrequency (RF) coil.

Imaging was performed at the Center for In Vivo Microscopy (CIVM) using a 9.4-Tesla, 89-mm bore, superconducting magnet (Oxford) equipped with actively shielded gradients capable of 850 mT/m. A number of MR contrasts and spatial resolutions were explored. Two- and three-dimensional spin-echo (SE) images were acquired with the echo time/repetition time (TE/TR) equal to 8/700, 65/3000, and 20/3000 msec for T1-, T2-, and proton density (PD)-weighted images, respectively. A number of spatial resolutions were explored for SE imaging; an in-plane resolution of 60 × 60 μm was used with a slice thickness of 230, 1000, or 2000 μm. For T2*-weighted images, a gradient-recalled acquisition steady-state (GRASS) three-dimensional sequence was used with a TE of 4 msec, a TR of 50 msec, pulse flip angle of 20°, and a spatial resolution of 60 × 60 × 230 μm. A Stejkal and Tanner (19) three-dimensional spin-echo pulse sequence was used for diffusion imaging with a 20 msec TE, 800 msec TR, and diffusion gradients of 500 mT/m applied in either the x, y, or z direction, and spatial resolution of 60 × 60 × 230 μm.

Correlative Histopathology

After imaging, the specimens were prepared for a histopathologic analysis. Specimens were cut from front to back every 1 mm. Contiguous sections (20 μm) from each block were stained with H&E to assess cellular infiltration and with SoloChromatic-R-cyanin (SCR) to demonstrate myelin. Histologic and MRM images were matched by locating corresponding landmarks.

RESULTS

Normal Guinea Pig Brain

In both the two- and three-dimensional images, many neuroanatomical features of normal guinea pig brain were apparent in the MRM proton stains we examined. In PD-weighted images, a large amount of anatomical detail and very good contrast was observed between gray matter (GM) and WM; fiber tracts appeared with very low SI. In Figure 1, a labeled PD stain (60 μm × 60 μm × 1 mm) is illustrated along with the corresponding histological image (20 μm) of a corresponding view. The WM fibers of the corpus callosum (splenium [sec], cin-
gulate [cg], and external capsule [ec]) are clearly visualized as a continuous band of very low SI. The optic tract (ot), cerebral peduncle (cp), and acoustic radiation (ar) are visible with similar clarity in the MR and histological images. Certain small tracts of fibers, for example the medial lemniscus (ml), superior cerebellar peduncle (scp), and mammillothalamic tracts (mt), appear to be more readily visualized in the PD MR stain as compared to the corresponding SCR histological stain.

Anatomic details of the hippocampus were better visualized on T1 stains of normal guinea pig brain, as shown in Figure 2. The hippocampus is bordered dorsally by the corpus callosum (cc) and ventrally by the thalamic nuclei. The hippocampal fissure (hf) appears as a low SI band centrally located in the hippocampus. On the corresponding SCR stain, the pyramidal cell layer (pcl) of the CA1–CA4 regions and the granule cell layer (gcl) of the dentate gyrus appear as pink lines. These structures become more conspicuous using MRM on three-dimensional T1 proton stains (60 μm × 60 μm × 230 μm), as shown in Figure 3. The pyramidal (pcl) and granule cell layer (gcl) appear as faint bright

Figure 1. Corresponding PD MRM stain (a, b) and SCR-stained brain section (c) from a normal guinea pig. The resolution of the PD stain was 60 μm × 60 μm × 1 mm. Various structures are labeled. cg = cingulate, scc = splenium, ec = external capsule, ar = acoustic radiation, ml = medial lemniscus, scp = superior cerebellar peduncle, cp = cerebral peduncle, ot = optic tracts, mt = mammillothalamic tracts. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
lines. Additional layers of the hippocampus are apparent as bands above and below the hippocampal fissure. A number of small vessels (arrows) are visible in the MRM and analogous SCR stains (c), though not all vessels emerge on the T1 stain.

**EAE Guinea Pig Brain**

In Figure 4, T1, T2, T2*, PD, and diffusion proton stains are shown for the same image slice from an EAE brain example. For the most part, the same anatomical features are visible in EAE and control brain images. Compared to T1 and PD stains, T2 proton stains in Figure 4b revealed less microanatomic detail. Some hippocampal anatomy is apparent in three-dimensional T2* stains, and small vessels in this region are well demonstrated (Fig. 4d). In Figures 4e and 4f, corresponding two- and three-dimensional diffusion stains are shown for comparison. In these examples, the diffusion gradients were applied in the x direction, as shown in the figure. As a result, many of the WM fiber tracts of the corpus callosum, which travel parallel to x, are not discernible. The splenium of the corpus callosum, on the other hand, which is made up of fibers that run perpendicular to the direction of the diffusion gradients, is visible in these images.

Of particular interest was that three-dimensional T1 proton stains revealed pathologic features in EAE brain tissue. Figure 5 shows corresponding H&E stained brain section (20 μm) and T1 proton stain (60 μm × 60 μm × 230 μm) from a representative EAE brain. In the H&E stain, perivascular cuffs of inflammation can be seen in multiple locations within the WM areas of the
hippocampus. A high power view of this location (Fig. 5a, inset) reveals that the cuffs are several cells deep and measure from 50–150 μm across. In the T1 proton stain, a corresponding row of perivascular cuffs can be identified and measures 100–150 μm across (Fig. 5b and 5c). These features were apparent in each of the EAE brains examined. These structures could not be identified in any of the other MR stains of equivalent spatial resolution. In normal guinea pig brain, small vessels in this region are visible histologically (see Fig. 3); they are in the same location but are not surrounded by inflammatory cells.

DISCUSSION

In this study, MRM was used to examine the in vitro appearance of normal and EAE guinea pig brain. Several MR proton stains were explored and various macro- and microanatomical brain regions were characterized. All MRM images were compared with H&E and SCR-stained brain sections. Some anatomic features were more easily recognized in the MRM stains as compared to the common histological stains. In general, three-dimensional T1 proton stains demonstrated hippocampal anatomy, while PD stains defined several fiber tracts with very good anatomic detail. Three-dimensional T2* stains did not provide good contrast between brain tissue regions for these samples. Previous work at the CIVM by Benveniste et al (22) showed that perfusion fixation of neurologic tissue with a formalin/Magnevist® solution improved the delineation of certain microanatomical details on T2* stains in the C57BL mouse brain.

Figure 3. Normal hippocampus anatomy is revealed by a three-dimensional proton stain (a, b), and the corresponding SCR-stained tissue section (c) is shown. The hippocampal fissure appears as a low SI band centrally located in the hippocampus. Layers of the hippocampus are apparent as bands above and below the hippocampal fissure. Small blood vessels (arrows) appear as solid black structures. Not all vessels apparent in the histologic stain appear in the MR image. This may represent mismatch between the 20 μm-thick SCR stain and the 230-μm-thick T1 stain. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
The assessment of EAE brain showed that it is possible to directly visualize perivascular cuffing in vitro by MRM. The quantitation of individual perivascular cuffs of inflammation on histological sections of brain tissue is a common pathological scoring method for the estimation of the extent of inflammation in studies of EAE and MS. In this study, individual perivascular cuffs could be readily visualized on three-dimensional T1 proton stains. The spatial resolution required for detection of individual perivascular cuffs was 60 μm × 60 μm × 230 μm. In T1 proton stains acquired with a thicker slice, the individual perivascular cuffs were not detected, probably because they were obscured by partial volume artifacts.

Why the perivascular cuffs were detected only with T1 proton stains is not completely understood. It appears that their prominence is related to opposing contrasts of the low SI normally associated with the blood vessel and the high SI associated with leukocytes surrounding the vessel. The inflammatory exudate typically consists of leukocytes and a highly proteinaceous fluid, which has a short T1. The exact mechanisms for these are under investigation. Vessels that did not have inflammatory cells surrounding them were not as conspicuous on equivalent high-resolution T1 proton stains of normal guinea pig brain. The presence of morphologically normal vessels in this brain location was confirmed histologically.

On three-dimensional T2* proton stains of EAE brain, there is some indication of vessels in the same region, though it is less conspicuous than on the T1 proton stains. Regions of low SI can be observed in the corresponding location of the perivascular cuffs observed on the T1 stain. This may be a reflection of the presence of paramagnetic compounds associated with red blood cells (RBCs), which would contribute to susceptibility-induced signal loss in T2* weighted images.

Figure 4. Representative MRM proton stains of the analogous brain slice for acute EAE guinea pig brain. a: T1 (60 μm × 60 μm × 1 mm); b: T2 (60 μm × 60 μm × 1 mm); c: PD (60 μm × 60 μm × 1 mm); d: T2* (60 μm × 60 μm × 230 μm); e: Diffusion (60 μm × 60 μm × 1 mm); f: Diffusion (60 μm × 60 μm × 230 μm).
Ahrens et al (23) have also investigated EAE using MRM and have shown that small, 100-μm wide EAE WM lesions in the spinal cord could be clearly visualized by diffusion contrast. These spinal cord lesions appeared as areas of hyperintensity, which were non-specific and may reflect demyelination, inflammatory cell infiltration, or edema. In this study, we investigated brain tissue during acute EAE. During the acute phase of this disease, inflammation is the predominant pathologic feature. Demyelinating lesions in the brain are not expected, though demyelination in the spinal cord has been shown to occur in the acute phase. No lesions were detected during our histologic analysis. In EAE and MS, inflammatory cell infiltration is a prerequisite for the development of WM lesions. Thus, the ability to directly detect individual perivascular cuffs of inflammation may provide a useful means of monitoring the course of inflammatory events, as conventional histopathological scoring of perivascular cuffs is utilized, but in the absence of sectioning and staining.

A number of studies at the CIVM have successfully used MRM proton staining techniques to characterize normal and diseased human and animal tissue in specimens (22,24–26) and in live animals. MRM of postmortem specimens has become an important tool for investigating small animal phenotyping and pathology (27). The acquisition of three-dimensional MR image volumes of the soft tissue structure of an animal, obtained without destroying the animal or specimen, has certain advantages over conventional histological evaluations of tissue morphology. The three-dimensional nature of MRM allows for interpretation of complex spatial relationships between substructures without the problems typically associated with serial sectioning and histological processing, including sample distortion, processing artifacts, and the preservation of intact specimens. Because motion artifacts and imaging times are not a major consideration in specimens, images can be obtained with very high spatial resolution and very good SNR. The nondestructive autopsy approach has also been used for pathologic investigations of human cadavers (28).

Future experiments are aimed at developing this technology for in vivo examinations of EAE guinea pig brain. There are a number of challenges for high field in vivo MRM. To maintain similar voxel dimensions in the live animal will require novel approaches to recover SNR. One possibility is the use of high-temperature superconducting (HTS) RF coils. The HTS coil has been shown to yield a seven-fold increase in SNR compared with a conventional copper coil (29). A second approach involves the administration of MR contrast agents, such as gadolinium DTPA, before scanning to reduce T1 and allow equivalent image SNR in shorter scan times (22). The MR appearance of perivascular cuffs may be different in vivo compared with the brain specimens we examined in vitro. In the live animal, the presence of flowing blood vs. static RBCs, and the hemoglobin state and magnetic properties of the RBCs in vessels, may generate different contrast mechanisms.

Important and unresolved questions remain as to whether clinical symptoms associated with new lesions are due to damage to the myelin sheath or whether these changes can occur as a response to the inflammation without demyelination. Some evidence suggests that the degree of inflammation associated with some MS lesions is very closely related to the likelihood of progressing to demyelination (30). The spatial distribution or patterns of perivascular cuffing may represent important markers for early events in MS.

Direct visualization of these cellular events in specimens, and ultimately in vivo, will yield important new information about disease processes in MS, the cause of which still remains elusive.

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REFERENCES


