

DISAPPEARING “T1 BLACK HOLES” IN AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

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1. ABSTRACT

Brain MRI in multiple sclerosis (MS) frequently shows areas of hypointensity in the white matter on T1 weighted sequences (“T1 black holes”). These areas are thought to be consistent with irreversible axonal loss. In this study T1 black holes were characterized in Theiler's Murine Encephalitis Virus infection, an established model of demyelinating diseases in mice. The spectrum of TMEV is broad in different strains. C57BL/6J mice develop a self-limited brain disease, which resolves within 4-6 weeks. We followed six mice with serial MRI and MRS on days 0, 3, 7, 21 and 45. The studies were performed in a 7 Tesla magnet. Periventricular and parahippocampal T1 black holes seen as early as 3 days, with decreasing NAA/Cre ratio on MRS. The extent of pathology was most severe on days 3 and 7. T1 black holes are thought to be consistent with areas of irreversible axonal loss. This is challenged by our observations of resolution of T1 black holes by day 45. This was concomitant with the normalization of MRS findings in the areas of interest. We conclude that T1 black holes may represent a transient phenomenon in this model of MS. The recovery of these areas studied suggests an active repair mechanism.

2. INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). Since it is the leading cause of disability among young adults, it has a significant socioeconomic impact on the societies of the western world (1). Magnetic resonance imaging (MRI) has become accepted as the most sensitive paraclinical measure for the detection of lesion formation and dissemination in MS, and is being widely used in drug trials as a secondary outcome measure (2-6). The best

known MRI finding associated with MS lesions is the T2 lesional hyperintensity. However, high T2 signal, does not distinguish between edema, inflammation, demyelination, remyelination, gliosis and axonal loss. Therefore, high T2 signal is a sensitive, but non-specific finding in MS. The correlation between T2 weighted lesions and disability is only modest (7, 8).

A proportion of high signal T2 lesions also appear as hypointense areas on T1 weighted images. These lesions are thought to represent a more chronic form of the disease, characterized by axonal loss and more severe tissue destruction (9-13). These hypointense lesions also appear to correlate better with disability (14, 15). Histopathological studies have correlated these areas with extensive axonal loss, but not with demyelination or the number of reactive astrocytes. (10, 14).

Proton MR spectroscopy (1H-MRS) is a non-invasive, magnetic resonance-based method that enables *in vivo* quantification of metabolite concentrations in the studied tissue of interest. The principal peaks in a normal brain spectrum are produced by N-acetyl aspartate (NAA), myo-inositol (Ins), creatine/phosphocreatine (Cre) and choline-containing compounds (Cho). NAA is almost exclusively restricted to neurons, and its density is thought to reflect axonal/neuronal integrity (16, 17). Myo-inositol has been proposed as a glial marker (18-20). Creatine containing compounds are thought to be “constant”, and are related to energy metabolism in the cells of the nervous system. Choline containing compounds are related to membrane integrity. Higher membrane turnover may result in an elevated Cho peak in areas of remyelination (8, 16). In general, NAA is thought to be significantly lower in T1

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hypointense areas, also the same areas may contain higher Ins compared to normal white matter(21).

One of the best studied rodent models of MS is an infection induced by Theiler’s Murine Encephalitis Virus (22). Infection with this virus causes a strain-dependent progressive demyelinating disease in certain susceptible strains of mice(23, 24). Strains resistant to TMEV are able to effectively clear the infection, and recover fully after the initial encephalitogenic stage of the infection. We studied the MRI and MRS features of TMEV infection in C57BL/6J congenic mice, with special attention to T1 black hole formation, and corresponding spectroscopic findings. These mice effectively clear the infection, and recover in approximately 6 weeks after inoculation. Our research hypothesis was that these mice would develop MR findings of tissue loss/axonal loss, and that these findings should return close to baseline as these mice functionally recover from the illness.

3. MATERIALS AND METHODS

3.1. Mice

C57BL/6J mice (catalog # 000664) were obtained from Jackson Laboratories. The study was approved by the Institutional Animal care and Use Committee of the Mayo Clinic (approval number A291-01).

3.2. Theiler’s Virus Infection

TMEV infection was produced by intracerebral virus injection of 4-8 week old mice anesthetized with inhalational isoflurane. With a 27-gauge needle attached to a Hamilton syringe, 10 μ L volume containing 200,000 PFU purified Daniel’s strain of TMEV was injected intracerebrally. Intracerebral injection results in greater than 98% incidence of infection, with only a rare fatality following injection.

3.3. Magnetic resonance imaging (MRI)

Six C57BL/6J mice were studied longitudinally with MRI and MRS at time points 0, 3, 7, 21 and 45 days following their intracranial inoculation with TMEV. MRI was performed in a Bruker Avance 300 MHz (7 Tesla) vertical bore NMR spectrometer (Bruker Biospin, Billerica, MA) equipped with “miniimaging” accessories. The \sim 37 $^{\circ}$ C core temperature of the studied animals was maintained by a thermocouple based system. Inhalational isofluran anesthesia (1.5% in oxygen) was delivered via a nose cone during the imaging procedure. T1 weighted volume-acquisition spin-echo sequences were used to visualize the brain and proximal cervical cord (TR: 200ms, TE: 6.2ms, NA: 2; FOV: 4cm x 2.5cm x 2.5cm, matrix: 256x160x160). By using 3D datasets, we were able to generate arbitrarily oriented slices. Image analysis, reconstruction, and slice selection were done using Bruker Xtip Image (part of Bruker’s original ParaVision software package) and Analyze 5.0, developed by Mayo Biomedical Imaging Resource.

3.4. MR Spectroscopy

MRS data was obtained from a 27 mm³ voxels (3mm x 3mm x 3mm) placed over the parahippocampal

area of each animal. The parahippocampal area was chosen for this investigation based on our previous observations suggesting that this is the area of most significant pathology(25). To maintain the best possible uniformity within the MRS measurements, the same investigator selected the studied voxel, based on anatomical landmarks in the murine brain. Bruker’s VSEL sequence, an implementation of the standard PRESS sequence, was used for voxel-based spectroscopy, with built-in water suppression pulses (TR 3000ms, TE 120ms, 512 averages). For shimming, both manual and the automated techniques (FASTMAP) were used(26). The acquired spectra were processed and analyzed using X-win NMR, Bruker Biospin’s proprietary NMR software.

3.5. MRI volumetry

To characterize the changes in total T1 hypointense lesion load (“T1 black holes”), 3D volumetric studies were done using Analyze 5.0. The datasets were segmented, and the brains were extracted using the Object Extractor and Image Edit tools. To correct for potential misalignment of the animals at different scanning time points, 3D surface matching and voxel-matching algorithms were used on the extracted 3D brain image data. These algorithms generated a rotation matrix. The rotated and co-registered 3D images were generated with windowed sinc interpolation of the original images. The extracted and co-registered brain images were then segmented using the 3D ROI Analysis Tool. A semi-automated intensity-based seed-growing algorithm was used to generate object maps. These object maps define sub-volumes of the brain. Each sub-volume was a stand-alone, numbered, individually identified, three-dimensionally represented “T1 black hole” lesion. The generated object maps were then applied to the image set representing the subsequent time point. These object maps were corrected as appropriate with the same seed-growing algorithm so that changes in lesion number, volume and shape could be accounted for. Since we used semi-automated tools, there was a human factor involved in this analysis. To minimize that, the involved investigators were trained for several weeks on test datasets, and their intra- and inter-rater reliability was repeatedly tested and was found to be superior (>95%).

After the generation of object maps describing both time points in every animal, the 3D ROI Scan Tool was used to calculate the “T1 black hole” lesion volumes.

4. RESULTS

4.1. Changes in total “T1 black hole” volume

In four C57BL/6J mice, the formation of “T1 black holes” reached its peak 7 days after TMEV infection, in two of them the same phenomenon peaked at 3 days. By 45 days after TMEV infection, the average T1 black hole volume was only 12.13% of the maximum value observed (figure 1). A statistically significant difference in T1 black hole volume was observed between the individual time points ($p < 0.001$, ANOVA test) (table 1). We concluded that TMEV infected C57BL/6J mice recover remarkably well by day 45, with only minimal observable abnormality on T1 weighted images (figure 3).

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Table 1. MRI findings in C57/BL6J mice infected with Theiler’s Murine Encephalitis Virus

Days following TMEV infection	Mean “T1 black hole” volume	SD
3	542.7	321.0
7	843.7	224.5
21	455.8	233.3
45	102.3	59.0

Tukey-Kramer ANOVA test, between group comparison: $p < 0.001$

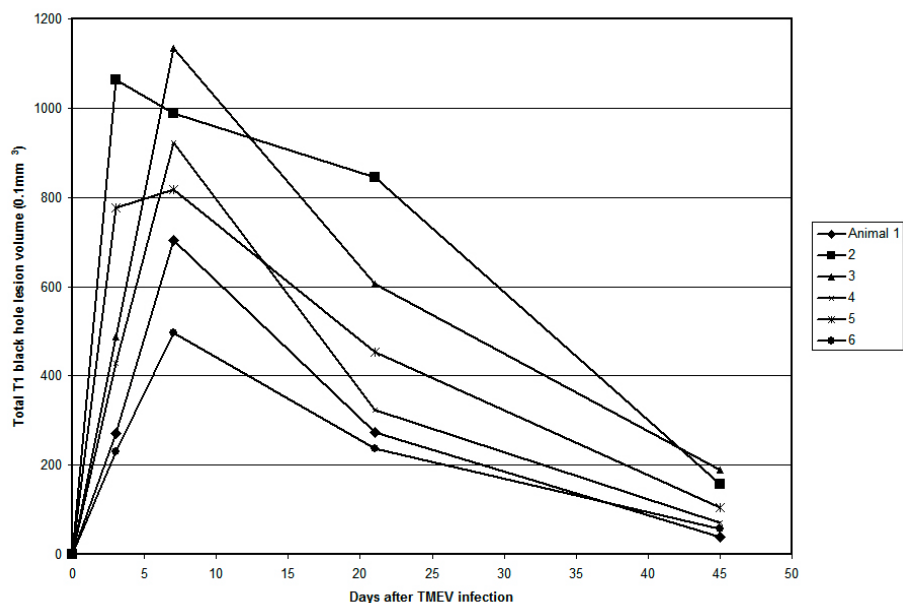


Figure 1. Total T1 black hole volume changes in individual TMEV infected C57BL6J mice. Six C57BL6J mice were infected with TMEV on day 0, and were followed serially with volumetric MR imaging. The peak of T1 black hole formation was seen on day 3 in two mice, and day 7 in four mice. By day 45, the animals recovered close to baseline.

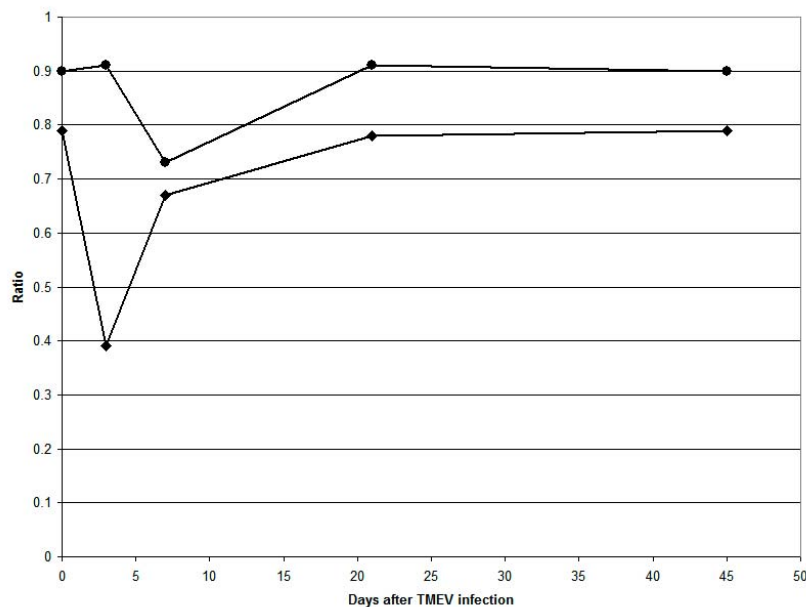


Figure 2. Changes in NAA/Cr and Cho/Cr ratios in a 27 mm³ parahippocampal voxel of TMEV infected C57BL/6J mice. The NAA/Cr ratio was found decreased on days 3-7, and recovered by day 45. This suggests recovery of the compromised axonal integrity.. The Cho/Cr ratio showed a temporary decrease on day 7, suggesting decreased membrane turnover at that time point.

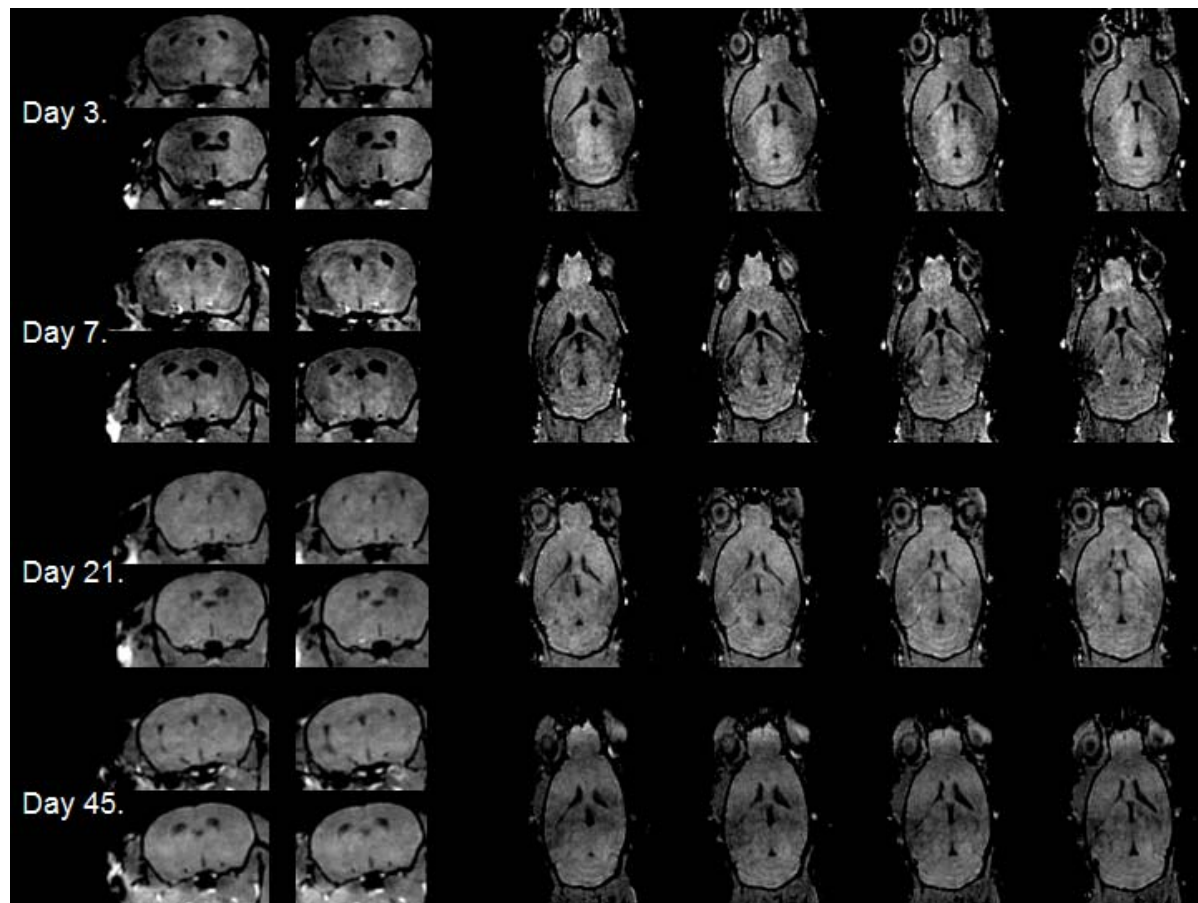


Figure 3. Representative brain MRI images. From the 3D MRI datasets representing post-infection days 3, 7, 21 and 45, we extracted representative examples of coronal (left) and axial (right) images. Note the strong hypointensity near the ventricles, especially in the parahippocampal areas on days 3 and 7. By day 45, the phenomenon is almost fully resolved.

4.2. Changes in NAA/Cre ratio

At each time point in all six C57BL/6J mice, a 27mm^3 voxel was studied in the parahippocampal area with MR spectroscopy. The NAA/Creatine ratio (a marker of axonal integrity) was found most decreased on post-infection days 3 and 7 (table 2, figure 2). By day 45, this was increased by 202.6% compared to the lowest observed average value ($p < 0.01$, ANOVA test). We concluded that the neuronal and axonal integrity of the parahippocampal area improved as measured by MRS.

4.3 Changes in Cho/Cre ratio

The Cho/Cre ratio, a marker of membrane turnover, showed a significant decrease on day 7, otherwise remained stable until the 45 day time point (figure 2, table 2).

5. DISCUSSION

Even though T1 black holes are generally thought to be areas of irreversible axonal loss, several observations have challenged the irreversibility of this process in human MS. While most T1 black hole areas represent a chronic process, they may also be observed in newly forming lesions as a transient phenomenon(9-13). To our knowledge, ours is the first animal study to investigate the

phenomenon of T1 black holes in a mouse model of MS. By using 3D techniques for MRI data acquisition, we were able to accurately measure the extent of T1 black hole formation in our animals. Partial volume averaging or “missed” lesions are virtually eliminated by the use of this technique. Our finding of resolving T1 black holes in the TMEV model in C57BL/6J mice corresponds well with the human studies describing the partial reversibility of the process that leads to this imaging phenomenon (27, 28).

The early decrease of NAA/Cre ratio is a known observation in human MS plaques. This has been observed not only in the early stages of lesion formation, but also in normal appearing white matter(29, 30). Inside T1 black holes, most previous studies have described a definite decrease of the NAA/Cre ratio compared to the surrounding parenchyma (6, 10-12, 14, 21, 31-33). When observed longitudinally during the process of acute lesion formation, the partial resolution of the decreased NAA/Cre ratio has also been described(27). A recent study by Li *et al* (28) also suggested that the formation of T1 black holes is neither final-stage nor a static pathologic abnormality. In this study, the investigators found considerable heterogeneity of the NAA/Cre peak in lesions that otherwise all appeared hypointense on T1 weighted images. Our study underlines that even in lesions that both look hypointense on T1

Table 2. MRS findings in C57/BL6J mice infected with Theiler’s Murine Encephalitis Virus

Days following TMEV infection	NAA/Cre ratio	Chol/Cre ratio
0	0.79+/- 0.13	0.9+/-0.11
3	0.39+/- 0.08	0.91+/-0.09
7	0.67+/- 0.09	0.73+/-0.1
21	0.78+/-0.11	0.91+/-0.09
45	0.80+/-0.12	0.9+/-0.14

weighted images and have significantly decreased NAA/Cre ratios, the process that leads to the observed phenomena does not necessarily represent an irreversible change at the tissue level.

The Cho/Cre ratio is considered to be an excellent marker for membrane turnover. It is usually thought to represent active demyelination and remyelination in the studied white matter voxel. In a recent study in human MS (28), the Cho peak was increased in 71% of all T1 hypointense lesions. In our study, at the peak of the T1 black hole formation, the Cho/Cre ratio was actually decreased. This may represent that at the peak of the process that leads to the formation of T1 black holes, the turnover of cells may come to a temporary “halt”, only to be followed by increased cell turnover again as the lesion recovers.

One possible explanation for the observed changes, including the imaging findings, NAA/Cre and Cho/Cre findings, is active neuronal regeneration from precursor cells. This may explain why the imaging phenomenon resolves, why the membrane turnover marker increases as the restorative process takes place, and why the NAA/Cre peak recovers. As an alternative explanation, resolution of local edema that may decrease the local NAA and Cho concentration by “dilution” effect is a possibility, although appears less likely. Further investigations of the observed changes with histological studies are warranted.

6. ACKNOWLEDGMENT

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