Gadofluorine M enhancement allows more sensitive detection of inflammatory CNS lesions than T2-w imaging: a quantitative MRI study

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Magnetic resonance imaging plays a pivotal role in the diagnosis and treatment monitoring of multiple sclerosis. Currently available magnetic resonance-techniques only partly reflect the extent of tissue inflammation and damage. In the present study, application of the experimental magnetic resonance-contrast agent Gadofluorine M significantly increased the sensitivity of lesion detection in myelin-oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis. Gadofluorine M-enhancement on T1-weighted (T1-w) images utilizing a clinical 1.5 T magnetic resonance unit showed numerous lesions in optic nerve, spinal cord and brain, the majority of which were not detectable on standard T2-weighted (T2-w) and Gd-DTPA enhanced T1-w sequences. Quantitative assessment by pixel counts revealed highly significant differences in sensitivity in favour of Gadofluorine M. Gadofluorine uptake closely corresponded to inflammation and demyelination on tissue sections. These unique features of Gadofluorine M in visualizing inflammatory CNS lesions hold promise for future clinical development in multiple sclerosis.

Keywords: multiple sclerosis; CNS inflammation; experimental autoimmune encephalomyelitis; magnetic resonance imaging; Gadofluorine

Abbreviations: BBB = blood–brain barrier; EAE = experimental autoimmune encephalomyelitis; Gf = Gadofluorine M; Gd = Gadolinium; ON = optic nerve; MOG = myelin–oligodendrocyte–glycoprotein; MRI = magnetic resonance imaging


Introduction

Multiple sclerosis is the most prevalent inflammatory CNS disorder in young adults. Despite modern immunomodulatory treatment options multiple sclerosis is still a major cause of disability. The diagnosis of multiple sclerosis is based on the typical clinical pattern and lesion dissemination in time and space. Recently new diagnostic criteria have been defined (Polman et al., 2005). According to these criteria, multiple sclerosis can now be diagnosed after a single clinical bout in combination with a defined number, localization and contrast enhancement of lesions on MRI (Barkhof et al., 1997; Polman et al., 2005). This is important because it was demonstrated that early treatment can delay relapses in relapsing-remitting multiple sclerosis and probably improves long-term outcome (Jacobs et al., 2000; Comi et al., 2001; Kappos et al., 2006). Based on comparative histological and MRI studies in autopsy and biopsy cases it became apparent, however, that conventional MRI visualizes only a proportion of multiple sclerosis lesions (Filippi et al., 2002). Thus, there is a demand for improving the sensitivity of non-invasive lesion detection by MRI.

By use of the novel experimental MR-contrast agent Gadofluorine M (Gf) (Bayer Schering Pharma AG) we now
could significantly increase the diagnostic yield in MRI lesion detection in myelin–oligodendrocyte–glycoprotein (MOG) induced relapsing experimental autoimmune encephalomyelitis (MOG-EAE) (Storch et al., 1998). Gf is an amphiphilic macrocyclic Gd complex with a molecular weight of about 1530 g/mol originally developed for MR lymphography and imaging of atherosclerotic plaques and gives a bright contrast on T1-weighted (T1-w) MRI (Barkhausen et al., 2003; Misselwitz et al., 2004). We show that Gf accumulated extensively in inflammatory-demyelinating lesions in the spinal cord, brain and optic nerve in this animal model of multiple sclerosis. By direct quantitative comparison, lesion load was significantly higher than on T2-weighted (T2-w) and Gd-DTPA enhanced T1-w sequences which is the standard MR protocol in clinical practice. Thus, Gf has the potential to markedly improve the detection of multiple sclerosis pathology by MRI.

Material and Methods
Experimental model
A total of 63 Dark Agouti (DA) rats were used. Animal studies were approved by the governmental agencies for animal research and performed in accordance with institutional guidelines. Chronic relapsing EAE was induced in 10- to 12-week-old female DA rats by immunization with 55 μg truncated soluble human recombinant MOG (kindly provided by Dr A Weishaupt, Würzburg, Germany) (Hilton et al., 1995; Devaux et al., 1997) emulsified 1:1 in incomplete Freund’s adjuvant into the tail base and both hind footpads (Storch et al., 1998). The first peak of disease occurred 10–14 days after immunization. Animals first recovered and relapsed with another bout of severe EAE around day 20 followed by a secondary progressive disease state thereafter. Rats were weighed and examined daily for clinical signs of EAE and both hind footpads (Storch et al., 1998). The first peak of disease occurred 10–14 days after immunization. Animals first recovered and relapsed with another bout of severe EAE around day 20 followed by a secondary progressive disease state thereafter. Rats were weighed and examined daily for clinical signs of EAE that were scored on the following scale: 0.5 = partial loss of tail tone, 1 = complete tail atony, 2 = hind limb weakness, 3 = hind limb paralysis, 4 = moribund state, 5 = death (Storch et al., 1998).

Contrast agents
Gadofluorine M (Gf; Bayer Schering Pharma AG, Berlin, Germany) is an amphiphilic Gadolinium (Gd) complex with a molecular weight of 1528 g/mol and a concentration of 250 mmol Gd/l (Barkhausen et al., 2003; Misselwitz et al., 2004). To be used for autofluorescence in histological studies and for macroscopical detection Gf was prelabelled with a carbocyanine dye which has optical absorption and fluorescence of the kind of Cy3. The dye was covalently attached to the amino group of the lysine backbone of Gf exactly at the position of mannose. Thus, mannose is replaced by the dye based on a structurally identical attachment. The dye has similar properties in terms of hydrophilicity rendering the Gf-carbocyanine similar to Gf itself (Henning et al., 2007). Gf was applied i.v. at a dose of 0.1 mmol/kg body weight. Gf binds to serum albumin at an affinity of kD = 2 μmol/L. Moreover, Gf reveals a similar binding affinity to the extracellular matrix (ECM) components collagen, proteoglycan and tenasin. The driving force of binding and accumulation is the hydrophobic moiety of the Gf molecules interacting with hydrophobic ECM constituents (Meding et al., 2007). Most of the injected dose of Gf is eliminated from the body in 7 days after intravenous injection. About one-third of the dose is excreted by glomerular filtration, and two-thirds are excreted in the feces. On the basis of the survival patterns observed in the acute toxicity experiments (Misselwitz et al., 2004), the gross acute systemic tolerance (lethal dose) after a single intravenous injection of Gf in mice may be in the order of magnitude of the medium dose of 5 mmol/kg body weight. This reflects about 100 times the expected diagnostic dose of 0.05 mmol/kg body weight. For comparison: Gd-DTPA (Magnevist) features a safety margin of 50 (LD50: 5 mmol/kg; diagnostic dose: 0.1 mmol/kg).

As an established marker for the integrity of the blood–brain-barrier (BBB) Gadolinium (Gd)-DTPA (Magnevist®, Bayer Schering Pharma AG, Berlin, Germany) was injected at a dose of 0.2 mmol/kg body weight.

Magnetic resonance imaging
MRI was performed on a clinical 1.5T MRI unit (Magnetom Siemens Vision, Erlangen, Germany) under inhalation anaesthesia with 2.5% isoflurane in a 2:1 nitrogen/oxygen atmosphere. A custom made dual channel surface coil was used for all measurements (A063HACG; Rapid Biomedical, Rimpar, Germany). The MR protocol included a coronal T1-w SE sequence (TR 476 ms, TE 28 ms, slice thickness 2 mm) and a coronal T2-w TSE sequence (TR 2370 ms, TE 92 ms, slice thickness 2 mm) for imaging of the entire brain and the optic nerves. Subsequently, the animal position was changed for imaging of the cervico-thoracic spine. Here, sagittal and axial T1-w and T2-w sequences (slice thickness 2 mm) were applied. MR images were read blinded to the time of examination after immunization and blinded to the contrast agent applied by an experienced neuroradiologist. On a separate work-station (Leonardo, Siemens, Erlangen, Germany) every contrast-enhancing lesion or hyperintensity on T2-w images was delineated manually and the respective area was calculated (as pixel count) by an experienced neuroradiologist. Moreover, the enhancement in the periventricular region or optic nerves was assessed as present or absent.

Experimental groups
Interindividual comparison of Gd-DTPA and Gf-enhancement (group 1)
To assess cerebral lesions on T2-w images and contrast enhancement, Gd-DTPA (n = 5 animals) or Gf (n = 32 animals) was applied prior to cranial MRI at the first clinical event or at the first relapse. Gd-DTPA was applied twice 30 min before MRI in the same animals both at the first clinical event and at the first relapse. Gf was applied 24 h before MRI only once at the first clinical event (n = 13) or at the first relapse (n = 19). All animals except of five were sacrificed after MRI. In five rats repeat MRI was performed 5 days after initial MRI without further application of contrast medium.

Intraindividual comparison of Gd-DTPA and Gf-enhancement (group 2)
In seven rats, Gd-DTPA was injected at the first relapse, followed by MRI 30 min later. Two hours after MRI, when Gd-DTPA was largely eliminated from the circulation, Gf was applied in the same animals. Repeat MRI was performed 24 h later. For both time points, cranial and spinal MRI was performed encompassing T1-w and T2-w sequences.
To compare identical time frames between injection and MRI for both agents, in five additional animals Gd-DTPA was applied 3 h before MRI. After MRI, Gf was injected followed by MRI another 3 h later.

Assessment of the relationship between Gf-enhancement and inflammatory lesions on tissue sections (group 3)

In 14 animals Gf was applied at the first clinical event 24 h before spinal MRI. After MRI, all animals were perfused (see later) and spinal cord segments were removed for embedding in paraffin. Overall 35 separate tissue specimens were removed from spinal cord segments devoid of macroscopically visible Gf and another 43 specimens with the typical pink appearance of labelled Gf.

Histology and immunohistochemistry

After MRI rats were deeply anaesthetized and perfused through the left ventricle with Ringer solution, followed by cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4). In a first gross histological evaluation cerebral hemispheres, brainstem with cerebellum, cervical and thoracic spinal cords were exposed after removal of bones and connective tissue for photography, areas showing Gf-enhancement were then removed, postfixed for 2 h in 4% paraformaldehyde at 4°C, and routinely embedded in paraffin. In addition, the intracranial and intraorbital portions of ON were prepared and processed accordingly. Photographs of tissue specimens in situ were taken to document sites of labelled Gf accumulation (appearing pink) and these areas were cut out and processed for histochemistry for direct comparison of MRI, Gf accumulation and inflammation/demyelination. Five micrometre thick sections were stained with haematoxylin/eosin and luxol fast blue to assess inflammation and demyelination, respectively. In adjacent serial sections immunohistochemistry was performed with antibodies against following targets: macrophages/activated microglia (ED1, 1:500; Serotec, UK), T cells (B151-1, 1:500; HyCult Biotechnology, Holland), rat albumin (1:200) (Cappel, Lot 37201) and myelin basic protein (1:1000; MBL International Corporation, USA). Deparaffinized sections were preincubated with 10% BSA and then incubated overnight with the primary antibody optimally diluted in Tris-buffered saline (TBS)/1% BSA. After washing, sections were incubated with biotinylated secondary Ab (Vector Laboratories, Burlingame, CA), and avidin–biotin–peroxidase complex reagent (Dako), according to the manufacturer’s instructions with the exception of anti-rat albumin abs which were peroxidase labelled. Finally, 3,3-diaminobenzidine (DAB; Kem En Tec Diagnostics, Copenhagen, Denmark), in the presence of 0.03% H2O2, was used as substrate for staining reactions. For negative controls, the primary antibody was omitted from the diluent. In addition, 5 μm thick sections from snap-frozen spinal cord lesions were cut and analysed for the presence of carbocyanine labelled Gf by red fluorescence on a Zeiss Axiohot microscope (Zeiss, Thornwood, NY) and serial sections were stained with fluorescein-conjugated antibodies against ED1. In a second histological series (animal group 3 involving the 14 animals described earlier) spinal cord segments of regions with macroscopically visible Gf (n=43) and devoid of Gf-accumulation (n=35) were selected, embedded in paraffin, and entire cross-sections were stained for macrophages by ED-1 immunocytochemistry as a measure for inflammation. All 78 spinal cord blocks were evaluated histologically based on cross-sections showing the entire spinal cord and inflammation was assessed semiquantitatively by a simple score: (0) no inflammation, (1) mild, (2) moderate and (3) severe. Examples are shown together with the results in Fig. 8. Thereafter, the extent of inflammation was compared to the macroscopical analysis of Gf-positive versus Gf-negative tissue blocks.

Statistical analysis

Statistical analysis used STATA 9 SE (StataCorp, Texas, USA). Mean areas including SD of T2-lesions, Gd-DTPA enhancement or Gf-enhancement were calculated in groups 1 and 2. Similarly, lesion areas in animals receiving both Gd-DTPA and Gf (n=12 measurements for each contrast medium, group 2) were analysed. Paired comparisons were done with the Mann–Whitney U-test and Wilcoxon’s signed rank test was used for two sample comparisons.

Results

Interindividual comparison of Gd-DTPA and Gf-enhancement (group 1)

In five rats, Gd-DTPA was injected 30 min before MRI including T1-w and T2-w sequences, closely resembling clinical routine imaging in multiple sclerosis. Gd-DTPA enhanced MRI was performed at the first clinical event and repeated at the first relapse about 10 days later. At the first clinical event (mean clinical score 3.0) no lesions were present on T2-w images, and there was no enhancement of Gd-DTPA in these animals (Fig. 1A). At the first relapse about 10 days later (mean clinical score 4.0), the same animals underwent the initial MR protocol again. Two of five animals demonstrated small foci of hyperintensity in the cerebellum and brain stem on T2-w MRI (mean lesion area: 62.2 pixel, SD 74.2), one of which showed uptake of Gd-DTPA (mean lesion area 45.5 pixel, SD 52.8). In none of these animals, uptake of Gd-DTPA was present in the ON or in the periventricular brain parenchyma.

A total of 32 rats received Gf 24 h before MRI including T1-w and T2-w sequences at the first (n=13; mean clinical score 3.6) or second (n=19; mean clinical score 3.6) bout. In all animals with clinically manifest MOG-EAE Gf-enhancement was present in the brain and ON to varying degrees, leading to a bright signal on T1-w images. The degree and pattern of contrast enhancement did not differ between the first clinical event and the first relapse. A common pattern in all animals was contrast enhancement of the ON and the periventricular regions (Fig. 1B and C). In addition, a patchy enhancement pattern was present in the supratentorial brain parenchyma, brainstem and cerebellum (Fig. 1D and E, mean lesion area: 757.9 pixels, SD 422.1). Importantly, the vast majority of these Gf-enhancing lesions were not visible on T2-w images, which are considered the standard sequence in the clinical setting when visualizing multiple sclerosis lesions (Fig. 1F–H, mean lesion area: 93.5 pixels, SD 174.7).
Gf-enhancement persisted in MOG-EAE lesions

In five animals, Gf was applied at the first relapse of clinical symptoms, followed by cranial MRI 24 h later. The first MR examination revealed contrast enhancement in the ON and periventricular regions, as well as patchy contrast uptake in the CNS parenchyma (Fig. 2A). Arrows indicate contrast medium in venous sinuses. After application of Gf (B–E) intense contrast enhancement is present in the optic nerves (B), in the periventricular region (C), in the deep white matter (D) and cerebellum and brain stem (E) (arrows in B–E). Importantly, none of these lesions shown in (C–E) presented on corresponding T2-w images (lower row, F–H) which is considered as the gold standard in the detection of multiple sclerosis lesions.

Intraindividual comparison of Gd-DTPA and Gf-enhancement (group 2)

In seven rats, Gd-DTPA was injected at the first relapse (mean clinical score 3.6), followed by MRI 30 min later. Two hours after MRI, when Gd-DTPA was largely eliminated from the circulation, Gf was applied. Repeat MRI was performed 24 h later. For both time points, cranial and spinal MRI was performed encompassing T1-w and T2-w sequences.

In only two animals hyperintense lesions on cranial T2-w images were visible (Fig. 3A; mean lesion area: 45.6 pixel, SD 52.1) accompanied by a smaller area of Gd-DTPA enhancement (Fig. 3B; mean lesion area: 37.3 pixel, SD 47.9). Spinal lesions on T2-w images were more abundant and seen in six of the seven animals examined serially (Fig. 3D; mean lesion area: 230.5 pixel, SD 126.6). Again, T2-w positive spinal cord lesions showed less Gd-DTPA enhancement on T1-w MRI (Fig. 3E, mean lesion area: 148.1 pixel, SD 128.0). In contrast, after application of Gf significantly more enhancing cerebral and spinal lesions were seen in all seven rats as areas of bright signal on T1-w images (Figure 3C and F). All animals exhibited Gf-enhancement of the ON and periventricular regions,
Fig. 2 Persistence of Gf-enhancement. Coronal T1-w image 24 h after application of Gf demonstrating Gf-enhancement laterally in the mesencephalon on day 14 after induction of EAE (arrow in A), but no lesion on T2-w MRI (B). Repeat MRI on day 18 without further application of Gf shows persistent GF accumulation in this lesion (arrow in C) which is still not visible on T2-w MRI (D).

Fig. 3 Intra-individual comparison of Gd-DTPA and Gf-enhancement demonstrates increased sensitivity of Gf. (A–C) Coronal images of the brainstem and cerebellum demonstrate a small lesion in the cerebellar vermis on the T2-w image (A) with slight uptake of Gd-DTPA on the T1-w image (B). Contrast enhancement is much more widespread in the vermis (C, arrow) and also present in the brainstem and cerebellum after application of Gf (C, arrowheads). (D–F) Axial images of the cervical spine demonstrate a small lesion on the T2-w image in the dorsal column (D, arrow) with no uptake of Gd-DTPA (E, arrow). In this animal, there is intense Gf-enhancement of the entire posterior aspect of the cervical spine (F, arrow).
a patchy contrast accumulation in supratentorial brain regions, cerebellum and brainstem with a mean cerebral lesion area of 502.9 pixel (SD 244.2), and even more extensive spinal contrast enhancement (mean lesion area: 726.1 pixels, SD 571.1).

In order to compare identical intervals after contrast medium application, five rats received Gd-DTPA 3 h before MRI. After this first Gd-DTPA enhanced MRI Gf was injected followed by a second MRI 3 h later. On Gd-DTPA enhanced MRI no spinal or cerebral lesions were present on T1-w or T2-w images. In contrast, 3 h after Gf-enhanced MRI spinal cord enhancement was present in all animals (mean lesion area 583.8 pixel, SD 366.2) and cerebral enhancement in two animals (mean lesion area 216.4 pixel, SD 129.0).

**Statistical analysis**

Quantitative data for the lesion areas on T1-w and T2-w images (data presented as pixel count) for animals receiving either Gd-DTPA or Gf (group 1) as well animals receiving both Gd-DTPA and Gf subsequently (group 2) are shown in Figs 4 and 5.

In group 1 (interindividual comparison of contrast agents, 10 measurements in the Gd-DTPA group, 32 measurements in the Gf group, Fig. 4) no statistically significant difference ($P = 0.31$) was observed for the mean T2 cerebral lesion area between animals receiving Gd-DTPA and the experimental group in which Gf was applied (22.6, SD 47.7 versus 93.5, SD 174.7). However, there was a substantial difference ($P < 0.0001$) between the extent of cerebral T2 area (93.5, SD 174.7) and lesion area detected by Gf-enhancement (757.9 ± SD 422.1). Furthermore, there was a marked difference ($P < 0.0001$) in the extent of lesion area after Gd-DTPA (18.5, SD 39.06) and after Gf (757.9, SD 422.1).

In group 2 (intraindividual comparison of contrast agents, $n = 12$ animals, Fig. 5) cerebral T2 lesion area (26.7, SD 62.1) was significantly smaller ($P = 0.004$) compared to the cerebral lesion area detected by Gf-enhancement (382.5, SD 303.8). Similarly, the extent of cerebral Gd enhancing area on T1-w MRI (21.7, SD 51.5) was significantly smaller ($P = 0.004$) than the lesion area detected by Gf-enhancement (382.5, SD 303.8). Similarly, spinal T2 lesion area (134.33, SD 150.6) was significantly smaller ($P = 0.002$) than lesion area on Gf-enhanced images (666.5, SD 481.3). Finally, lesion area on Gd-DTPA enhanced T1 images (86.2, SD 121.5) was smaller ($P = 0.002$) than the lesion area on Gf-enhanced images (666.5, SD 481.3).

**Deposition of Gf in tissue specimens and its correlation with Gf-enhancement on MRI**

In a subgroup of Gf-injected animals spinal cord, ON, brainstem and brain hemispheres were examined macro- and microscopically for foci of Gf deposition. These could be easily detected macroscopically during tissue preparation due to the pink appearance of the carbocyanine-prelabelled Gf (Figs 6 and 7). This first histological analysis was biased because we only analysed Gf-positive lesions. Tissue containing Gf was further processed for histological and immunocytochemical analysis. Gf-enhancing lesions in the brainstem and cerebellum (Fig. 6A), the spinal cord (Fig. 6E and F) as well as ON (Fig. 7A and B) closely corresponded to histological lesions showing macrophage infiltration, loss of myelin as revealed by reduced MBP or luxol fast blue staining, and albumin extravasation (Fig. 6B–D, G, J and K). Because of the asymmetric involvement similar to human multiple sclerosis, it was possible to compare ON abnormalities side-to-side. In a representative rat, the ON on the left showed Gf-enhancement on MRI (Fig. 7A) and corresponding Gf deposition macroscopically (Fig. 7C),
Fig. 6  Histological correlates of Gf-enhancing brainstem/cerebellar (A) and spinal cord lesions (E, F and I) on T1-w MRI: (A) shows two cerebellar foci and one brainstem lesion with bright contrast on Gf-enhanced MRI. Arrows point to corresponding paraffin sections. (B) represents a subcortical lesion infiltrated by ED1-positive macrophages, (C) and (D) represent sections through the cerebellar white matter lesion with perivascular demyelination as revealed by staining with anti-MBP antibodies (C) and corresponding breakdown of the BBB as revealed by extravasation of albumin (D). (E) Sagittal T1-w MRI of a representative Gf-enhancing dorsal column lesion of the cervical spinal cord. Arrows point to the corresponding axial MRI (F) showing locally restricted Gf uptake, to a histological section from this region showing macrophage infiltration in the dorsal column (G), and, finally, a micrograph of the spinal cord preparation before embedding which confirms Gf accumulation (appearing pink due to the coupled carbocyanide dye) exactly at the site of MR enhancement (H). On the left in (H) another large thoracic spinal cord lesion exhibits accumulation of pink Gf. Arrows again point to the corresponding axial T1-w MRI showing Gf-enhancement of almost the entire spinal cord cross area only sparing the left anterior part (I). The other arrows refer to corresponding histological sections with severe macrophage infiltration of almost all white matter tracts except in the left anterior part as revealed by ED1 antibody staining (J), and to a 5 μm serial paraffin section stained with luxol fast blue (K); note the reduced blue staining in areas of macrophage infiltration and Gf-enhancement indicating myelin loss by focal demyelination. Bars in B, C, D and G represent 200 μm; in J, K 500 μm.
the other ON did not. Accordingly, macrophage infiltration and myelin loss was restricted to the ON with enhancing lesions on MRI (Fig. 7D and E). In contrast, more distally both ON showed Gf-enhancement on MRI (Fig. 7B), Gf deposition (Fig. 7C) and inflammatory demyelination (Fig. 7D and E).

In a separate set of experiments we compared the morphological features of Gf-enhancing and non-enhancing tissue specimens. Overall, 78 spinal cord specimens from 14 EAE rats were collected, 35 from regions devoid of Gf accumulation and 43 from areas with macroscopically visible Gf accumulation. The nine specimens without evidence for macrophage infiltration as revealed by examining whole cross-sections stained with ab ED-1 did not show Gf accumulation (Fig. 8). All 43 Gf-positive specimens contained inflammatory EAE lesions, indicating that Gf-enhancement is specific. However, there was also mild to moderate inflammation in Gf-negative tissue blocks, while all severe lesions (8/8) showed Gf accumulation. Out of 23 mild lesions, 13 were Gf-negative, while more moderate lesions were Gf-positive than Gf-negative (25 versus 13) (Fig. 8). Figure 9 shows serial cryostat sections stained for macrophages by ED1-immunofluorescence and corresponding deposition of labelled Gf exhibiting a large overlap. Taken together these findings indicate that there was no false positive Gf-enhancement in MOG-EAE, but the sensitivity in lesion detection is still far below 100% when compared to histology, but significantly increased compared with Gd-DTPA (see quantitative analysis in Figs 4 and 5).

**Discussion**

In clinical practice the number of lesions on T2-w images is regarded as a measure for the overall disease burden in multiple sclerosis, and Gd-DTPA enhancement is taken as an indicator of acute disease activity. Our novel findings in an established animal model for multiple sclerosis indicate that the lesion burden is dramatically underestimated by the conventional MR-techniques. Cerebral lesion area as demonstrated with Gf-enhancement was significantly higher compared with the gold standard of T2-w MRI, and a large number of Gf-enhancing lesions were completely undetectable on T2-w images. The increased sensitivity of this new
MRI approach was even more evident by direct intraindividual comparison between conventional Gd-DTPA- and Gf-enhanced MRI since most Gf-enhancing lesions did not show Gd-DTPA uptake. In support of our experimental findings, comparative studies using autopsy and biopsy material from multiple sclerosis patients have revealed an intriguing discrepancy between T2-w lesion load and histologically verified tissue damage (Filippi et al., 2002).

In particular, visualization of spinal cord and ON lesions in multiple sclerosis patients may fail despite unambiguous clinical involvement (Bergers et al., 2002; Rocca et al., 2005). Since Gf showed strong focal contrast enhancement even in the spinal cord and ON lesions in MOG-EAE at only 1.5 T field strength, this novel technique has the potential to dramatically increase lesion detection in CNS inflammatory disorders including human multiple sclerosis.

Gd-DTPA has a lower molecular weight (MW) of 928 compared to Gf (MW 1528) (Misselwitz et al., 2004). Thus, if the mechanism of entry and local accumulation through a disrupted BBB were identical one would rather expect more extensive Gd-DTPA enhancement in MOG-EAE lesions than Gf-enhancement. Gf is an amphiphilic molecule revealing a high-binding affinity to albumin but not to other serum proteins such as low density lipoprotein (Meding et al., 2007). Under normal conditions, the complex of albumin and Gf formed in the endovascular compartment does not cross the intact BBB. Gf enters the CNS in MOG-EAE most likely by passive diffusion through a disrupted BBB. Gf could in principle also have gained access to the CNS by an active crossing mechanism through the intact endothelium, but there is no evidence for this alternative explanation.

A recent biochemical study provides a possible explanation for the persistence of Gf-enhancement in the inflammatory lesions (Meding et al., 2007). In addition to albumin, Gf has a high-binding affinity to proteins of the extracellular matrix such as collagens I and IV, proteoglycan, decorin and tenascin (kD ~ 2 μM/l) (Meding et al., 2007). These components are likely to be exposed and modified by the inflammatory insult upon immigration of immune cells into the CNS. Specifically, activated T-cells and macrophages secrete matrix metalloproteinases (Naparstek et al., 1984) that induce enzymatic clips in the extracellular matrix components of the basal membrane (Madri and Graesser, 2000). Thus, Gf may dissociate from its albumin binds and interact with local extracellular matrix proteins thereby being locally trapped. Another local trapping mechanism of Gf may be phagocytosis of
Gf-labelled myelin debris by macrophages as seen in Wallerian degeneration (WD) of the peripheral nervous system (Bendszus et al., 2005). In WD, Gf accumulation was, however, mainly associated with extracellular structures and reversal of Gf-enhancement in nerves depended on the reversal of inflammation-related breakdown of the blood–nerve barrier by regeneration. Similar to most EAE lesions, degenerating nerves during WD did not show Gd-DTPA-enhancement. Most probably, the difference in Gd-DTPA and Gf-enhancement in both EAE lesions and WD is caused by different diffusion kinetics: Gd-DTPA enhancement is caused by a passive extravasation via a disturbed BBB along a concentration gradient. After the rapid renal excretion Gd-DTPA re-diffuses back to intravascular space along a reversed concentration gradient and, thus, does not accumulate at the foci of inflammation. Similarly, Gf bound to albumin most probably also enters the extracellular space along a disturbed BBB. In contrast to Gd-DTPA, however, Gf has a high-binding affinity to extracellular matrix proteins (Meding et al., 2007). Thereby, it gets trapped at these foci and can reach a higher local concentration than Gd-DTPA, thus enabling visualization on MRI.

Our initial histological analysis was focussed on identifying the underlying pathology of Gf-enhancing lesions which were selected for tissue processing. In all these preselected Gf-enhancing lesions we saw ED1-positive macrophages, albumin extravasation and myelin loss on tissue sections. In a second experimental set of experiments we asked whether Gf-enhancement is able to cover all lesions. Specimens of both Gf-positive and -negative spinal cord segments were embedded in paraffin and the extent of inflammation assessed on whole cross sections by ED1-immunocytochemistry. All specimens devoid of macrophages were negative for Gf, and, vice versa, all Gf-enhancing lesions showed inflammation indicating a 100% specificity.
Sensitivity, however, was much less. Lesions classified as severe by almost complete infiltration of the spinal cord cross area by macrophages always showed Gf accumulation macroscopically, while a considerable number of moderate and mild lesions were Gf-negative. Additional complex studies are necessary to further define the precise lesion characteristics in MOG-EAE that allow detection by Gf-enhanced MRI. Similarly, it remains unclear why most of these Gf-enhancing lesions escape detection by T2-w MRI.

In multiple sclerosis patients with a clinically isolated syndrome the diagnosis of multiple sclerosis can already be established upon demonstrating defined lesion numbers and characteristics on MRI and, as a consequence, immunomodulatory treatment may be installed early (Polman et al., 2005). Attempts to improve lesion detection by MRI included use of higher Gd-DTPA doses (Filippi et al., 2002), higher magnetic field strengths (Kangarlù et al., 2007), and novel high resolution MR-sequences (Pouwels et al., 2006). Development of novel contrast agents is another approach. Small and ultrasmall superparamagnetic iron particles (SPIO/USPIO) have been successfully used to follow macrophage infiltration in experimental disorders of the nervous system (Bendszus and Stoll, 2003; Kleinschnitz et al., 2003; Brochet et al., 2006). In multiple sclerosis, recent clinical trials clearly showed that Gd-DTPA enhancement and USPIO-enhancement are separate events (Dousset et al., 2006; Vellinga et al., 2008). It appears at least in most experimental settings that disruption of the BBB is not a prerequisite for the entry of iron laden cells into the nervous system (Kleinschnitz et al., 2003; Bendszus et al., 2007). Similar findings have been reported in clinical trials in multiple sclerosis (Vellinga et al., 2008). While SPIO/USPIO enhanced MRI mostly indicates recent macrophage infiltration Gf appears to label extracellular matrix proteins by its high-binding affinity which become accessible due to breakdown of the BBB. It will be interesting to further investigate the spatiotemporal relationship between BBB disturbances as revealed by Gf-enhancement and macrophage infiltration.

Our present study shows that the novel MR contrast agent Gf has the potential to dramatically improve sensitivity in lesion detection in CNS autoimmunity since even small ON and spinal cord lesions in rodents could be visualized on a clinical 1.5T MR scanner. The unique binding properties of Gf therefore hold promise for future clinical application in multiple sclerosis.

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References