8. A new USPIO contrast agent (P904), designed for clinical application, in EAE Lewis Rat


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In preparation
Abstract

Purpose: To investigate whether P904, a new magnetic resonance (MR) contrast agent based on ultra small super paramagnetic particles of iron oxide (USPIO) will show comparable contrast enhancement as Sinerem or perhaps shows other characteristics in experimental autoimmune encephalomyelitis (EAE), an established animal model for Multiple Sclerosis (MS). Defining what aspects of the disease process in EAE are visualized by P904 will be important regarding possible clinical application in MS.

Material and Methods: Five Lewis rats were immunized with EAE and administered P904 at disease onset (n=3) or disease peak (n=2). Ultra high field MRI exams using multiple contrasts were performed on three consecutive days to follow contrast development. After the last examination animals were sacrificed and brain and cervical lymph nodes were studied by immunohistology for inflammation and iron accumulation.

Results: Disease development showed large variation between animals regarding clinical symptoms. Contrast enhancement was observed in one animal at disease onset on a predilection location for EAE inflammation by a gadolinium constrast agent. After administration of P904, 24 hours later this area showed hypointense on T2* weighted images and T2* relaxation time maps. Immunohistology on cryosections of the brain did show inflammation, but no iron could be detected in the MR enhanced areas. However, iron clustering was observed on cryosections of cervical lymph nodes of all animals that were administered P904 at disease onset.

Conclusion: Although iron accumulations were seen in the cervical lymph nodes by histology, that may have resulted from drainage by the brain, this routing is not expected to be exclusive. Therefore with the given setup and administered P904 doses MRI contrast enhancement could not be confirmed.
Introduction

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS). Its pathology is characterized by impairment of the blood brain barrier (BBB) and leukocyte accumulation resulting in the formation of inflammatory lesions that may dissolve over time. Today, magnetic resonance imaging (MRI) is used to visualize lesions at various locations throughout the brain with and without the use of contrast agents(1). The use of gadolinium based contrast agents has significantly improved the diagnostics of MS and has contributed to detection of more lesions. These contrast agents passively pass an impaired BBB, revealing inflammation in the brain and spinal cords by positive contrast on T₁-weighted (T₁w) sequences (2). This enhancement is considered to predominantly represent active inflammatory lesions, while lesions with less pronounced inflammation may not be enhanced by this method and appear dark on T₁w sequences, as so called black holes (3; 4).

Novel contrast agents based on iron oxides were developed to reveal other pathological events. Contrast induced by ultra-small super paramagnetic particles of iron-oxide (USPIO) was shown to co-localize with inflammation that was revealed by gadolinium, although not exclusively. It was also shown that gadolinium enhancement, caused by leakage of the contrast agent over an impaired BBB, preceded USPIO enhancement (5–8). Histological analysis of animals with experimental autoimmune encephalomyelitis (EAE), an established animal model for MS, injected with these iron particles revealed that the contrast agent is accumulated particularly in infiltrated macrophages within the lesions (5; 8–11). Typically, post contrast USPIO images are collected 24 hours after USPIO injected. It is believed that within this time frame USPIOs are phagocytised by blood born macrophages. These macrophages then migrate to neuroinflammatory tissue and become visible in the brain by MRI. In addition, clustering of USPIO inside lesions results in contrast enhancement on MRI. The observed patterns obtained after the administration of USPIO were complementary to the gadolinium enhanced contrast and may therefore be used as biomarkers to give insight in underlying pathology. One of the complementary effects resulted from the observation that not all gadolinium enhanced lesions showed USPIO enhancement. This suggests that USPIO enhanced MRI would visualize a different lesion type or lesion development stage.

Next to the mechanism of USPIO uptake by macrophages in the bloodstream, two other possible routes are considered for USPIO to reach inflammatory brain lesions. The first is the passive passage of free USPIO over an impaired BBB (7; 11–15). For long it remained unclear whether USPIO particles are able to pass the BBB passively in EAE. However, a study by Oude Engberink et al. (8) showed contrast enhancement within one hour after administration, that would confirm passive passage. It would suggest that BBB impairment and macrophage infiltration are independent processes during the disease stages. The second route would be uptake of USPIO by macrophages inside lesions or by activated microglia once they have passed the BBB (16).

Several of the described clinical results using USPIO contrast agents were found by use of Sinerem, a contrast agent with a neutrally charged dextran-coated iron oxide crystalline core with a mean hydrodynamic diameter of 20-30 nm (factsheet Guerbet). Its half time is 5 to 6 hours in rats and typically an incubation time of at least 24 hours is observed before imaging. These characteristics differ from super paramagnetic particles of iron oxide (SPIO) like Endorem with hydrodynamic diameters of 80-150 nm and typical half life values smaller than 6 minutes. Surprisingly contrast enhancement by Sinerem was already observed within 1.5 hours after administration in EAE implying that this agent passes a disturbed BBB (8). The agent was initially applied for imaging metastatic lymph nodes. At this moment, this agent is no longer available on
the market, due to efficacy reasons in cancer imaging as communicated by Guerbet (17).

A new agent, P904, was developed by Guerbet that has similar characteristics, but is coated by an amino alcohol that results in a charged coating. Its blood pool half life is lower (ca. 2.5 hours vs. 5-6 hours for Sinerem), while the hydrodynamic size is comparable to Sinerem (factsheet Guerbet). The in vitro uptake of P904 for macrophages is slightly higher after 24 hours of incubation compared to Sinerem (personal communication). However, no in vivo data is available yet on how this new agent performs in EAE rats. The coating differences, more specifically the charge, may result in the observed increased in vitro uptake by macrophages. This would enhance the efficacy of P904 compared to Sinerem.

A study by Floris et al. (13) showed that macrophage infiltration occurred after the first onset of symptoms in EAE and maximum macrophages presence was observed during disease peak by histology. However, presence of macrophages does not equal the influx of macrophages. The influx, which is believed to be visualized by USPIO enhanced MRI, may be low during peak of the disease, whereas the presence of macrophages can still remain high. This may question the exact time point of administration of USPIO. One could hypothesize that a “pure” macrophage contrast agent, which is not confounded by a possible leakage over an disrupted BBB, will be most effective during the maximal macrophage influx. With or without passive leakage therefore administration of P904 at disease onset would seem favourable to obtain maximum contrast enhancement.

The aim of this explorative study is to investigate whether this new contrast agent will perform comparable with Sinerem or perhaps shows other characteristics. Also trafficking of the contrast agent to the will be studied by histology. Defining what aspects of the disease process in EAE are visualized by P904 will be important regarding possible clinical application in MS.

Material and Methods
This animal study was approved by the local animal ethical committee. International guidelines regarding handling of laboratory animals were followed and local protocols for induction of EAE, anaesthesia, administration of contrast agents and MR imaging in rats were applied.

Procedures
EAE induction
In 17 male Lewis rats (252 - 264 grams, Charles River, the Netherlands) EAE was induced according to the protocol described by Schreibelt et al.(18). This protocol consisted of injection of a suspension of guinea pig myelin basic protein (MBP) in phosphate-buffered saline (PBS), complete Freund’s adjuvant (CFA, Difco Laboratories, Detroit, USA) and heat shocked Mycobacterium tuberculosis (37HRa) subcutaneously in one hind footpad under free breathing isoflurane (~2%) and Air/O₂ anaesthesia. After recovery, animals were kept under standard housing conditions and given food and water ad libitum. The day of immunization was defined as day 1.

Animals were daily monitored and checked for behaviour, condition and weight as well as other EAE induced clinical symptoms. These symptoms were scored from 1 to 5: 0, no clinical signs; 0.5, partial loss of tail tonus; 1, complete loss of tail tonus; 2, unsteady gait; 2.5, partial hind limb paralysis; 3, complete hind limb paralysis; 4, paralysis of the complete lower part of the body up to the diaphragm; 5, death due to EAE. According to previous experiences with this protocol, disease onset is expected at day 10 after immunization, showing the first clinical symptoms. At
day 14 disease peak is then encountered with maximum clinical scores. A score of 3 for three successive day as well as scores higher than 3 were considered as ethical end points.

**Immunization protocol optimization**
Three groups of four animals were used to optimize the amount of MBP necessary to induce EAE while not exceeding humane endpoints regarding clinical symptoms. Group 1 received 10 μg MPB, group 2, 15 μg MBP and group 3, 20 μg MBP (the standard dose normally applied for animals provided by supplier Harlan). All other conditions were kept the same for all animals.

**Contrast agent administration**
In this study we used the P904 MRI contrast agent (Guerbet, France) that consists of particles with a crystalline core of iron oxide with a 25-30 nm hydrodynamic diameter and a blood pool half-life of 145 minutes. Also a Gadolinium contrast agent (Gadovist, Bayer, Germany) was used to confirm BBB leakage. Both agents were administered intravenously through a canulated tail vein. Gadolinium was given during each MRI examination to obtain pre and post-administration images within each session. P904 was applied immediately after the first MRI examination as the active uptake of the agent by macrophages and trafficking into the brain was expected to take many hours. Table 2 shows administered concentrations per animal. P904 concentrations represent concentrations of iron.

**MR imaging**

Setup
Animals were examined in a 9.4T horizontal bore Varian MRI system (Varian, Palo Alto, California, USA) using an 72 mm quadrature volume coil (Rapid Biomedical, Würzburg-Rimpar, Germany) and an actively decoupled quadrature surface coil (Rapid Biomedical, Würzburg-Rimpar, Germany) for respectively transmission and signal detection. After the animals were anesthetized they were endotracheally intubated and the tail vein was canulated for MR contrast agent injections. Animals were fixated on a dedicated cradle in the bore using a stereotactic holder. Mechanical ventilation was performed using isoflurane (2%) and a mixture of Air/O₂ (70/30). During sedation animals were monitored for expiratory CO₂, heart rate and oxygenation. Body temperature was monitored by a rectal probe and controlled through a heated water pad.

Acquisition
Scan positions were outlined on transversal scout images with a central slice just caudal of the cerebellum. For all 2D sequences, T₂w, T₂⁺w and T₁w, 31 slices were acquired with an in plane resolution of 0.12x0.25mm and a slice thickness of 0.75 mm. Sequence details are shown in table 1. A T₁ map was acquired by using multiple 3D sequences (T₁m) with different flip angles (19) with a voxel size of 0.25x0.25x0.25 mm³. Data from the T₁w and T₁⁺w sequence were multi-echo and could also be used to obtain T₂ and T₂⁺ relaxation times respectively . Other sequence parameters are listed in table 1. Every MRI session finished with T₁ weighted imaging with a pre- and post-contrast administration of Gadolinium (10 minutes in circulation). Animals underwent MRI examinations on three successive days: from day 10 (disease onset, n=3) to 12 or from day 13 to 15 (disease peak, n=2). P904 was administered either at day 10 post immunization (first group) or day 12 (second group)
Table 1
MR Sequence parameters

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<th>Contrast</th>
<th>Seq</th>
<th>TR</th>
<th>TE</th>
<th>FA</th>
<th>Matrix</th>
<th>FOV</th>
<th>nex</th>
<th>ns</th>
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</thead>
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<td>5.1-40.</td>
<td>90</td>
<td>256x128</td>
<td>32x32x0.75</td>
<td>2</td>
<td>31</td>
</tr>
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<td>2750</td>
<td>6-82.5</td>
<td>90</td>
<td>256x128</td>
<td>32x32x0.75</td>
<td>2</td>
<td>31</td>
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<tr>
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<td>GE</td>
<td>1600</td>
<td>4</td>
<td>90</td>
<td>256x128</td>
<td>32x32x0.75</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
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<td>2.5</td>
<td>4,18,26</td>
<td>256x128x128</td>
<td>64x32x32</td>
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</table>

Post-processing

Image data was used to calculate T₁-, T₂- and T₂*-maps (DESPOT1 (19) or echo time array) by in-house programmed tools. The percentual increase of T₂<sub>w</sub> signal intensity, as a result of GdDTPA presence, was also calculated.

Analysis

MRI

On both qualitative and quantitative image types USPIO and gadolinium contrast enhancement was analyzed by comparing data from different imaging days. T₂<sub>w</sub> and T₂*<sub>w</sub> images as well as data from relaxation time maps were analyzed using mean intensities of regions of interest (ROI) in areas with clear USPIO presence.

Immunohistochemistry

Immediately after the last MRI exam, animals were sacrificed. The brain and cervical lymph nodes were removed and snap-frozen in liquid nitrogen. All samples were then stored at -80°C. Parts of the brain were cut into 5 and 10 μm cryosections (-16°C) and fixed in acetone for 10 min. Half of the sections were then stained for inflammation by incubation in PBS with the monoclonal antibody ED1 that recognizes (infiltrated) monocytes. Procedures were followed as described by Oude Engberink et al (20): Monocyte infiltration was detected by incubation of the sections with the monocyte/ macrophage marker ED1 (1/100, Serotec, Oxfordshire, UK) for 1 hour at 20°C, followed by a rabbit -α- mouse IgG-peroxidase conjugate (1 μg/ml) second antibody. The procedure was finished by showing peroxidase activity.

The other half of the cryosections received a Perls’ Prussian blue staining to show iron accumulation. Therefore the samples were incubated for 30 minutes with fresh Perl’s solution (1:1 v/v of 2N HCl and 2% ferrocyanide in ultra pure water). Then samples were rinsed and counterstained by nuclear fast red for 5 minutes. Samples were qualitatively analyzed by microscopy for presence of infiltrated monocytes and iron particles.

Results

EAE induction

Figure 1 shows the results from an experiment to optimize the concentration of MBP on EAE induction for clinical symptoms and weight. Historically, a concentration of 20 μg MBP was used to induce EAE in animals provided by Harlan. At day 12 the experiment was ended for group 3 (20 μg MBP) for ethical reasons as 3 out of 4 animals reached score 4. Disease onset was also
earlier for group 3 than for the other groups. As the development of clinical symptoms for group 1 (10 μg MBP) matched expected results, this was considered a suitable dose to continue EAE induction in the other animals for the P904 uptake experiments. Results are shown in figure 1.

Figure 1, Animal group weight and clinical score after EAE induction. Horizontal axis shows the number of days after EAE immunization while the vertical axes show mean weight (left) and mean clinical score (right) for 3 different groups: 50% of the normal dose (_10), 75% (_15) and 100% (_20).

**MRI**

Two animals (A1, A2) were induced with a suspension with 20 μg MBP and scanned from day 13 p.i. (one day before expected disease peak). Progression of EAE in these animals was more severe than initially anticipated and subsequently the MBP immunization protocol was optimized after this initial experiment. The animals examined at disease onset (B1, B2, B3) were immunized with 10 μg MBP in suspension after optimization of the immunization protocol and scanned from day 10 after induction (expected disease onset). Animals A1 and A2 showed very severe symptoms at disease peak (up to score 4), while animals B1-3 only showed mild symptoms after day 10 (see table 2 for animal details). After the first MRI exam, rats A1 and A2 were administered P904 with 0.3 μmol Fe/g respectively. Animal A2 received an extra dose of 0.6 μmol Fe/g P904 after the second MRI exam. B1 and B2 were administered P904 with 0.6 μmol Fe/g Fe while rat B3 received 0.3 μmol/g Fe. These concentrations were comparable with Sinerem.

**Disease peak imaging**

Just before MR examinations on day 14 animal A1 died and successive experiments could not be performed. Animal A2 died during the third scan session. Acquired images did not show hyper or hypo intensities that could represent lesions, also no contrast enhancement was observed, even not after administration of the second higher dose of P904 in animal A2.

**Disease onset imaging**

Animals B1-3 were immunized with 10 μg MBP in suspension. EAE developed more slowly and at the first day of MRI examinations (day10) the animals only showed weight loss. At day 11 and 12 experiments were repeated including gadolinium administration. MR images from animals B1 and B3 did not show any contrast enhancement or obvious inflammation nor for P904 neither for gadolinium.
These animals had a maximum score of 1 for clinical symptoms at day 12. Rat B2 developed more severe symptoms from day 10. The first MRI exam showed gadolinium contrast enhancement on T₁w images. From day 11, 24 hours after P904 administration, a hypo intense area was barely visible on T2w image but particular visible on the T2*-w images as well as on T₂ and T₂*-maps (see figure 2). The hypo intense area co-localized with the gadolinium difference images on day 10 (top row figure 2).

Analyses of the T₂* maps of the hypointense area in rat B2 showed statistically significant changes over time (day 10-12, figure 3). Analyses showed that GdDTPA leakage was significant maximal before the administration of P904 (day 10). Quantification of other image types did not show lesions or statistically significant contrast agent enhancement. All animals (B1-3) lost weight as expected during the development of EAE (-11.5 ± 1 g/day), from day 10-12.

![Figure 2](image-url)

**Figure 2**, T₁w_post-T₁w_pre, T₁-map, T₂w, T₂-map, T₂*-w, T₂*-map MR images of rat B2 for day 10-12 showing the development of a hypointense area on day 11 (arrows), which almost returned to iso-intensity on day 12. Gd enhancement preceded USPIO enhancement as can be observed on ΔT₁w on day 10 (open arrow). Note: T₁ map image for day 11 was not available.
Figure 3, ROI analysis of the hypo intense area shown in figure 2 shows significant decrease of T2* relaxation times on day 11 and 12. ROI analysis of percentual GdDTPA T1w difference images showed only contrast enhancement before administration of P904, however no enhancement on day 11 and 12.

Table 2, Clinical scores[-], relative weights [%] and P904 administration of animals A1, A2 and B1-B3 from day 10 (expected disease onset) to day 15

<table>
<thead>
<tr>
<th>Animal</th>
<th>Day 10</th>
<th>Day 11</th>
<th>Day 12</th>
<th>Day 13</th>
<th>Day 14</th>
<th>Day 15</th>
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<tbody>
<tr>
<td></td>
<td>Scr. RW [-] [%]</td>
<td>P904 [μmol Fe/g]</td>
<td>Scr. RW [-] [%]</td>
<td>Scr. RW [-] [%]</td>
<td>P904 [μmol Fe/g]</td>
<td>Scr. RW [-] [%]</td>
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<td>Peak</td>
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<td>98</td>
<td>3</td>
<td>93</td>
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<td>3</td>
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<tr>
<td>Onset</td>
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<td>4</td>
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<td>B3</td>
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<td>0.3</td>
<td>0</td>
<td>104</td>
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</table>

Scr.: Score [-], RW: Relative Weight (based on weight at immunization) [%]
† Animal died before MR experiment
* Animal received an additional dose P904 (0.6 μmol/g Fe)
✝✝ Animal died during MR experiment

Immunohistochemistry
Results of both ED1 as a marker for monocytes and Perls’ Prussian blue staining for iron are shown in figure 4 for the brain and in figure 5 for the cervical lymph nodes. Inflammatory areas were visible in a number of brain sections, however no iron could be shown by the Perls’ staining. Cervical lymph nodes did show presence of iron particles on several locations. Besides iron clustering also diffuse staining could be seen.
Figure 4, Examples of Prussian Blue staining: A: Rat B2, B,C: Rat B3 and ED1 staining for macrophages: D: Rat B1, E, F: Rat B2.
Prussian Blue staining does not show any iron particles, while ED1 staining shows inflammation (by macrophages)

Figure 5, Examples of Prussian Blue staining of cervical lymph nodes of rat B2 (A-C) and B3 (D) showing iron clustering and diffuse staining (arrows in C and D)
Discussion

Development of EAE
The animals used in these experiments reacted differently to the EAE induction protocol as was observed in previous studies (8). Factsheets from both providers show sensitivity for EAE but provider Charles River describes this as highly sensitive, which is in agreement with our experiments. While both providers supply animals that originate from the same inbred strain dating back to the seventies, changes in phenotype are likely. Possible explanations for the difference in EAE sensitivity may be found in spontaneous developed differences over time or may be due to housing protocols. Examples of growth charts show that male Lewis rat from Charles River animals have a 25% higher weight after 12 weeks compared to Harlan animals (21; 22).

To be able to work with these animals out of a ethical and practical point of view, the protocol was optimized by changing the MBP dose between 10 μg and 20 μg MBP. The lowest dose of 10 μg resembled the expected disease course regarding clinical symptoms observed in the 20 μg MBP immunized animals provided by Harlan (13). Lower doses were not applied, as it was expected to increase the risk of animals not developing EAE at all (23; 24). Regarding our observations for animals B1 and B3 late onset may have occurred.

MRI Contrast
From five animals examined by MRI, only one showed clear P904 related abnormalities in the brain. In this animal a double dose of P904 (compared to standard concentrations used for Sinerem) was given at disease onset. Diffuse abnormalities were visible on MR images acquired after administration of P904 as well as gadolinium. Contrast analysis showed enhancement for gadolinium before the administration of P904, and significant lowering of T₂* relaxation time values from 24 hours after P904 administration. The abnormalities were best appreciated on the T2*w images. T2w images, which are less sensitive for iron presence, almost showed no USPIO presence.

Compared to Sinerem USPIO, P904 induced abnormalities were less outspoken. With Sinerem MRI abnormalities could already be visualized with the less sensitive T2w sequence (8; 13). The animals developed some typical clinical disease manifestations like (partial) paralysis which confirms the preferential attack on the spinal cord and cerebellum. The diffuse hypo-intensities observed in the animal are therefore expected to represent inflammatory areas. In other studies besides diffuse abnormalities also focal loci of inflammation were seen (8).

Immunohistochemistry
ED1 staining on cryosections of the brains of the animals showed a more diffuse inflammation type (figure 4) as compared to the historical experience that was gained on working with animals provided by Harlan. However, while (diffuse) inflammation corresponded with contrast enhancement on MR images, Perl’s staining did not show presence of iron particles in these areas.

Staining of cryosections of the cervical lymph nodes (figure 5) showed presence of iron concentrations. Although drainage of those particle from the brain to the cervical lymph nodes may have occurred, this routing is not expected to be exclusive. Therefore only presence of the
particles in the bloodstream can be confirmed but not trafficking into the brain parenchyma. P904 was presented by Guerbet as an alternative for Sinerem that was able to enter the brain within an hour after administration and co-localized with areas of impaired BBB (8). In vitro studies showed that small particles of iron oxide (SPIO) had an improved cellular uptake compared to smaller particles like USPIO (20; 25). However, in these studies dextran coated particles were used. We therefore think that coating properties may have a large influence on the ability of macrophages to phagocytise the particles.

Other studies showed that not all UPSIO agents were able to induce contrast in EAE: e.g. Manninger et al. (26) showed in humans, with a lower dose, that for several diseases uptake of ferumoxtran-10 was different and is possibly strongly dependent on both timing and concentration of administration during disease progression. Finding contrast enhancement in only one animal, using a double dose, we think effective concentrations may be even higher than the dose we applied. Creating a high concentration of USPIO in the blood during a prolonged period (using the increased half time compared to Sinerem) could then result in increased concentrations of USPIO laden macrophages. This effect may be created by a single high dose (e.g. triple or quadruple doses) or repeated administrations in days before imaging and should then be tested in a group of animals presenting consistent and comparable disease symptoms. In another study in ApoE -/- mice, concentrations up to 1 μmol Fe/g were used (15). However, these concentrations may result in background enhancement due to circulating contrast agent in the blood which should be considered.

Although previous models showed that disease onset shows some variation, severity of clinical symptoms should not increase above score 3 to be able to perform repeated MR examinations on successive days. At the moment we have no reason to explain the observed heterogeneity in development of EAE.

In conclusion, our experiments showed iron accumulation in the cervical lymph nodes of EAE rats, but no confirmation of uptake by the brain could be shown histologically. MRI did show contrast enhancement on predilection sites for EAE. However, the effect observed in a single animal is not conclusive.

Acknowledgements
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References


