Pluriformity of inflammation in multiple sclerosis shown by ultra-small iron oxide particle enhancement

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Gadolinium-DTPA (Gd-DTPA) is routinely used as a marker for inflammation in MRI to visualize breakdown of the blood–brain barrier (BBB) in multiple sclerosis. Recent data suggest that ultra-small superparamagnetic particles of iron oxide (USPIO) can be used to visualize cellular infiltration, another aspect of inflammation. This project aimed to compare the novel USPIO particle SHU555C to the longitudinal pattern of Gd-DTPA enhancement in multiple sclerosis. Nineteen relapsing-remitting patients were screened monthly using Gd-enhanced MRI. In case of new enhancing lesions, USPIO were injected and 24 h later, MRI was performed and blood was collected to confirm USPIO loading of circulating monocytes. Lesion development was monitored by 3 monthly Gd-DTPA-enhanced scans and a final scan 7–11 months after injection. USPIO-enhancement was observed as hyperintensity on T1-weighted images, whereas no signal changes were observed on T2-weighted-gradient-echo images. In 14 patients with disease activity, 188 USPIO-positive lesions were seen, 144 of which were Gd-negative. By contrast, there were a total of 59 Gd-positive lesions, 15 of which were USPIO negative. Three patterns of USPIO-enhancement were seen: (i) focal enhancement; (ii) ring-like enhancement and (iii) return to isointensity of a previously hypointense lesion. The latter pattern was most frequently observed for lesions that turned out to be transiently hypointense on follow-up scans, and ring-enhancing lesions were less likely to evolve into black holes at follow-up than lesions without ring-like USPIO-enhancement; we speculate this to be associated with repair. In 4% of the USPIO-positive/Gd negative lesions, USPIO-enhancement preceded Gd-enhancement by 1 month. USPIO-enhancement remained visible for up to 3 months in 1.5% of all USPIO-positive lesions. In 29% of the lesions enhancing with both contrast agents, USPIO-enhancement persisted whereas Gd-enhancement had already resolved. In conclusion, the new nano-particle SHU555C provides complementary information to Gd-enhanced MRI, probably related to monocyte infiltration. The use of USPIO-enhanced MRI is likely to lead to more insight in the pluriformity of inflammation in multiple sclerosis.

Keywords: MS; USPIO; cellular imaging; MRI; lesional patterns

Abbreviations: BBB = blood–brain barrier; BW = body weight; EAE = experimental allergic encephalomyelitis; EDSS = expanded disability status scale; Gd = gadolinium-diethylene-triamine pentaacetic acid (Gd-DTPA); Gd+ = Gd-DTPA-positive; Gd− = Gd-DTPA-negative; nm = nanometre; PB = Prussian blue; PBMC = peripheral blood mononuclear cells; PD = proton density; ROI = region of interest; ROS = reactive oxygen species; T1-w = T1-weighted; T2(-w) = T2-weighted gradient echo; TR = repetition time; TE = echo time; USPIO = ultra-small superparamagnetic particles of iron oxide; USPIO+ = USPIO-positive; USPIO− = USPIO-negative

**Introduction**

Multiple sclerosis is a multifocal disease of the CNS, characterized by inflammation, demyelination and axonal loss. Although MRI is highly sensitive in detecting multiple sclerosis lesions, it lacks histopathological specificity. The current MRI marker for inflammation is gadolinium-diethylene-triamine-penta-acetic-acid (Gd-DTPA), which visualizes blood–brain barrier (BBB) leakage (Grossman et al., 1988), occurring as a result of inflammation, but not inflammation in itself.

New contrast agents based on ultra-small superparamagnetic particles of iron oxide (USPIO) have recently been developed for clinical MRI. Their superparamagnetic iron oxide core decreases T1- and T2-relaxation times of water molecules, inducing signal increase on T1-weighted images (T1-w) and signal reduction on T2-weighted-gradient-echo (T2-g-e) images (Corot et al., 2006). Phagocytosis of USPIO by cells of the monocyte/macrophage system visualizes cellular infiltration in diseases with high macrophage activity (Corot et al., 2006). Thus, USPIO-enhancement, reflecting cellular infiltration, may complement Gd-DTPA enhancement in visualizing cellular aspects of inflammation in multiple sclerosis.

Most animal and human studies exploring USPIO in inflammatory CNS diseases have used ferumoxtran-10, (Dousset et al., 1999b; Floris et al., 2004; Rausch et al., 2004; Brochet et al., 2006), although other compounds were previously studied (Xu et al., 1998). In experimental allergic encephalomyelitis (EAE), USPIO enhancement was hypointense on T2-g-e images. Immunohistochemical analysis revealed USPIO in infiltrated monocytes in inflammatory lesions (Floris et al., 2004) (Dousset et al., 1999a). USPIO-enhancement patterns differed from Gd-enhancement in time, demonstrating that BBB leakage, as shown by Gd-enhancement, and cellular infiltration as shown by USPIO-enhancement are two separate mechanisms that can be distinguished in vivo (Rausch et al., 2003; Floris et al., 2004; Rausch et al., 2004; Bendszus et al., 2005; Dousset et al., 2006). The clinical relevance of this distinction is emphasized by data showing that USPIO-enhancement correlated with disability, axonal loss and response to therapy (Floris et al., 2004; Rausch et al., 2004; Brochet et al., 2006).

In humans, ferumoxtran-10 demonstrated the presence of phagocytic cells in the CNS in multiple sclerosis (Dousset et al., 2006) (Manninger et al., 2005), stroke (Saleh et al., 2004; Nighoghossian et al., 2007) and intracranial tumours (Neuwelt et al., 2004). USPIO-enhancement was hyperintense on T1-w images, matching with the expected distribution of macrophages, but not always with Gd-enhancement. This may suggest that USPIO are more specific for cellular infiltration. Immunohistochemical staining of USPIO-enhancing tumours demonstrated USPIO particles in macrophages and astrocytes (Neuwelt et al., 2004).

The present study aimed at visualizing cellular infiltration in multiple sclerosis lesions, using a novel USPIO particle, SHU555C, which is smaller than ferumoxtran-10 (25 versus 30 nm), has a shorter half-life (6–8 versus 24–30 h), and is negatively charged which has shown to enhance uptake by activated monocytes in vitro (Metz et al., 2004). USPIO-enhancement of multiple sclerosis lesions was compared to Gd-enhancement both in space and in time, and labelling of monocytes was confirmed in blood samples.

**Methods**

**Design**

In this clinical phase II study, 19 relapsing-remitting multiple sclerosis patients with active disease were included. The protocol was approved by the local ethics review board; all subjects gave informed consent. Subjects underwent monthly Gd-enhanced brain MRI screening for Gd-enhancing multiple sclerosis lesions. If present, USPIO were injected within 24–48 h, followed by another MRI 24 h after USPIO injection. Blood was then withdrawn to evaluate liver and kidney functions, and monocyte activity and labelling. Follow-up consisted of 3 monthly scans and one long-term follow-up scan 7–11 months after injection. Treatment status, relapses and adverse events were registered and disability was measured using the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983).

**Image acquisition**

For every scan, the same protocol was used on a 1.5T MR scanner (Siemens Vision, Erlangen, Germany): axial T1-w spin-echo before and after Gd-DTPA-administration (Magnevist®, Schering, Berlin, Germany, 0.2 ml/kg BW) (TR/TE 830/15; two acquisitions), dual-echo T2-w spin-echo (TR/TE 3837/1698; one acquisition), and T2 gradient-echo (T2-g-e) (TR/TE 613/27; one acquisition). In-plane resolution was 1 x 1 mm², slice thickness 4 mm. For the scan 24 h after USPIO-administration [SHU555C, Schering, Berlin (Germany), diameter: 25 nm, T1/2: 6–8 h, 40 μmol Fe/kgBW at 5 ml/s] no Gd was administered.

**Image analysis**

USPIO-enhancement was marked on consensus on post-USPIO T1-w images, using pre-USPIO T2- and PD-images for lesion identification, and pre-Gd T1-w images for comparison, blinded to post-Gd images. Then, Gd-enhancement was marked. T1-hypointensity of lesions was determined as described (van Walderveen et al., 1999) on historic, baseline and long-term-follow-up scans blinded to post-USPIO images. According to longitudinal appearance, black holes were classified as follows: ‘chronic black holes’ were hypointense on historical MRI and remained so throughout the study period. ‘Acute and persistent black holes’ were hypointense at USPIO-injection and follow-up. ‘Transiently, T1-hypointense lesions’ were hypointense around USPIO-injection, but neither on historical nor on long-term follow-up MRI. ‘USPIO-enhanced isointensity of a T1-hypointense lesion’ was defined by two criteria: firstly, post-USPIO signal intensity was in the range of signal intensity of the surrounding white matter, secondly, signal intensity of the lesion had changed at least 2 SD more than signal intensity of the surrounding white matter,
compared to pre-USPIO images. To determine associations between ring-like USPIO-enhancement and longitudinal T1 patterns of lesions, a control group of USPIO-negative (USPIO−) T2 lesions was created blinded for hypointensity status, after which their T1 pattern was analysed.

**Cell assays**
Peripheral blood mononuclear cells (PBMC) were collected 24 h after USPIO injection as described (Oude Engberink et al., 2007). Production of reactive oxygen species (ROS) by isolated monocytes was measured as a marker for cell activation using dihydrorhodamine (Schreibelt et al., 2006). Cell viability was routinely checked by 7-aminoactinomycin D (7AAD, Molecular Probes, Eugene, OR, USA) exclusion and the percentage of monocytes was determined by the number of CD14 (BD Pharmingen) positive cells using a FACScan flow cytometer (Calibur, Becton Dickinson, Mountain view, CA, USA). Cell spots were prepared from PBMC to detect the presence of intracellular USPIO by Prussian blue (PB) staining (Oude Engberink et al., 2007).

**Statistical analysis**
Pearson’s Chi-Square test was used to determine associations between USPIO-enhancement patterns and T1-hypointensity patterns, with a risk estimate for ring-like USPIO-enhancement.

**Results**

**Patients**
Five patients displayed no Gd-DTPA enhancing lesions within 5 months after inclusion, and therefore did not receive USPIO injection. Of the 14 patients receiving SHU555C [five males, nine females, median EDSS at inclusion: 3.0 (range 1.5–5.0)], the mean disease duration at the time of USPIO injection was 4.2 years (range 0.3–16) and the mean time from most recent relapse to USPIO injection was 16 months (range 0–84). Nine were on immunomodulatory treatment. No patients developed any adverse events.

**Three patterns of USPIO-enhancement**
USPIO-enhancement was hyperintense on T1-w images. On T2-w and T2* w sequences, no signal changes were observed in any of the lesions, but blood vessels appeared slightly hypointense. In the 14 patients given USPIO, there were 59 Gd+ lesions and 188 USPIO+ lesions (Table 1). Forty-four out of 59 Gd+ lesions were USPIO+; conversely, 144/188 of the USPIO+ lesions were Gd−. USPIO-enhancement occurred in three different patterns (Figs 1 and 2): focal enhancement, ring-like enhancement (mostly around pre-existing T2-hyperintense lesions) and return to isointensity of lesions that were hypointense on pre-contrast T1-w images. Each type of USPIO+ lesions could be either Gd+ or Gd−, but Gd enhancement was less common (6/70; 9%) in ring-like lesions than in lesions that were focal or returned to isointensity (24/79; 30% and 14/39; 36%, respectively). Use of immunomodulatory treatment did not appear to relate to the different patterns of enhancement (data not shown).

‘Return to isointensity’ was mostly detected in transiently T1-hypointense lesions
In total, 200 T1-hypointense lesions were observed around the time of the USPIO injection, which on follow-up could be classified as either chronic, or acute and persistent or transient (Table 2). Only eight (6%) of the 127 chronic black holes and four (13%) of the 32 acute and persistent black holes showed a return to isointensity pattern. In contrast, 27 (66%) of the 41 transiently T1-hypointense lesions, appeared isointense on post-USPIO images. There was a significant association between T1-patterns and USPIO-enhancement patterns of these lesions (P<0.001; Table 2). Hypointense lesions showing enhancement with both contrast agents were mainly transiently hypointense (12/14), whereas non-enhancing black holes were mostly chronic or acute and persistent (138/151; P<0.001; Table 3).

**Ring-like USPIO-enhancement**
Of all lesions showing ring-like USPIO-enhancement (n=70), 31% were hypointense around the time of USPIO imaging, similar to 33% of the control-group (n=76) of USPIO-negative T2 lesions (see Methods section). However, at long-term follow-up, 13% of the ring-enhancing lesions had evolved into chronic black holes, compared to 28% of the control lesions (OR 0.38, CI 0.16–0.90, P = 0.025).

**Long-term USPIO-enhancement**
After 1 month, 85% of the originally USPIO+/Gd− lesions showed no enhancement with either contrast agent; 11% of the lesions were still USPIO+/Gd−, and 4% had become Gd+ (see Fig. 3). Of the 15 USPIO−/Gd+ lesions, three (20%) were still Gd+ after 1 month, the remaining 12 (80%) showing no enhancement. For the 44 USPIO+/Gd+ lesions, 45% showed no enhancement with either contrast

<table>
<thead>
<tr>
<th>USPIO-enhancement</th>
<th>Gd-enhancement</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>144 (77%)</td>
<td>188</td>
</tr>
<tr>
<td>Focal</td>
<td>55 (70%)</td>
<td>79</td>
</tr>
<tr>
<td>Ring-like</td>
<td>64 (91%)</td>
<td>70</td>
</tr>
<tr>
<td>Return to isointensity</td>
<td>25 (64%)</td>
<td>39</td>
</tr>
<tr>
<td>No</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>144 (71%)</td>
<td>203</td>
</tr>
</tbody>
</table>

Italicized data represent subgroups under “yes”.

Table I Cross-sectional USPIO and Gd-enhancement of MS lesions: numbers of lesions in different enhancement patterns

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agent after 1 month, but 12% still enhanced with both agents. Twenty-nine percent enhanced with USPIO only, and 14% showed only Gd-enhancement. After 2 months, 92% of the 188 originally USPIO+ and/or Gd+ lesions did not enhance anymore, and after 3 months, only 1.5% of these lesions still appeared USPIO+. In these cases, USPIO-enhancement had completely resolved at long-term follow-up.

**USPIO are taken up by monocytes in the bloodstream**

Intracellular clusters of USPIO were detected after incubation in vitro as shown by PB staining. Qualitative analysis of the PB staining on patient PBMC obtained 24h after USPIO-injection revealed several iron-positive cells, providing evidence of cellular incorporation of USPIO in
monocytes in the circulation (Fig. 4). The monocyte fraction in patient PBMC 24 h after USPIO injection showed no elevated levels of ROS, indicating that USPIO uptake does not activate monocytes.

**Discussion**

This is the first study describing SHU555C lesion enhancement in multiple sclerosis patients. USPIO-enhancement occurred more than Gd-DTPA enhancement, remained visible for a longer period than Gd-DTPA enhancement and, in some cases, preceded Gd-DTPA enhancement. Lesions enhancing with both contrast agents at baseline were more prone to continue enhancing at follow-up, compared to lesions enhancing with only one of the contrast agents. USPIO-enhancement occurred in three patterns: focally, ring-like and returning to isointensity after having been $T_1$-hypointense. Taken together, this indicates

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**Table 2** Longitudinal $T_1$ patterns (rows) of the 200 identified hypointense lesions around USPIO injection, related to patterns of USPIO-enhancement (columns) of these lesions

<table>
<thead>
<tr>
<th>$T_1$ pattern</th>
<th>Pattern of USPIO-enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Return to isointensity</td>
</tr>
<tr>
<td>Chronic</td>
<td>8 (6%)</td>
</tr>
<tr>
<td>Acute and persistent</td>
<td>4 (13%)</td>
</tr>
<tr>
<td>Transient</td>
<td>27 (66%)</td>
</tr>
<tr>
<td>Total</td>
<td>39 (19%)</td>
</tr>
</tbody>
</table>

**Table 3** Longitudinal $T_1$ patterns (rows) of the 200 identified hypointense lesions around USPIO injection, related to crosssectional USPIO-and Gd-DTPA enhancement status (columns) of these lesions

<table>
<thead>
<tr>
<th>$T_1$ pattern</th>
<th>USPIO/Gd-DTPA enhancement status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>USPIO+/Gd--</td>
</tr>
<tr>
<td>Chronic</td>
<td>15</td>
</tr>
<tr>
<td>Acute and persistent</td>
<td>4</td>
</tr>
<tr>
<td>Transient</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
</tr>
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</table>

**Fig. 3** An USPIO+/Gd-- lesion becoming Gd+ at 1 month follow-up. (A) Post-Gd image at time point around USPIO injection showing no lesion enhancement (a lesion was present on the $T_2$SE image at that time point). (B) Post-USPIO image showing focal USPIO-enhancement. (C) Post-Gd image 1 month after USPIO injection showing Gd- enhancement. (data not shown: on the pre-Gd $T_1$-w image at that time point, no focal enhancement was visible, meaning that this focal hyperintense enhancement is Gd-enhancement, instead of remaining USPIO-enhancement).
that USPIO-enhancement provides relevant information over Gd-DTPA enhancement, and illustrates the pluriformity of inflammation in multiple sclerosis.

We have good reasons to assume that USPIO-enhancement reflects entrance of monocytes into the CNS. Blood samples revealed incorporation of USPIO into PBMC in the bloodstream. The ability of USPIO to visualize macrophage infiltration on MRI has been shown previously (Kooi et al., 2003; Neuwelt et al., 2004; Brochet et al., 2006; Corot et al., 2006; Douset et al., 2006; Jander et al., 2007). The major challenge in validating USPIO as a marker for cellular infiltration in multiple sclerosis lies in ruling out that USPIO may reach the brain parenchyma in ways not specifically related to macrophage infiltration. Leakage of non-macrophage-incorporated USPIO over a damaged BBB might play a role. However, as in the current study 77% of USPIO+ lesions were found in areas with an intact BBB (as marked by absence of Gd-enhancement), and 25% of the lesions showing BBB leakage were USPIO−, our data suggest that USPIO-enhancement is subject to a different, probably cell-specific mechanism. On the other hand, Gd-enhancement may not be fully sensitive to BBB damage. For example, it is known that sensitivity depends on dosage (Silver et al., 2001), and subtle BBB changes that do not enhance with Gd may occur in normal-appearing white matter (Vos et al., 2005).

Spatial and temporal discrepancies between BBB leakage as demonstrated by Gd-enhancement, and cellular infiltration as demonstrated by USPIO-enhancement have been reported previously using ferumoxtran-10 (Sinerem®) (Manninger et al., 2005; Douset et al., 2006). These discrepancies were smaller than in the current study, where SHU555C was used. SHU555C differs from Sinerem in size, plasma half-life and ionic charge. In vitro, the smaller, negatively charged SHU555C particle was incorporated more efficiently by monocytes than Sinerem (Metz et al., 2004), but its shorter half-life time may counteract this advantage in vivo. The differences between our results and previous results using ferumoxtran-10 may also be explained by patient characteristics. Comparative in vivo studies are needed to further explore the differences between SHU555C and ferumoxtran-10.

It is tempting to speculate about the three patterns of USPIO-enhancement found in this study. When lesions were USPIO+/Gd+, enhancement usually occurred in a similar focal pattern; this may implicate the co-occurrence of active and passive BBB leakage. Return to isointensity of a previously hypointense lesion was not noticed before as a type of contrast enhancement. This ‘change to isointensity’ of black holes on post-USPIO images may selectively indicate the presence of USPIO in macrophages within these lesions. Interestingly, this pattern of enhancement was seen especially in temporarily hypointense T1 lesions compared to chronic, persistent black holes. While the latter are associated with matrix destruction and axonal loss, the temporarily T1-hypointense lesions may reflect remyelination (van Waesberghe et al., 1998; Rovira et al., 1999; Barkhof et al., 2003). If so, USPIO-enhancement may be associated with a beneficial aspect of inflammation, possibly associated with repair mechanisms (Hohlfeld, 2007). Of course this hypothesis should be validated in future studies, but it may also explain our finding that lesions showing ring-like USPIO-enhancement were less prone to evolving into chronic black holes compared to USPIO-negative lesions.

Considering all USPIO+ lesions longitudinally, we found that in 85% of these lesions, USPIO-enhancement had resolved after 1 month (USPIO were injected only once), but some lesions (1.5%) remained USPIO+ for up to 3 months. USPIO-enhancement remained visible after Gd-enhancement had resolved, and USPIO+/Gd− lesions were more prone to keep enhancing at follow-up than lesions that enhanced with only one contrast agent. In a fraction (4%) of the USPIO+/Gd− lesions, USPIO-enhancement preceded Gd-enhancement by 1 month. Assuming that USPIO-enhancement reflects macrophages in inflammatory lesions, these results suggest that cellular inflammation can both precede and persist longer than BBB leakage. The inverse question however, how often BBB breakdown precedes cellular infiltration, cannot be answered in this

Fig. 4 Microscopy image: iron (arrow) positive cells were detected in patient PBMC 24 h after USPIO injection as well, though at low concentrations.
study. Due to study design, patients were screened for Gd-DTPA enhancement of lesions, followed by USPIO injection if Gd-DTPA enhancement was observed. Therefore, by definition, Gd+ lesions were never present 1 month prior to USPIO injection. Future studies are warranted to address the exact temporal relationship between BBB breakdown and cellular infiltration.

The lack of signal decrease on post-USPIO $T_2$-w images was interesting, as this is the most commonly described form of USPIO-enhancement. In previous studies, both $T_2$-hypointense and $T_1$-hyperintense enhancement were seen (Neuwelt et al., 2004; Manninger et al., 2005; Dousset et al., 2006). $T_2$ signal decrease is a susceptibility effect caused by clustering of USPIO particles (Dousset et al., 1999b; Floris et al., 2004). The lack of $T_2$ effects may be explained by concentration and cellular incorporation dependent effects that have been described previously using other USPIO particles (Corot et al., 2006; Simon et al., 2006). In the current study, due to dosage restrictions, USPIO concentrations were low as compared to animal experiments, possibly explaining the presence of $T_1$ effects without $T_2$ signal changes.

In conclusion, we have demonstrated that USPIO-enhanced brain MRI in multiple sclerosis shows patterns distinct from Gd-enhancement. These patterns provide complementary insight into the underlying pathology and are therefore clinically relevant as potential MRI markers for disease severity and possibly treatment efficacy. Further investigation should elucidate how sensitivity and specificity for disease severity and possibly treatment efficacy. Further investigation should elucidate how sensitivity and specificity for disease severity and possibly treatment efficacy. Further investigation should elucidate how sensitivity and specificity for disease severity and possibly treatment efficacy.

Acknowledgements

The authors wish to acknowledge Dr. Bernard M.J. Uitdehaag (MS Centre Amsterdam, VU University Medical Centre, Department of Clinical Epidemiology and Biostatistics) for assistance with statistical analyses. The contrast agent was kindly provided free of charge by Bayer Schering Pharma, Berlin, Germany. This work was supported by Dutch MS Research Foundation (Vooruitzichten, the Netherlands), grant no. 02-358b.

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