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Magnetic starch microspheres: Interactions of a microsphere MR contrast medium with macrophages in vitro

Anne K. Fahlvik [1], Per Artursson [2] and Peter Edman [2]

[1] *Department of Pharmacology, Institute of Pharmacy, University of Oslo, Oslo (Norway) and* [2] *Department of Pharmaceutics, Uppsala University, Uppsala (Sweden)*

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Summary

The cellular effects of a reticuloendothelial contrast agent for MR imaging were examined in cultured murine macrophages (J774 cells). The macrophage handling and toxicity of iron oxide loaded starch microspheres (MSM) were evaluated by radiotracer studies, relaxation time analysis, electron microscopy and a cytotoxicity assay (MTT test). Macrophages exposed to MSM showed a concentration-dependent and saturable accumulation of the microspheres. MSM were intracellularly localized to the lysosomes. Relaxation time measurements indicated that the iron oxide in MSM was mobilized and eliminated from the cells. The cellular toxicity of MSM was investigated after various incubation times and no cytotoxicity of MSM or components of MSM was detected, whereas ionic iron administred directly to J744 cells showed high cellular toxicity. This study has shown that starch microspheres containing iron oxide are nontoxic to macrophages in vitro. Consequently, MSM is an attractive delivery system for targeting of MR contrast agents to the reticuloendothelial system.

Introduction

The clinical usefulness of magnetic resonance (MR) imaging as a diagnostic tool has increased as the advantages over other imaging techniques have been demonstrated. Although MR imaging shows excellent soft tissue contrast, the diagnostic value can be improved further by use of contrast agents. Potential MR contrast enhancers are substances with magnetic properties such as magnetic (ferromagnetic or superparamagnetic) iron oxides and paramagnetic metal ions.

During recent years there has been great interest in the use of particles and liposomes as MR contrast agents, especially for gastrointestinal (Lønnemark et al., 1988) and reticuloendothelial imaging (Burnett et al., 1985, Kabalka et al., 1987, Stark et al., 1988, Schwendener et al., 1989). Intravenously administered particles are mainly distributed to the liver and spleen due to macrophage uptake. The macrophages in these organs comprise about 90% of the total intravascular phagocytic capacity of the reticuloendothelial system (RES) (Saba, 1970). Hepatosplenic clearance of magnetic particles facilitates the detection of space-occupying lesions such as malignancies, cysts and abscesses. Since these formations do not con-

Correspondence: A.K. Fahlvik, Dept of Pharmacology and Toxicology, R & D Division, Nycomed Imaging AS, PB 4220 Torshov, N-0402 Oslo, Norway.

tain macrophages, they exclude the contrast agent and are distinguished from the normal tissue parenchyma.

The pharmacokinetics and contrast enhancement of intravenously injected iron oxide loaded starch microspheres have been evaluated in animal studies (Fahlvik et al., 1990a,b). The kinetics showed a typical distribution pattern for particulate matter and the administered iron oxide was exclusively observed in the reticuloendothelial cells of the liver and spleen. A time-dependent solubilization of iron oxide within the macrophages followed by incorporation of the dissolved iron into the body iron pool was observed. Doses from 1 to 3 mg microspheres/kg gave good contrast enhancement and the safety index of the substance was of the order of several hundred.

Although the magnetic microspheres seemed to be well tolerated in animals, the effect on macrophages needs to be studied further. In this investigation some cellular interactions of iron oxide loaded starch microspheres on macrophages are presented. The microsphere-related, as well as the iron-related toxic effects, are studied in macrophages in cell culture by a well-known cytotoxic assay.

Materials and Methods

Test substances

Magnetic starch microspheres (MSM) (Table 1) containing iron oxide (Fe_3O_4) were prepared from ferrous and ferric chloride and hydrolyzed starch as previously described (Fahlvik et al., 1990a). Degradable epichlorhydrin-crosslinked starch microspheres (DSM) were obtained from Pharmacia AB, Sweden. Polyacryl starch microspheres (PSM) were a gift from Dr Peter Stjärnquist, National Board of Health and Welfare, Uppsala, Sweden. Glucan particles (Lichenan; prepared from Cetraria islandica) and polystyrene particles (Latex beads) were purchased from Sigma, U.S.A. Iron oxide crystals were obtained from Nycomed Imaging AS, Norway. Hydrolyzed potato starch (PS1) was obtained from Stadex AB, Sweden; ferric chloride was obtained from Merck, F.R.G., and Macofer^R (saccharated iron oxide) was obtained from Hausmann, Switzerland. All test substances were dissolved or suspended in physiological saline or phosphate buffered saline (PBS) and sterilized.

TABLE 1

Characterization of microspheres

Microsphere	Matrix material	Size (μm)
MSM [a]	hydrolyzed starch	0.1–0.5
DSM [b]	crosslinked starch	1.0–1.8
PSM [c]	acryloylated starch	0.6–2
PS1	hydrolyzed starch	n.m. [d]
Lichenan	glucan	n.m. [d]
Latex	polystyrene	0.9

[a] MSM, magnetic starch microspheres.
[b] DSM, degradable starch microspheres.
[c] PSM, polyacryl starch microspheres.
[d] Not measured.

Cells

The murine macrophage cell line J774 used in these studies is described as a reticulum cell sarcoma with the morphology, adherence and phagocytic properties of macrophages (Ralph et al., 1975). J774 cells were seeded into 75 cm^2 cell culture flasks (Costar, U.S.A.) and grown in Dulbecco's modifided Eagles medium (Gibco, U.S.A.) with 10 fetal bovine serum, benzylpenicillin (100 U/ml) and streptomycin (10 μg/ml) at 37 °C and 10% CO_2.

Phagocytosis

The macrophages were suspended to give a concentration of 1×10^6 cells/3 ml and seeded on to circular plastic coverslips (Thermanox 25 mm diametre, Miles Scientific, U.S.A.) in tissue culture wells (35 mm diametre, Costar, U.S.A.) and cultivated for 24 h. The uptake capacity of the J774 cells was quantified by cellular exposure to ^{14}C-labelled DSM (1 mg microspheres/ml) for up to 4 h at 4 and 37 °C. Coadministration of 1 mg/ml of radiolabelled DSM and variable concentrations of MSM and unlabelled DSM (1–10 mg microspheres/ml) was performed, and the uptake measured after 5 h at 37 °C. The coverslips were washed in PBS and the macrophage-associated

radioactivity was determined by liquid scintillation counting.

MTT assay

The macrophage toxicity of the test compounds was determined with a modified MTT assay (Mosmann, 1983). The assay is based on the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,3,-dipentyltetrazolium bromide) to the blue formazan product by the mitochondrial enzyme succinate dehydrogenase of viable cells. To determine the relationship of cell number to formazan formation in unexposed cells, plates containing cell numbers increasing from 240 to 124000/well were prepared. The optical absorption as a function of the number of viable cells was linear within the range of 240–31 000 cells/well ($r = 0.9886$). According to these results, 15 000 and 5 000 cells/well were seeded for the 24 and 96 h experiments, respectively.

J774 cells were plated in the wells of 96-well flat-bottom microtiter plates (Flow Laboratories, U.S.A.) for 24 h cultivation. Serial dilutions of the various test compounds were prepared in cell culture medium and added to each well. Incubations were performed either for 24 or 96 h, or for 24 h followed by rinsing to remove excess of the test substance and further cultivation for 72 h. MTT (Sigma, U.S.A.) (5 mg/ml in cell culture medium) was then added, followed by an additional incubation period of 45 minutes, during which MTT was transformed to formazan. To achieve solubilization of the formed formazan, an acid isobutanol/sodium lauryl sulphate solubilizer was added (Tada et al., 1986). The absorbance was measured by a multiwell scanning spectrometer (Labsystem Multiscan MCC/340) using a wavelength of 590 mm. The absorbance levels from exposed cells were corrected against the absorbance values of untreated cells as well as against the baseline absorption of the test compounds. The ratio of the absorbance of the exposed cells to the control absorbance was equated with the surviving fraction of the cells. The IC_{50} value was definded as the concentration of the test compound which produced 50% reduction of development of formazan compared to the control values. Wells containing medium but no cells served as blanks.

Relaxation time measurements

The proton relaxation times are important contrast parameters in MR imaging. The mechanism of action of MR contrast agents is to shorten the relaxation times and thereby enhance the contrast of organs or compartments containing these substances. To estimate the cellular uptake and metabolism of magnetic iron oxide, the T_1 and T_2 relaxation times of MSM exposed cells were recorded. Low concentrations of iron oxide selectively decrease T_2 while higher amounts will reduce both T_2 and T_1. Non-crystalline, dissolved iron will not alter the relaxation times at equivalent iron concentrations.

J774 cells cultivated in cell culture flasks were incubated with MSM in a concentration of 0.1 mg Fe/ml for 24 h followed by washing with PBS. The macrophages were harvested by trypsinization, or were further cultivated till 96 h before trypsinization and then resuspened in HSA-medium (0.1% protein) for relaxation time analysis. The measurements were performed with a MR spectrometer (Minispec PC/20 Series, Bruker, F.R.G.) operating at a field strength of 0.47 T. The T_1 relaxation time was determined by calculation from eight data points generated by a standard inversion recovery pulse sequence. The T_2 relaxation time was calculated from ten data points generated with a Carr-Purcell-Meiboom-Gill sequence. A standard curve was based on the relaxation values obtained from measurements of MSM suspended in HSA-medium.

Transmission electron microscopy

The macrophages were seeded and cultivated on coverslips as in the phagocytosis experiment. MSM or iron oxide crystals were added to the wells at a concentration of 0.1 mg Fe/ml and incubation was performed for 24 h before washing to remove the non-phagocitized fraction. The cells were then fixed directly on to the coverslips with 1.5% glutaraldehyde in phosphate buffer (pH 7.4) or further cultivated in cell culture medium for 72 h before fixation. Postfixation was performed for 1 h in 1% osmium tetroxide in phosphate buffer (pH 7.4). The cells were then dehydrated in ethanol, counterstained for 2 h with 1% uranyl acetate in 50% ethanol, and subsequently em-

bedded in Epon. The sections were cut with diamond knives on an LKB Ultratom III, stained with uranyl acetate and lead citrate, and examined at 60 kV in a Philips 420 microscope.

Statistics

The two-tailed Student's *t*-test was used to analyse the data and a difference was considered significant if $P < 0.05$. All results are presented as mean values standard deviation (SD).

Results

Phagocytosis

The phagocytic capacity of the J774 cells was evaluated by uptake of radiolabelled DSM. The experiment was performed at 4 and 37 °C for up to 4 h. The amount of cell-associated material was significantly higher at 37 °C than at 4 °C for all the corresponding time points. The calculated difference between the microsphere quantities recorded at 37 °C and 4 °C represented the internalized fraction (Fig. 1A). After 3 h of incubation the uptake reached a plateau representing 9–10 μg microspheres per 10^6 cells.

As both MSM and DSM are made of starch, the microspheres may be internalized into the macrophages via the same receptor-mediated, saturable phagocytosis mechanism. A fixed amount of radiolabelled DSM and variable concentrations of unlabelled DSM and MSM were coadministred to study the phagocytic process. Although MSM significantly inhibited the uptake of radiolabelled DSM in a dose-dependent manner, the unlabelled DSM was a more efficient competitor (Fig. 1B). For instance, when equal concentrations of labelled and unlabelled microspheres were added, the unlabelled DSM and MSM resulted in 53 ± 12 and 80 ± 16% uptake of radiolabelled DSM, respectively.

MTT assay

The macrophage toxicity of the test compounds was investigated by exposing the macrophages to increasing doses of the various materials. Some results obtained with the MTT assay after 24 or 96 h incubation are plotted in Figs. 2 and 3 and the IC_{50} values are shown in Table 2. The cytotoxicity of MSM was compared with the toxic effects of equivalent concentrations of iron given as iron oxide crystals, saccharated iron oxide, and ferric chloride. After 24 h incubation no statistically significant alterations in the MTT reduction of cells treated with MSM, iron oxide crystals, or saccharated iron oxide, were detected. However, ferric iron was highly toxic showing an IC_{50} value of 0.4 mM Fe. After 96 h of continuous drug exposure, a decrease in cell viability was observed for both MSM and iron oxide crystal treatment, but the applied concentrations were too low to detect 50% reduction of formazan production. The apparent sensitivity of the cells to saccharated iron oxide increased with time, giving a IC_{50} of 0.9

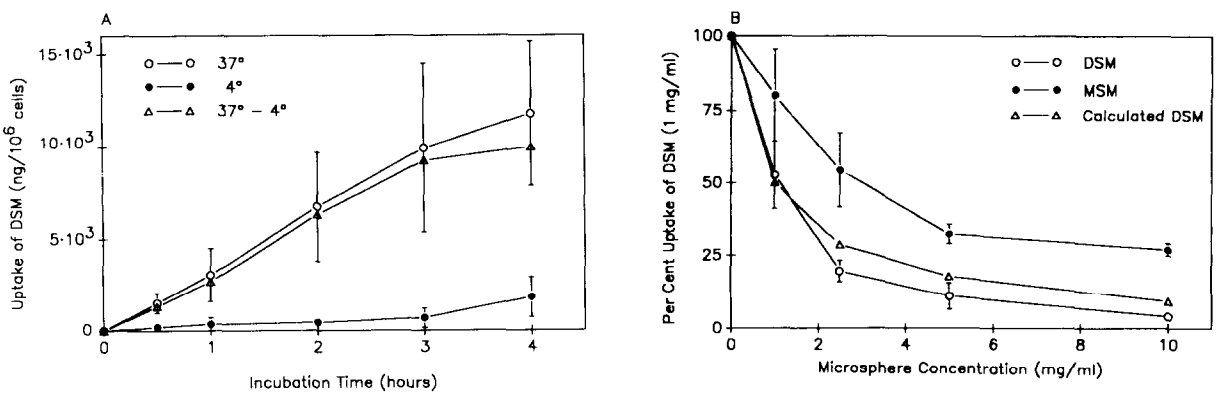

Fig. 1. Uptake of ^{14}C-labelled degradable epichlorhydrin-crosslinked starch microspheres (DSM) by J774 cells (A) at 4 and 37 °C for up to 4 h and (B) after coincubation with magnetic starch microspheres (MSM) or unlabelled DSM. $N = 4$, mean ± SD are given.

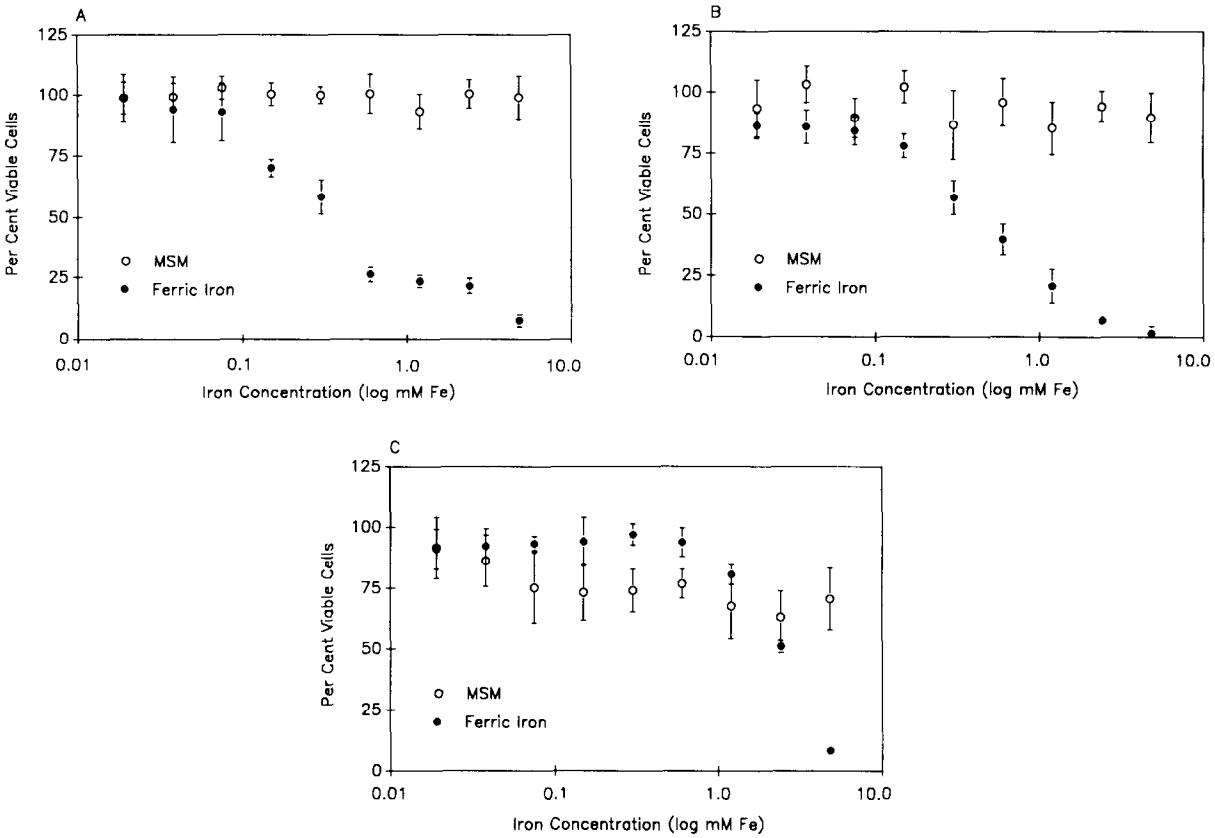

Fig. 2. Iron related cytotoxicity against J774 macrophages in the MTT assay. Cellular sensitivity is shown after (A) 24 h incubation, (B) after 24 h exposure followed by rinsing to remove the test substances and additional cultivation ad 96 h, and (C) after 96 h continous exposure. $N = 6–8$, mean $\pm$ SD are given.

mM Fe after 96 h of continuous exposure. The IC_{50} value of ferric iron was 0.5 mM Fe at this time point. When the cells were washed after 24 h to remove non-phagocitized material and then cultivated for an additional 72 h, the cells were insensitive to both MSM and the iron oxide crystals. Ionic iron was active in the same concentration range as observed previously, while saccharated iron oxide showed lower cytotoxicity than after 96 h of continuous exposure (Table 2).

The cellular effects of MSM on macrophages were compared to the cellular response obtained with DSM, PSM, PS1, glucan microspheres and polystyrene microspheres. A high degree of cellular tolerance to the particles was detected after 24 h incubation and only the polystyrene particles turned out to be toxic, with an IC_{50} of 0.4 mg microspheres/ml. Particulate matter in general reduced the surviving fraction of macrophages after 96 h of continuous exposure. A slight decrease in viability was observed after treatment with MSM, DSM, PSM and PS1, while the effects of glucan and polystyrene microspheres were more pronounced. The IC_{50} dose of polystyrene was decreased six-fold as compared to the result of the 24 h experiment. After 24 h exposure of MSM and DSM followed by rinsing and further cultivation till 96 h, no effects on macrophage viability were

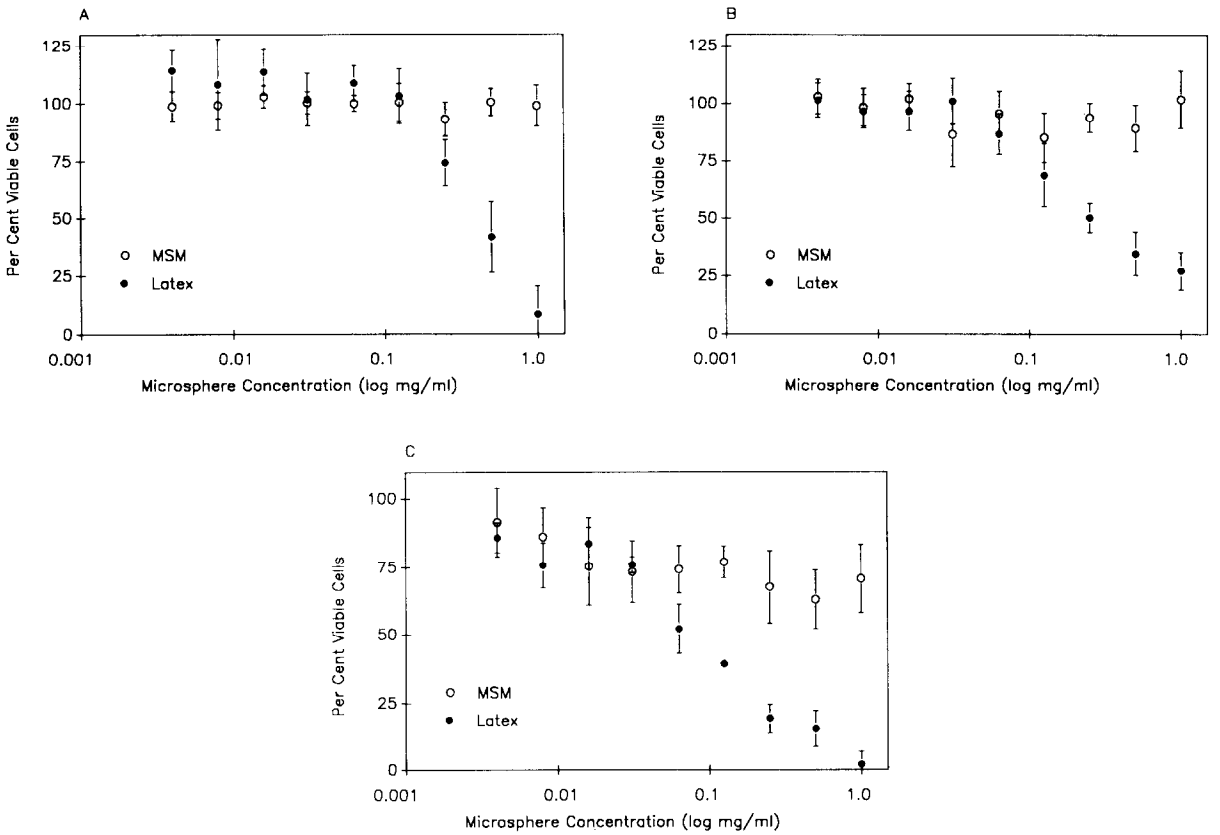

Fig. 3. Particle related cytotoxicity against J774 macrophages in the MTT assay. Cellular sensitivity is shown after (A) 24 h incubation, (B) after 24 h exposure followed by rinsing to remove the test substances and additional cultivation ad 96 h, and (C) after 96 h continous exposure. $N = 6–8$, mean $\pm$ SD are given.

observed. The polystyrene cytotoxic concentration was in the same range as detected after 24 h incubation.

Relaxation time measurements

The relaxation parameters of MSM-treated cells and controls are given in Table 3. The T_1 and T_2 of the cell suspensions were dramatically shortened after 24 h incubation with MSM, indicating efficient uptake of magnetic material. Based on the standard curve relaxation measurements, the cell-associated MSM concentration corresponded to about 2.5% of the administered MSM amount. After 24 h of exposure followed by further cultivation and relaxation analysis at 96 h, only changes in T_2 were significant. Although T_2 was still decreased compared to the control cells, the T_2 effect at 96 h was not as strong as that recorded after 24 h of incubation.

Transmission electron microscopy

Due to the electron density of iron oxide, the material could be seen directly in the electron microscope. After 24 h exposure large amounts of both MSM and the iron oxide crystals were ingested by the J774 cells. The material was accumulated in the lysosomal regions of the cytoplasm and enclosed by a limiting membrane. Many iron oxide crystals formed clusters, and aggregates were clearly detectable in the macrophages. The number of lysosomes loaded with iron oxide decreased with further cultivation. The electron density of the affected regions was high, indicating accumulation of magnetic material. Compared to the ul-

TABLE 2

Cytotoxicity of J774 macrophages based on IC_{50} [a] values of (A) iron containing test substances and of (B) different microspheres

(A) IC_{50} [a] (mM Fe)	24 h [b]	24/96 h [b]	96 h [b]
MSM [c]	> 5	> 5	> 5
Iron oxide crystals	> 2	> 2	> 2
Saccharated iron oxide	> 7	6	0.9
Ferric iron	0.4	0.3	0.5
(B) IC_{50} (mg microspheres/ml)	24 h	24/96 h	96 h
MSM	> 1	> 1	> 1
DSM [d]	> 2	> 1	> 2
PS1 [e]	> 2	n.m. [f]	> 2
PSM [g]	> 2	n.m. [f]	> 2
Lichenan	> 1	n.m. [f]	1
Latex	0.4	0.3	0.07

[a] IC_{50}, concentration which produces 50% reduction in development of formazan compared to control values.
[b] 24 h = 24 h of continuous drug exposure. 24/96 h = 24 h of continuous drug exposure followed by washing of the cells to remove the test substance, and additional cultivation ad 96 h. 96 h = 96 h of continuous drug exposure.
[c] MSM, magnetic starch microspheres.
[d] DSM, degradable epichlorhydrin-crosslinked starch microspheres.
[e] PS1, hydrolyzed potato starch.
[f] Not measured.
[g] PSM, polyacryl starch microspheres.

trastructure of the control cells, cells treated with MSM, as well as those treated with iron oxide crystals, showed increased autophagic activity.

TABLE 3

Relaxation time measurements of J774 cells incubated with magnetic starch microspheres

	T_1 [a] (ms)	T_2 [a] (ms)
HSA-medium	3330 ± 70	1802 ± 56
Unexposed cells	3411 ± 15	1664 ± 53
Cells, 24 h [b]	1439 ± 134	99 ± 1
Cells, 24/96 h [b]	3393 ± 32	833 ± 63

[a] $T_1 = T_1$ relaxation time, $T_2 = T_2$ relaxation time. $N = 2$. Each relaxation time value is based on 5 measurements performed at 0.47 T and 37 °C.
[b] Cells, 24 h = 24 h of continuous exposure. Cells, 96 h = 24 h of continuous exposure followed by rinsing and additional cultivation ad 96 h.

Discussion

The iron oxide loaded starch microspheres (MSM) were developed as a targeting device for the delivery of iron oxide to the liver and spleen for the purpose of contrast enhancement. Intravascular administration of iron oxides is a new medical application and the biological handling of iron oxide is a question of concern. The organ and cellular distribution systems for particulate iron oxide introduce the compound to the RES, which is an important participant in internal iron exchange. The macrophages of RES have proved to have sufficient capacity to solubilize iron oxide and to distribute the iron to the circulation pool of iron (Weissleder et al., 1989, Fahlvik et al., 1990b).

The aim of this report is to focus on macrophage interactions of MSM. The macrophage properties of the J774 cell line used in these studies were confirmed during the phagocytosis experiment. The uptake of DSM and PSM have been studied previously in macrophages at 37°C (Artursson et al., 1987, 1989). The plateau levels of phagocytosis were 3 and 14 μg microspheres/10^6 for resident and inflammatory macrophages, respectively. The maximum uptake of DSM in J774 cells was in the same range as in inflammatory macrophages, probably due to the tumour line properties of the J774 cells.

According to the inhibition experiment, DSM were taken up to a larger extent than were MSM. The explanation for this could be that DSM and MSM are taken up by the macrophages by different receptor mechanisms. However, this is not convincing because of the similartity in matrix material and surface characteristics of MSM and DSM. A more plausible explanation is related to the differences in microsphere size.

The effect of MSM and the control compounds in the MTT test varied with the incubation times. After 96 h of continuous exposure, all the samples, independent of physio-chemical properties, decreased the survival fraction of the macrophages. Besides the above described non-specific effects after 96 h of continous exposure, no toxic effects of MSM on the J774 macrophages were observed. Iron oxide crystals and hydrolyzed potato starch, as isolated components of MSM, also appeared to

be non-toxic. An effective MR imaging dose of MSM in the rat is 1 mg Fe/kg or about 3 mg MSM/kg, and 85% of the MSM is localized to the macrophages of liver (Fahlvik et al., 1990a, 1990b). In the liver of rats about 12×10^6 macrophages (Kupffer cells) per g of tissue are present (Knook and Sleyster, 1980; Bouwens et al., 1986)). According to the in vivo results, the MSM concentration in the liver is about 0.05 mg per g of tissue or 0.004 mg per 10^6 Kupffer cells. In the MTT experiments, the J774 cells were exposed to MSM doses about thousand times the amounts of MSM accumulated by macrophages in vivo. According to the uptake study and the relaxation experiment, it is reasonable to assume that the fraction of MSM phagocitized at the higher doses is in the range of 1% of the total dose. Based on these calculations, the J774 macrophages showed no toxic reactions after uptake of MSM in doses about 10-fold the imaging dose, for up to 96 h

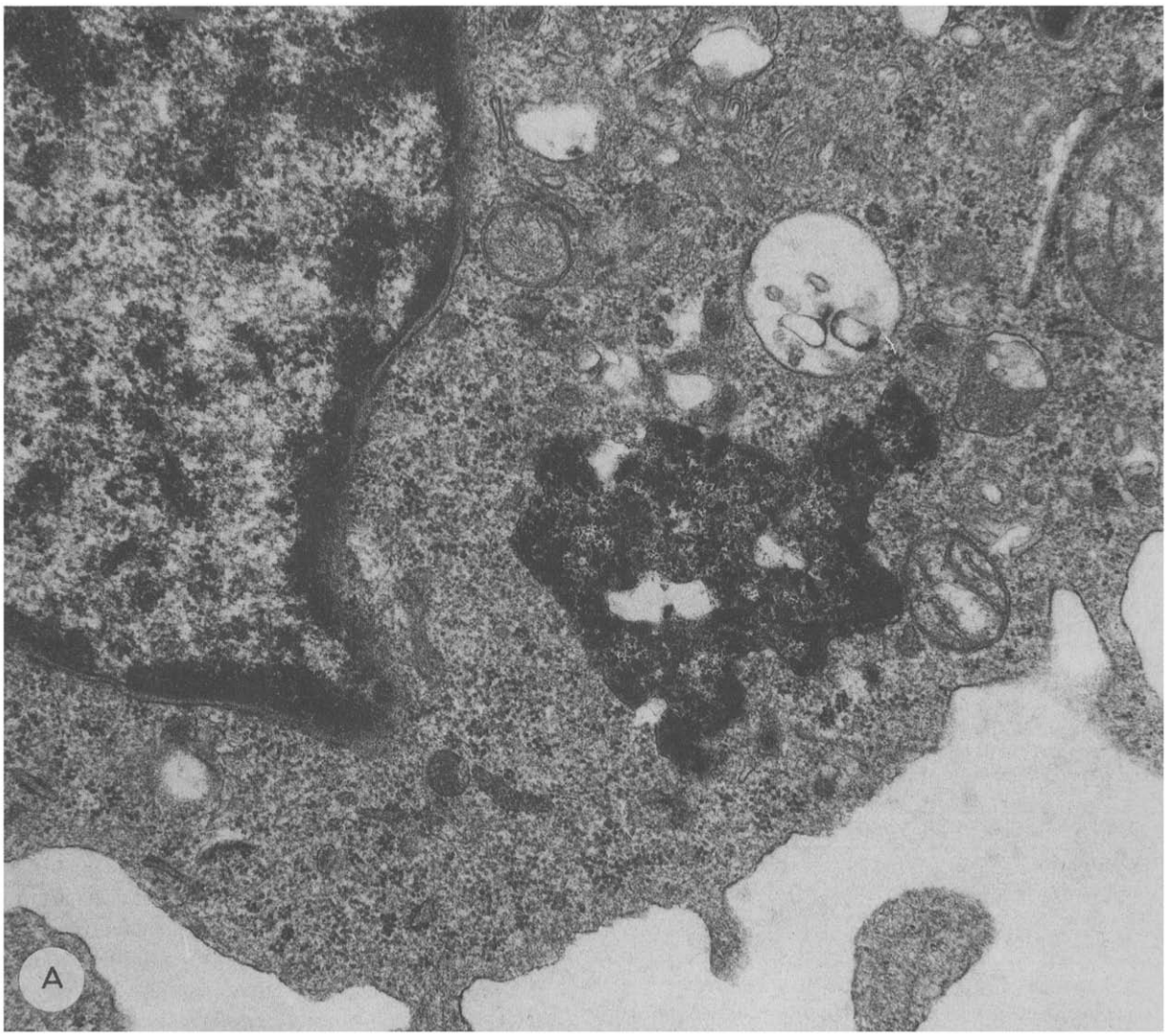

Fig. 4. Transmission electron micrographs of macrophages exposed to (A) magnetic starch microspheres (MSM) and (B) iron oxide crystals for 24 h and after rinsing cultivated further 72 h. Electron dense iron oxide is localized to lysosomal regions of the cytoplasm. Note the lysosomal clustering of iron oxide in the cells.

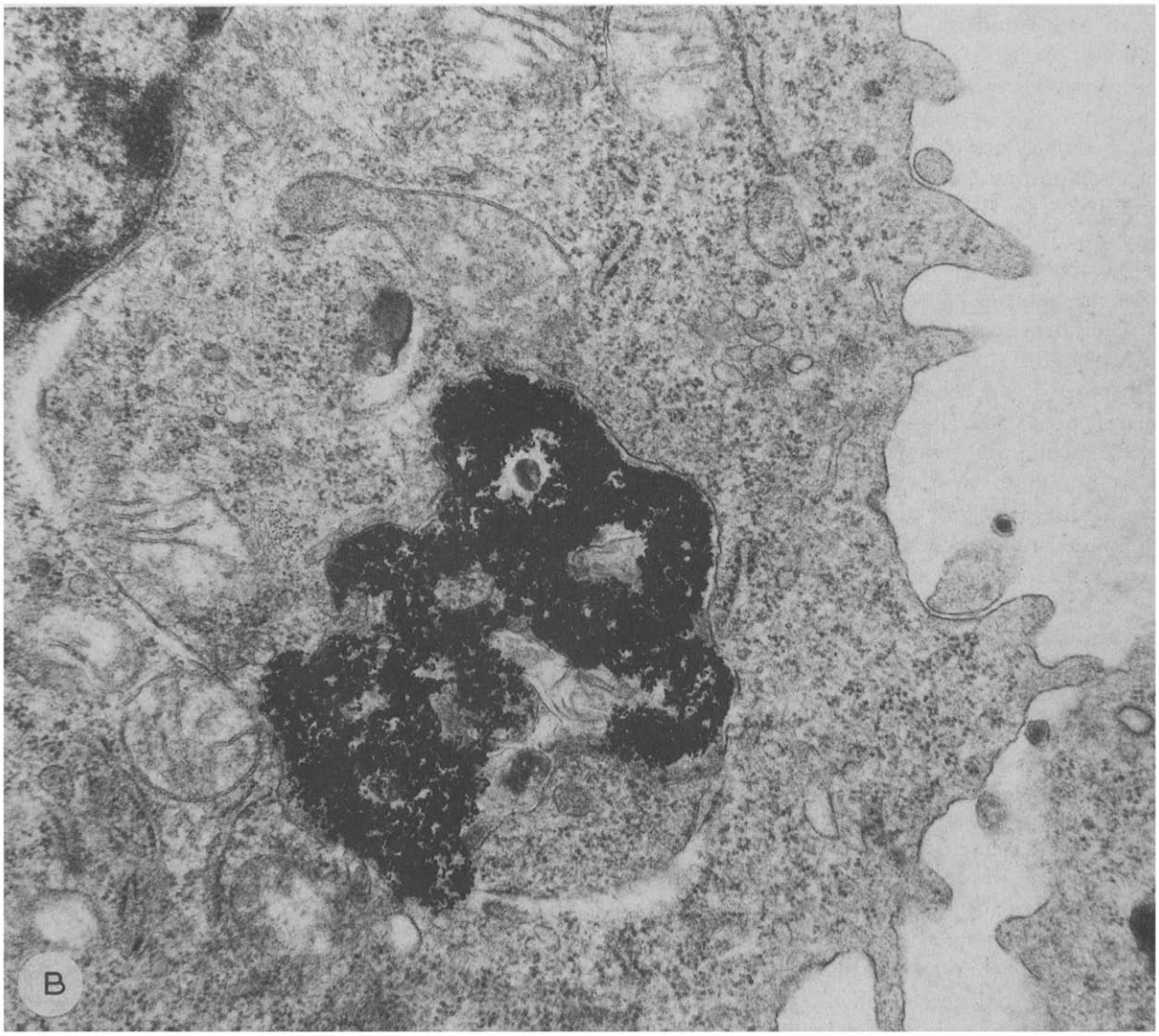

Fig. 4B.

after MSM administration.

However, the cellular sensitivity to the other tested iron compounds was different. Ferric iron was included in this test to serve as a model of the dissolved iron degradation product of MSM. The cytotoxicity in response to ionic iron was strictly dose-related and the IC_{50} value was about the same at all the measuring time points. The effect of ferric iron on the survival rate of peritoneal macrophages has been studied previously and morphologic evaluations showed a concentration-dependent lysosomal accumulation of iron (Abok et al., 1983). Saccharated iron oxide is a commercially available parenteral iron preparation. It is an iron-sucrose complex with ferric or ferrous iron suspended colloidally as iron hydroxide and contains no magnetic iron oxide crystals. This iron complex showed lower toxicity than ferric iron but was significantly more toxic then MSM and pure iron oxide crystals after 96 h of contineous exposure.

The detected differences in macrophage toxic-

ity of MSM and pure iron crystals versus ferric iron and saccharated iron oxide may be related to the physio-chemical properties of the substances. The iron oxide crystals are slowly solubilized and intracellularly presented as a sustained release preparation of ionic iron. Thus, the ferric iron protein complexes and the iron-sucrose complex will probably rapidly liberate ionic iron and, because of the consequent concentrations, saturate the cellular mechanisms of iron handling.

The ultrastructural study showed accumulation of both MSM and pure iron oxide crystals within lysosomal organelles of the macrophages. The appearance of iron oxide was more diffuse after MSM-incubation compared to that of pure crystals. Starch is metabolized by lysosomal enzymes and the matrix of MSM is probably rapidly eliminated (Artursson et al., 1984). However, the carrier may increase the dispersion and decrease the aggregation of iron oxide crystals within the lysosomal compartment. Consequently, the turnover of iron oxide could be higher when starch is used as a drug delivery system of magnetic material compared to administration of pure iron oxide. The concentration of iron oxide in affected areas seemed to rise during 96 h of incubation, while the number of organelles containing the material was decreased. Due to this process, it was impossible to prove any cellular elimination of the iron oxide during the test period from the transmission electron micrographes.

The only observed morphologic effect of the treated macrophages was enhanced autophagocytosis. Autophagy is part of the normal regeneration and turnover of cellular components and may be greatly increased in certain conditions. Induced autophagocytosis has previously been reported in macrophages after exposure to large doses of slowly degradable microspheres (Hamberg and Edman, 1983, Edman et al., 1984). High lysosomal concentrations of exogenous material in general may affect the fragility of the organelle membrane. Together with the ability of ionic iron to destabilize lysosomal membranes and enhance leakage of hydrolytic enzymes, the induced autophagy after accumulation of iron oxide can be explained. The observed dose-correlated cytotoxicity in response to accumulation of non-degradable polystyrene microspheres, is probably also related to increased lysosomal fragility followed by a broad variety of cell damaging processes.

In summary, it is evident from this study that starch microspheres containing iron oxide (MSM) are non-toxic to macrophages in vitro. Relaxation time measurements indicate that iron oxide can be metabolized when presented to macrophages in the MSM form. This means that MSM is an attractive delivery system for MR contrast agents aimed at the reticuloendothelial system.

Acknowledgements

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