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MR Receptor Imaging: Ultrasmall Iron Oxide Particles Targeted to Asialoglycoprotein Receptors

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Previously we have reported that ultrasmall superparamagnetic iron oxide (USPIO) particles migrate across capillary endothelium, a prerequisite for the design of particulate pharmaceuticals for MR receptor imaging. In the current study, USPIO particles are directed specifically to asialoglycoprotein (ASG) receptors by coupling galactose terminals in the form of arabinogalactan (AG) to these particles. Biodistribution data showed that ASG-directed, AG-coated USPIO (AG-USPIO) particles selectively accumulate in the liver but not in other organs. Electron microscopy of liver showed electron-dense iron oxide particles bound to hepatocyte cell-surface membranes and in large numbers within intracellular lysosomes. The specificity of AG-USPIO for asialoglycoprotein receptors was confirmed by incubation experiments with and without ASG-blocking agents such as D(+)galactose and asialofetuin. In vivo MR imaging in rats showed a significant decrease in liver signal intensity at low doses (2 μmol Fe/kg); no significant changes were observed in the spleen. This decrease in signal intensity is larger than that observed with conventional iron oxides at equal doses.

These initial data suggest that, for the first time, superparamagnetic agents can be directed to specific sites for MR imaging by strategies such as receptor targeting.

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Superparamagnetic iron oxides in the size range of 30–1000 nm have been used to reduce relaxation times of liver and spleen [1–9] and lymph nodes [10]. After IV administration, these agents are cleared from the blood within minutes, rapidly accumulating in cells of the mononuclear phagocytic system [9]. As superparamagnetic iron oxides possess high relaxivity, arguments have been made for their use in antibody-labeled and receptor-directed MR imaging. Although antibodies have been labeled with iron oxide particles in vitro [11–15], in vivo results are less encouraging because rapid clearance of these agents by cells of the mononuclear phagocytic system occurs before significant antibody antigen binding can take place.

We recently developed an ultrasmall superparamagnetic iron oxide (USPIO) that is not phagocytosed immediately by liver and spleen [16]. These particles have a mean size of 11.5 ± 6.3 nm, high R2 relaxivity (i.e., 1/T2 as a function of molar concentration, 44.1 $(mmol/l)^{-1}sec^{-1}$), a relatively long blood half-life (81 min), and they migrate through capillary endothelium [16]. We have reported previously on the IV use of USPIO to enhance lymph nodes in rodents [17]. These favorable characteristics suggest that, with appropriate modification, tissue biodistribution of USPIO can be altered selectively.

In this report, we present data on asialoglycoprotein (ASG) receptor-directed, arabinogalactan (AG)-coated USPIO (AG-USPIO). The ASG receptor system was chosen as a target because it is biochemically well characterized [18], it is an abundant receptor protein with as many as 500,000 surface ligand-binding sites per cell [19], the receptor can be targeted easily by attaching terminal galactose sugars to the desired pharmaceutical [20, 21], and the method has been used

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successfully to separate membrane vesicles of hepatocytes with ferromagnetic iron oxide microspheres [22]. The physical properties, pharmacologic behavior, and initial MR receptor imaging studies with AG-USPIO are reported.

Materials and Methods

Iron Oxide Preparations

USPIO was obtained by size fractionation of a heterogeneous dextran-stabilized iron oxide preparation as previously described [16] and was used at a concentration of 8.6 mg/ml (Advanced Magnetics, Inc., Cambridge, MA). USPIO has a molecular weight of approximately 700,000 by gel filtration, similar to that of endogenous ferritin (11 nm; molecular weight, 440,000–600,000) [23].

AG-USPIO was prepared by stabilizing iron oxide particles [24] with arabinogalactan, a galactose-terminated polysaccharide [25]. AG-USPIO (Advanced Magnetics, Inc.) was supplied at a concentration of 17 mg/ml [26].

Relaxation Time Measurements

T1 and T2 relaxation times were measured by using a nuclear magnetic resonance (NMR) spectrometer operating at 0.47 T at 37°C (PC-20 Minispec, IBM, Danbury, CT) and are reported in milliseconds. Before each measurement, the spectrometer was tuned and calibrated. T1 was measured from eight data points generated by an inversion-recovery pulse sequence. T2 was measured from 10 data points by using a Carr-Purcell-Meiboom-Gill pulse sequence with a tau of 1 msec. Timing intervals for different tissues were always optimized so that the multiexponential decay was sampled adequately. Each value is the mean of three separate measurements.

To determine the relaxivity of USPIO and AG-USPIO, we prepared solutions of decreasing concentrations (0.4, 0.2, 0.1, 0.05, 0.0025, and 0.00125 mg/ml) and measured their relaxation times. T1 and T2 relaxation times were expressed as the inverse (1/T1, 1/T2) and plotted against the concentration. The slopes ($[mmol/l]^{-1}sec^{-1}$) of these two curves correspond to R1 and R2 relaxivity. In all experiments, the linear correlation coefficient (r) of the curve fit was larger than .95.

Blood half-life of AG-USPIO was calculated after IV injections of 10 μmol Fe/kg into Sprague-Dawley rats. Blood samples were drawn at multiple time points up to 4 hr from six different animals. Blood relaxation times were determined at 37°C. Blood half-life was calculated by fitting mean 1/T1 values of blood to a single exponential equation: $1/T1_t = 1/T1_{t0} + C \times e^{-kt}$ (Systat Inc., Evanston, IL), where $1/T_{t0}$ is the spin-lattice relaxation rate of blood before injection of the preparation, $1/T1_t$ is the relaxation rate at time t after injection, C is the concentration in the blood at the moment of injection, and k is the rate constant for the decay.

The effect of AG-USPIO on various tissues was determined by injection of AG-USPIO at a dose of 10 μmol Fe/kg. Thirty minutes (> three half-lives) after injection of the agent, animals (n = 15) were sacrificed by exsanguination, and tissues were removed and blotted dry. Then tissues were placed in 5-ml NMR tubes for measurements of relaxation times. Values were expressed as mean plus or minus standard deviation of the mean.

AG-USPIO (10 μmol Fe/kg) was injected into 15 animals to study liver relaxation times as a function of time. Previous experiments had shown that these time-response curves are an accurate method for determining biodegradability of iron oxide particles [9]. T1 and T2 relaxation times of liver were determined at 0.5 and 6 hr and at 1, 2, 4, and 14 days after injection of the agent.

Pathologic Studies

Liver specimens were examined histologically to study the cellular distribution of AG-USPIO in a rat 30 min after injection of 150 μmol Fe/kg. The high dose was chosen because cellular iron oxides are barely visible on histologic specimens when lower doses are used. After removal, the liver was fixed in buffered 10% formaldehyde for 24 hr. Subsequently, tissue samples were embedded in paraffin and stained for iron with Perls' Prussian-blue stain. In this stain, ferric iron (Fe^{3+}) is dissolved from organic iron-containing complexes by hydrochloric acid and then reacted with potassium ferrocyanide [$K_4Fe(CN)_6$] to form a visible blue precipitate: ferric ferrocyanide [$Fe_4(Fe(CN)_6)_3$].

Electron microscopy of hepatic parenchymal cells was performed to study the receptor membrane and intracellular distribution of AG-USPIO. A rat was injected with 150 μmol Fe/kg of AG-USPIO and sacrificed after 30 min. Liver tissue was fixed in situ by perfusion with cold fixative and then studied by electron microscopy without previous staining. The fixative (2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.4, cooled to 3 C°) was perfused through the rat's vascular system and dripped into the abdominal cavity. Electron microscopy also was used to determine the particle size of USPIO and AG-USPIO as previously described [16]. After removal and successive dehydration in graded alcohols, specimens were embedded in Epon (Fluka Chemical Corp., Ronkonkoma, NY), and sections 60-nm thick without previous staining were obtained by using an ultratome. Electron microscopy was performed on a JEOL Jelectron microscopy 100S (JEOL Instruments, Palo Alto, CA). The voltage was 60 kV. Micrographs were taken at magnifications of 3000–100,000× and enlarged photographically 2.6 times.

Receptor Experiments

Uptake of asialoglycoproteins by the liver is mediated specifically by ASG receptors on hepatocytes and can be blocked with D(+) galactose and asialofetuin [27–29]. For in vitro experiments, rodent hepatocyte membranes with ASG surface receptors were isolated by using a modification of a previously described technique [30, 31]. Livers from Sprague Dawley rats (n = 2) were perfused in vivo for 5 min with Hanks solution (Sigma Chemicals, St. Louis, MO) buffered with 0.05 mol/l HEPES and 0.5 mmol/l EGTA (Sigma Chemicals). After this initial perfusion to remove erythrocytes, the livers were perfused with collagenase solution (100 U/ml) in Williams E medium (Sigma Chemicals) buffered with 0.05 mol/l HEPES. Livers then were washed in complete medium and sieved through a number 60 stainless steel mesh (Sigma Chemicals). The resulting tissue fragments and cells were incubated in complete medium containing 0.3 mg/ml collagenase. Subsequently, the cells were washed, centrifuged at 1500g for 10 min, sonicated (10 W for three 20-sec cycles on ice, Branson sonifier 450; Branson Ultrasonics Corp, Danbury, CT) and washed again.

Samples of the membrane suspensions enriched with ASG receptors were then incubated for 30 min at 37°C with D(+) galactose (0.1 mg/ml; Sigma Chemicals) and asialofetuin (2 mg/ml; Sigma Chemicals) to saturate ASG binding sites on the membrane or complete medium (control). After this incubation, USPIO or AG-USPIO was added to the test tubes (10 μmol Fe), and samples were washed and ultracentrifuged twice to remove excess iron oxide particles. Finally, T1 and T2 measurements were obtained as described earlier.

MR Imaging

All in vivo MR imaging experiments were performed on anesthetized rats (intraperitoneal phenobarbital, 35 mg/kg). Rats (n = 4) were imaged before and after administration of AG-USPIO (2 and 10 μmol

Fe/kg) or USPIO (10 μmol Fe/kg). One of the rats received asialofetuin (4 mg/100 g body weight) 30 min before administration of AG-USPIO to show the feasibility of receptor blockade in vivo.

MR imaging was performed on a 0.6-T (25.1 MHz) Technicare superconducting magnet. Coronal multislice images were obtained by using a slice thickness of 4 mm and a 128 × 256 matrix. When the head coil with a field of view (FOV) of 16 cm was used, pixel dimensions were 0.6 × 1.2 × 4.0 mm. Images were acquired by using a variety of spin-echo (SE) pulse sequences, including SE 250/20/6 (TR/TE/number of signal averages), SE 500/40/4, and SE 1500/40/2. Homogeneous regions of interest (21 pixels) within individual livers and spleens were used to calculate signal-to-noise ratios (SNR, mean signal intensity divided by the standard deviation of noise). MR images of ASG-receptor distribution were obtained by subtracting AG-USPIO images obtained before and after administration of contrast material.

Animals

A total of 43 male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used in the study. One animal was used for histopathologic studies, six for determination of blood half-life, 15 for biodistribution studies, 15 for time course studies, two for receptor studies, and four for MR imaging experiments.

Statistical Analysis

Differences in measurements of relaxation time and half-lives were evaluated statistically by using the nonparametric Wilcoxon test [32].

Results

Physical Properties

Magnetic properties.—The T1 relaxivity (R1) of USPIO was 21.6 $(mmol/l)^{-1}sec^{-1}$, and the T2 relaxivity was 44.1 $(mmol/l)^{-1}sec^{-1}$ (37°C and 0.47 T). The R1 of AG-USPIO was 23.3 $(mmol/l)^{-1}sec^{-1}$, and the R2 was 48.9$(mmol/l)^{-1}sec^{-1}$. The r value of the linear curve fits was greater than .95 for both substances. The R2/R1 ratio of both preparations was approximately two, which is lower than ratios obtained with larger iron oxide particles.

The mean particle sizes of USPIO (11.5 ± 6.3 nm) and AG-USPIO (12.2 ± 6.5 nm) were similar. For both agents, 96% of the particles were smaller than 20 nm, 70% were smaller than 10 nm, and 26% were smaller than 5 nm. USPIO and AG-USPIO were present as polycrystalline (each particle contained several electron-dense crystals), multidisperse particles (i.e., particles of different sizes) showing a more heterogeneous size distribution than ferritin (measured particle size, 9.3 ± 3.6 nm). USPIO has an apparent molecular weight by gel filtration of approximately 700,000 [16].

Pharmacology

Blood half-life.—The blood half-life of the T1 effect of USPIO is 81 min [16]. Compared with conventional superparamagnetic iron oxide preparations (AMI-25: half-life of 6 min), this half-life is significantly longer ($p < .001$), indicating that USPIO are recognized, opsonized, and/or phagocytosed less readily by the mononuclear phagocytic system of liver and spleen [16]. The half-life of AG-USPIO of 8 min was significantly shorter than that of USPIO ($p < .001$) despite the similar size, suggesting that AG-USPIO are effectively removed from the circulation by a high-affinity receptor system.

Biodistribution.—Thirty minutes after IV administration of 10 μmol Fe/kg of AG-USPIO, the only significant ($p < .001$) decrease in tissue relaxation times was noted in liver (Table 1). T1 decreased from 260.0 ± 6.2 msec to 224.3 ± 5.9 msec, and T2 decreased from 35.3 ± 2.1 to 19.7 ± 1.9 msec. Specifically, the spleen did not show a significant change in tissue relaxation time (Table 1), indicating selectivity of this agent for hepatocytic parenchyma rather than phagocytic cells. Relaxation times of small intestine and contents were not changed after IV administration of AG-USPIO, suggesting that this agent is not excreted via the biliary system and is therefore not a hepatobiliary contrast agent.

Time course (biodegradation).—To determine the intrahe-

TABLE 1: Biodistribution Studies

Tissue	T1 Control	T1 After AG-USPIO[a]	T2 Control	T2 After AG-USPIO[a]
Liver	260.0 ± 6.2	224.3 ± 5.9[b]	35.3 ± 2.1	19.7 ± 1.9[b]
Spleen	614.7 ± 37.8	605.7 ± 9.5	61.6 ± 1.0	60.0 ± 0.6
Muscle (flank)	590.5 ± 12.0	579.0 ± 4.2	39.0 ± 2.1	38.6 ± 0.4
Fat (peritoneal)	183.5 ± 2.1	183.0 ± 1.4	97.7 ± 6.2	107.3 ± 0.9
Kidney	458.0 ± 8.5	466.5 ± 6.4	48.6 ± 2.2	53.1 ± 1.5
Heart	620.5 ± 6.4	626.5 ± 17.7	42.9 ± 0.9	42.5 ± 1.7
Lungs	749.0 ± 2.8	745.5 ± 19.1	60.8 ± 4.7	52.8 ± 4.0
Testes	1002.0 ± 33.9	969.5 ± 40.3	128.2 ± 3.5	128.7 ± 1.0
Urine	1532.5 ± 40.3	1569.5 ± 35.7	203.2 ± 4.5	246.0 ± 20.2
Small intestine	366.3 ± 7.6	373.3 ± 7.2	50.9 ± 1.3	50.3 ± 0.5
Large intestine	428.5 ± 31.8	442.0 ± 67.9	42.4 ± 1.3	42.4 ± 2.3

Note.—Tissue relaxation times (in msec) without ($n = 7$) and with ($n = 8$) IV injection of 10 μmol Fe/kg of AG-USPIO in rats.

[a] AG-USPIO = arabinogalactan ultrasmall superparamagnetic iron oxide.

[b] Significantly ($p < .001$) lower values after administration of AG-USPIO.

patic degradability (i.e., loss of magnetic properties) of AG-USPIO, we determined liver relaxation times at various intervals after injection of 10 μmol AG-USPIO (Fig. 1). Thirty minutes after injection, liver T2 had decreased 44%. Twenty-four and 48 hr after administration, T2 was 24% and 14% lower than precontrast values, respectively. After 14 days, liver T2 had returned to precontrast values. These data suggest a liver half-life of AG-USPIO of approximately 24 hr. This rapid return to normal values indicates efficient degradation of the ultrasmall particles by hepatocytes.

Receptor Experiments

Cellular and intracellular distribution.—Histologically, stainable iron was detected in the cytoplasm of hepatic parenchymal cells (Fig. 2A). The iron stain also was shown in Kupffer cells, but not to the degree expected with conventional iron oxides [9].

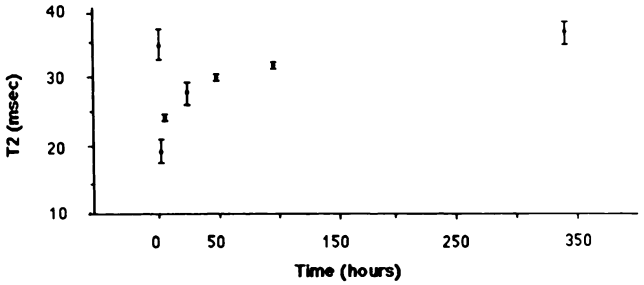

Fig. 1.—Degradation of ultrasmall iron oxide particles coated with arabinogalactan (AG-USPIO). T2 relaxation times of liver are mapped as a function of time before and after administration of 10 μmol Fe/kg of AG-USPIO. 30 min after administration, T2 decreases 44%. Within 24 hr T2 is 24%, and after 48 hr it is 14% lower than control values. Liver T2 has returned to baseline values after 14 days.

By electron microscopy, AG-USPIO was identified on the surface of hepatocyte membranes, presumably attached to ASG surface receptors (Fig. 2B). This distribution was such that most particles were seen close to coated pits. In addition, large amounts of electron-dense deposits were identified within cellular lysosomes. No particles were seen in the nucleus or the hepatic cytoplasm.

Membrane receptor studies.—T2 relaxation times of medium containing ASG membrane receptors were significantly ($p < .005$) shorter after incubation with AG-USPIO than after incubation with USPIO alone or with AG-USPIO after blockade (Fig. 3). This is best explained by the retention of AG-USPIO on membrane receptors despite repeated washing cycles. When ASG membrane receptors were blocked with D(+) galactose and asialofetuin before incubation with AG-USPIO, T2 values were significantly higher ($p < .001$). Membranes incubated with USPIO with and without blocker showed similar T2 values, confirming the hypothesis that there is no specific attachment of USPIO to ASG receptors. T2 values of membrane solutions incubated with USPIO and blocked with AG-USPIO are somewhat lower than those of the control experiment, presumably because not all particles had been removed during the two washing cycles.

MR Imaging Experiments

AG-USPIO vs USPIO imaging.—On unenhanced images, liver SNR was 42.9 ± 0.9 (SE 250/14) and spleen SNR was 24.5 ± 1.8 (Fig. 4A, spleen darker than liver). After administration of 10 μmol Fe/kg of AG-USPIO (Fig. 4B), liver SNR decreased from 42.9 ± 0.9 to 13.1 ± 0.2 ($p < .001$), whereas spleen SNR did not change significantly (24.5 ± 1.8 compared with 23.3 ± 0.2) (spleen brighter than liver). This biodistribution was different from that after administration of USPIO (Fig. 4D), which is known to be cleared by the mononuclear phag-

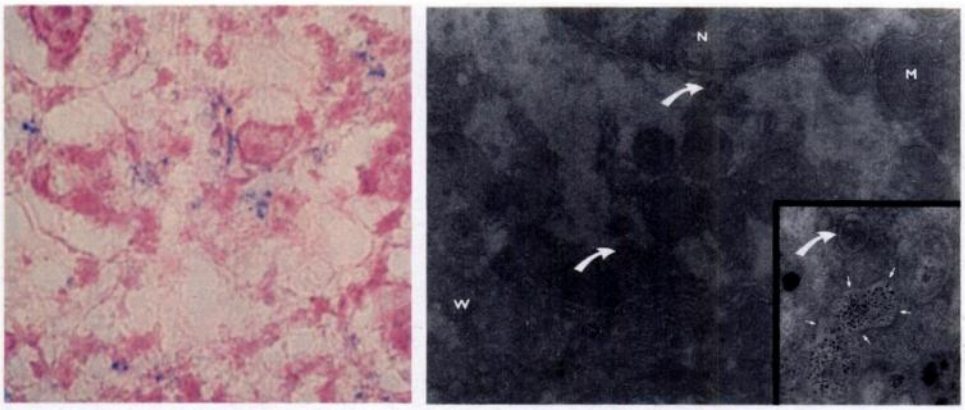

Fig. 2.—Cellular and intracellular distribution of ultrasmall iron oxide particles coated with arabinogalactan (AG-USPIO).

***A*, Photomicrograph of a liver specimen stained with Prussian blue shows iron preparation in blue and overall distribution of AG-USPIO particles. Blue $Fe_4(Fe(CN)_6)_3$ complex is seen within cytoplasm of polygonal hepatocytes. Cell nuclei do not contain stainable iron.**

***B*, Transmission electron micrograph (×20,000) of hepatocyte shows intracellular distribution of AG-USPIO. Electron-dense particles are seen in large numbers within lysosomes (*curved arrows*). Cell nucleus (N) is seen at top, mitochondria (M) to right and center, and cell wall (W) at lower left. At high power magnification (×100,000, inset) high-density AG-USPIO particles can be seen clearly along a hepatocyte cell membrane (*straight arrows*). Uptake by hepatocytes occurs by invagination of receptor-bound particles into membrane vesicles (*curved arrow*, inset).**

ocytic system. After administration of 10 μmol Fe/kg of USPIO, liver SNR decreased from 42.9 ± 0.9 to 20.4 ± 0.1 ($p < .001$) and spleen SNR decreased from 24.5 ± 1.8 to 18.6 ± 2.1 (Fig. 4C, spleen and liver are hypointense). The signal intensity of the liver after administration of contrast material was lower in animals injected with AG-USPIO than in animals that had received USPIO (13.1 ± 0.2 compared with 20.4 ± 0.1; $p < .05$).

In vivo blockade of ASG-receptors.—An experiment was performed to show in vivo that ASG receptors can be blocked competitively and that subsequently the biodistribution of the IV administered AG-USPIO (Fig. 4C) is altered. When ASG receptors were blocked with asialofetuin before administration of AG-USPIO, liver SNR was higher (31.5 ± 0.5 compared with 13.1 ± 0.2), and spleen SNR was lower (20.7 ± 0.7 compared with 23.3 ± 0.2) (compare Figs. 4B and 4C). These SNR changes in spleen and liver suggest that AG-USPIO had been redistributed into cells of the mononuclear phagocytic system. Indeed, this biodistribution was similar to that of USPIO (Fig. 4D).

MR receptor image.—In order to map the receptor distribution of the ASG receptor in vivo, coronal images were acquired as SE 500/40 and displayed as subtraction images ("receptor images") before and after administration of 2 μmol Fe/kg of AG-USPIO (Fig. 5). This dose is 10 times lower than that used in conventional iron oxide imaging [33]. On these receptor images, areas of high signal intensity represent regions with ASG receptors, whereas low-signal-intensity areas indicate the absence of receptors. The heart and some bowel loops appear bright because of motion-induced subtraction errors. No receptors were detectable in spleen, brain, lung, kidney, and muscle (Fig. 5). This biodistribution of receptors is consistent with previous reports of ASG receptor biodistribution [21].

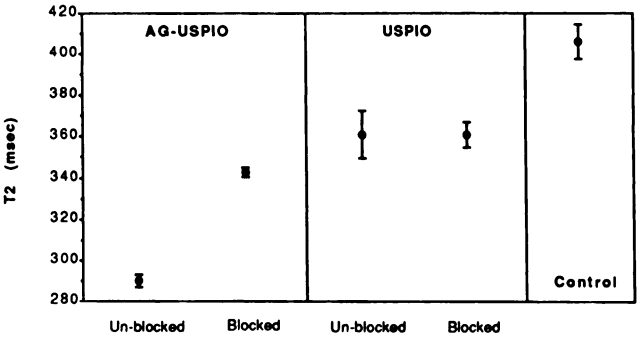

Fig. 3.—Graph shows T2 relaxation times (37°C, 0.47 T, Carr-Purcell-Meiboom-Gill sequence) of aqueous solutions containing asialoglycoprotein membrane receptors before (control) and after incubation in vitro with different iron oxide preparations. The solution of membrane receptors incubated with ultrasmall iron oxide particles coated with arabinogalactan (AG-USPIO) has a significantly lower T2 than that of the solution incubated with USPIO or AG-USPIO after the receptor was blocked with D(+)galactose and asialofetuin. T2 values of membrane solutions incubated with USPIO and blocked AG-USPIO are somewhat lower than those in control experiment, as not all particles had been removed during the two wash cycles.

Discussion

We have established that iron oxide particles can be targeted specifically to ASG receptors on hepatocytes. The findings are supported by biodistribution and MR imaging data and confirmed by in vitro and in vivo ASG blockade experiments. The characteristics of this novel receptor-specific iron oxide preparation that cause these results are the smallness of the particle and the receptor targeting.

We recently have shown that the biodistribution of superparamagnetic iron oxide particles can be altered by reducing the size of the particles [16]. USPIO particles have a mean size of 11.5 ± 6.3 nm compared with 30–100 nm for conventional iron oxide particles. The smaller size of USPIO and prolonged blood half-life (81 min vs 6 min for conventional iron oxide preparations) facilitate delivery into the interstitium by nonspecific vesicular transport and through transendothelial channels [16]. It has been shown that the transcytosis of USPIO into the interstitium results in significant delivery of the agent to regional and central lymph nodes [17]. The ability of an iron oxide particle to enter the interstitium is a prerequisite for successful MR receptor or antibody imaging.

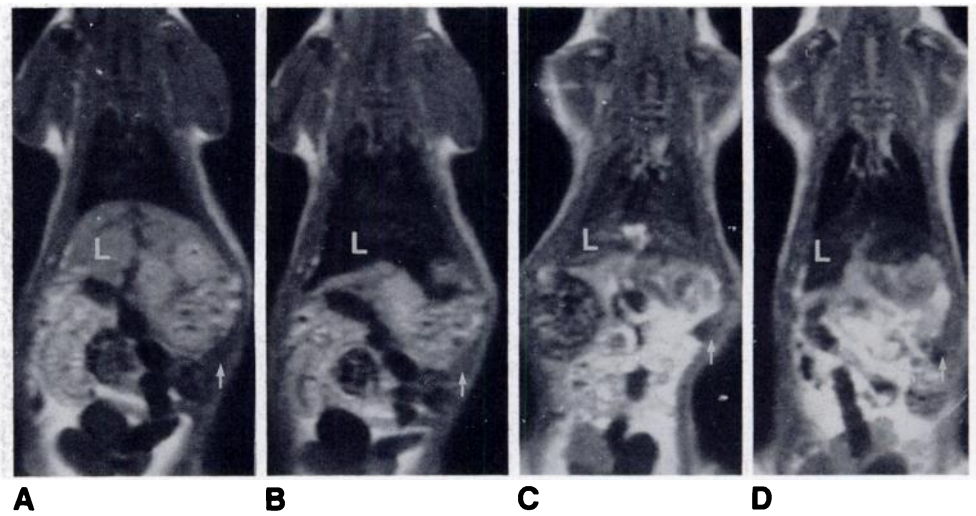

Fig. 4.—A–D, MR images of receptor blockade in rat. Images (SE 250/20) were obtained before (*A*), after 10 μmol Fe/kg of ultrasmall iron oxide particles coated with arabinogalactan (AG-USPIO, *B*), after blockade of asialoglycoprotein (ASG) receptors with D(+)galactose and asialofetuin and subsequent injection of 10 μmol Fe/kg of AG-USPIO (*C*), and after administration of 10 μmol Fe/kg of USPIO (*D*). Administration of AG-USPIO decreases liver (L) but not spleen (*arrow*) signal intensity (*B*), unlike USPIO, which decreases both (*D*). After in vivo blockade of ASG receptors (*C*), reduction of liver signal intensity is less pronounced (compare *B* and *C*) but still is present, as AG-USPIO now is diverted to Kupffer cells; similarly spleen (*arrow*) signal intensity is decreased, indicative of reticuloendothelial biodistribution.

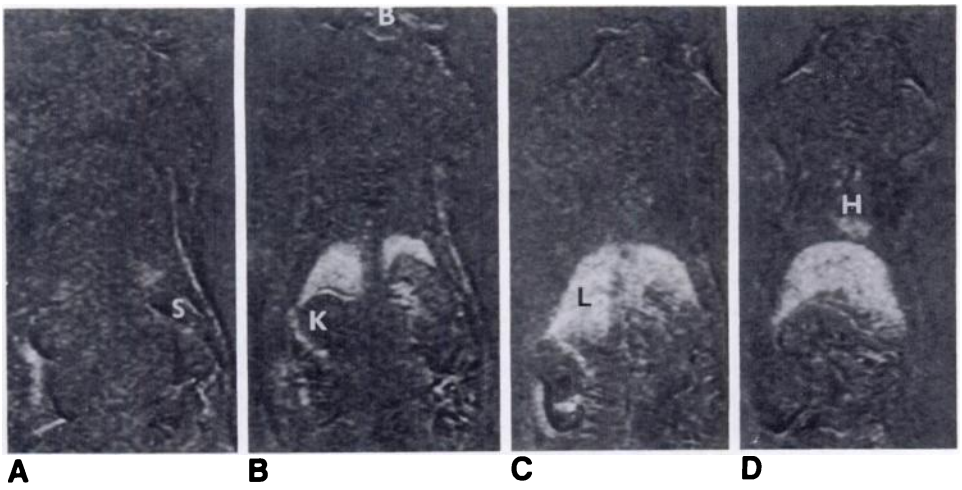

Fig. 5.—A–D, Receptor images of rat. MR images (SE 500/40) were obtained before and immediately after IV administration of 2 μmol Fe/kg of ultrasmall iron oxide particles coated with arabinogalactan (AG-USPIO). Images then were subtracted electronically and are displayed as four consecutive coronal slices from posterior to anterior (*A–D*). Only liver (L) appears white (indicating presence of receptor), whereas brain (B), lung, heart (H), kidney (K), muscle, and spleen (S) are dark (no receptor). Heart and some bowel loops appear bright because of motion-induced subtraction errors.

In the current study, USPIO was targeted to ASG receptors. Of the many different carbohydrate receptor systems on hepatocytes, the ASG receptor system was chosen because it is the most completely investigated and best understood carbohydrate-specific receptor system [18]. Asialoglycoprotein receptors are responsible for the plasma clearance of desialylated glycoproteins by the liver. The discovery of the ASG receptor system has led to the development of both diagnostic [21, 34] and therapeutic agents [35]. The ASG receptor has specificity for a large variety of desialated serum proteins in vivo, including follicle-stimulating hormone, human chorionic gonadotropin, transferrin, IgG, prothrombin, fetuin, and orosomucosoid [18]. The number of ASG binding sites per hepatocyte (as high as 500,000) [19] and the rapid internalization and reutilization of the receptor [36] indicate that a single dose of AG-USPIO will not interfere significantly with receptor binding of biological ligands.

Pharmaceuticals with terminal galactose groups are recognized by ASG receptors and thus can be directed to hepatocytes [18]. AG-USPIO particles differ from conventional USPIO in that they have been stabilized with arabinogalactan, a galactose-containing polysaccharide [25]. The terminal galactose groups are recognized by the ASG receptors, and the agent is taken up rapidly by hepatocytes before major uptake by the mononuclear phagocytic system occurs. The avidity of AG-USPIO uptake by ASG receptors is reflected by the reduction in blood half-life of these small particles from 81 to 6 min.

The initial interaction of the AG-USPIO with receptors in the course of endocytosis is at the hepatocyte plasma membrane. Our electron microscopy experiments confirm that this interaction occurs at least in part in coated pits, a membrane structure that has been implicated in adsorptive endocytosis [37]. It is well documented that internalization of the ASG-receptor-bound ligand is rapid. A first-order rate constant of ASG-receptor-bound ligands at 37°C has been calculated to be 3.7×10^{-3} sec^{-1} [36]. This corresponds to a half-life of approximately 3 min for the occupied receptor on the cell surface. The intracellular catabolic site of ASG-bound ligands is in lysosomes [38, 39]. Our electron microscopy results are in accordance with these observations, as electron-dense AG-USPIO could be shown within lysosomes (Fig. 2). Time-response curves support the observation that AG-USPIO are degraded completely in vivo and that liver relaxation times return to normal baseline values. The liver organ half-life of AG-USPIO as determined by T2 measurements is approximately 24 hr.

In vivo receptor imaging experiments and biodistribution studies confirm that AG-USPIO is taken up by liver tissue, with little or no uptake by the spleen, bone marrow, or lymph nodes, as would be expected for a mononuclear phagocytic system agent such as USPIO (Fig. 4). Proof that AG-USPIO is a receptor-specific agent was established with in vitro (Fig. 3) and in vivo (Fig. 4) receptor blockade experiments. MR imaging studies (Fig. 4) indicate that after the ASG receptor is blocked with D(+) galactose and asialofetuin, the agent redistributes to organs of the mononuclear phagocytic system (spleen, bone marrow), where it decreases signal intensity of these organs.

ASG receptor targeting of USPIO has several advantages over the use of conventional iron oxide preparations. First, because the majority of the dose reaches the liver, the injection dose can be decreased considerably (Fig. 3). Good liver enhancement was achieved with a dose of 2 μmol Fe/kg at 0.6 T, a dose 10 times lower than that proposed for MR imaging with conventional iron oxide preparations (Fig. 5). Second, AG-USPIO may provide a quantitative in vivo measure of ASG-receptor function that may be clinically useful for monitoring disease states and therapeutic response. Third, because most malignant tumors are devoid of ASG receptors, AG-USPIO is expected to provide excellent contrast between normal liver and metastases.

The current data on AG-USPIO provide new directions in development of tissue-specific contrast enhancement with MR imaging. Soon after the introduction of contrast agents for MR imaging, investigators proposed strategies for attaching antibodies to paramagnetic [40] and subsequently to iron oxide particles [13]. Early in vivo attempts were unsuccessful because of the combination of the low relaxivity of paramagnetic metal ions and the low concentration of antigenic sites.

Attempts at attachment of multimetal ions to organic backbones (e.g., polylysine) have been successful in vitro, but not in vivo (B. A. Khaw, personal communication). Conventional iron oxide particles also have been attached to antibodies [11, 12, 15]; however, in vivo these large particles are taken up quickly by cells of the mononuclear phagocytic system, and in vivo antibody binding to tumor cells is not reproducible in murine models with large tumors [14]. Ultrasmall iron oxide particles have the characteristics (small size, high relaxivity, extended blood half-life, and transcytosis) that are essential for receptor-directed MR imaging. Although AG-USPIO is specific for ASG receptor imaging, surface modification of these small particles should enable the development of a variety of receptor- and antibody-specific agents.

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