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Selective modification of NMR relaxation time in human colorectal carcinoma by using gadolinium·diethylenetriaminepentaacetic acid conjugated with monoclonal antibody 19-9

(contrast agent/tumor localization)

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ABSTRACT **Monoclonal antibody 19-9 (mAb 19-9) against human colon adenocarcinoma was conjugated with gadolinium·diethylenetriaminepentaacetic acid (Gd·DTPA) and used as a contrast agent in nuclear magnetic resonance (NMR) in an effort to improve tumor target selectivity in nude mice. The data indicate that Gd·DTPA-mAb 19-9 in solution decreased the T_1 relaxation of water protons at 90 MHz in direct proportion to the gadolinium concentration, and this effect was greater than in Gd·DTPA solutions. T_1 relaxation time at 90 MHz, measured in tumors removed from nude mice 24 hr after injection of Gd·DTPA-mAb 19-9 (Gd, 20 μmol/kg; 16 DTPA molecules per mAb molecule), was significantly decreased (by 15%) as compared with the control group. Similar results were obtained in tumors from mice injected with Gd·DTPA-mAb 19-9 solutions in which Gd was used at 2, 6, or 10 μmol/kg (16 DTPA molecules per mAb molecule). These doses are lower than those commonly used for Gd·DTPA (10–100 μmol/kg) as contrast agent. Tumor localization by the Gd·DTPA-mAb 19-9 complex containing radioactive Gd (0.3 μCi/μg of ^{153}Gd) to confirm scintigraphy revealed significant concentrations of the complex (5% of the injected dose per gram of tissue) in the tumor. Scan images recorded in planar scintigraphy at day 5 showed good visualization of tumors.**

Nuclear magnetic resonance (NMR) proton imaging provides sharp contrast between tissues based on their intrinsic T_1 and T_2 relaxation times when varied pulse sequences are used. By exploiting the differences in relaxation times, images have been produced that provide previously unobtainable anatomic and physiologic information. Early investigators felt that differences in relaxation times between tumors and normal tissue made contrast agents unnecessary. However, despite this intrinsic tissue contrast, the absence of oral or intravenous contrast agents has proved disadvantageous (1, 2). Various intravenous and oral contrast agents have been proposed, including paramagnetic metal ions and their chelates and complexes (3, 4). Recently, attention has focused on gadolinium·diethylenetriaminepentaacetic acid (Gd·DTPA), a complex that produces a marked reduction in the proton relaxation ratio *in vitro* and *in vivo* and appears to have minimal acute toxicity in imaging doses (5, 6). One approach to increasing the specificity of NMR image contrast is to use paramagnetic contrast agents coupled with a monoclonal antibody (mAb).

The use of radiolabeled mAbs has permitted scintigraphic detection of human tumors (7–9). Koprowski *et al.* (10) have obtained hybridomas secreting mAbs that bind specifically to human gastrointestinal cancers in cell culture. One of these antibodies, designated 19-9, recognizes a monosialoganglioside (11) that is shed into the circulation.

The present work describes results of studies using Gd·DTPA conjugated with mAb 19-9 as an NMR contrast agent in nude mice bearing a human colorectal adenocarcinoma (CRC). The biodistribution of the conjugate in various organs was assessed and the relaxation time T_1 was measured in tumor tissue.

MATERIALS AND METHODS

Mouse mAb. Murine mAb 19-9 specific for human gastrointestinal tract adenocarcinomas was provided by The Wistar Institute. Its pattern of reactivity with various cell lines *in vitro* has been described (10). mAb 19-9 detects a monosialoganglioside antigen (11) present on the surface of gastrointestinal adenocarcinomas and absent from other tumors and normal tissues. This antigen is shed by the tumor into the circulation, where it can be assayed by immunoradiometric methods (12).

DTPA-mAb 19-9 Preparation. mAb 19-9 was covalently bound to the DTPA chelating agent as described by Hnatowich *et al.* (13). Mole ratios of cDTPA to mAb (cDTPA is the cyclic anhydride of DTPA) of 2, 10, and 50 were used, and 0.6, 4.5, and 16 DTPA molecules, respectively, were conjugated to mAb 19-9. The high cDTPA-to-mAb ratio (50 cDTPA per mAb) led to some aggregate formation (17%), which was considerably reduced by gel filtration through a Sephadex G-200 column. The purified solution contained less than 5% aggregates, and an average of 16 DTPA molecules were conjugated per mAb 19-9 molecule. The number of DTPA molecules conjugated with the antibody was determined by using a titration method based on a competition between $InCl_3$ and radioactive, carrier-free, $^{111}InCl_3$. Several aliquots of DTPA-mAb 19-9 (25 μl) were incubated for 30 min with 100 μl of $InCl_3$ at various concentrations, approximating the estimated DTPA concentration conjugated by mAb 19-9. The same aliquots were incubated for 30 min with 100 μCi (1 Ci = 37 GBq) of $^{111}InCl_3$. Free indium ions were complexed with 10 μl of 0.1 M DTPA to prevent formation of indium hydroxide. An aliquot of each solution was chromatographed on a silica ITLC (Gelman) support, using 0.1 M sodium citrate, pH 5, as eluent. The $InCl_3$ concentration in which no ^{111}In·DTPA-mAb 19-9 was found corresponded to the concentration of DTPA conjugated with the mAb. The immunoreactivity of the three ^{111}In preparations measured by a

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Abbreviations: Gd·DTPA, gadolinium·diethylenetriaminepentaacetic acid; mAb, monoclonal antibody; CRC, colorectal adenocarcinoma.

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sandwich assay on an affinity column (Sepharose coupled to mAb 19-9) was 75% for the preparation containing 0.6 DTPA molecule conjugated per mAb 19-9 molecule, 70% for the preparation containing 4.5 DTPA conjugated per mAb 19-9, and 60% for the preparation containing 16 DTPA conjugated per mAb 19-9.

Gd·DTPA-mAb 19-9 Preparation. $GdCl_3$ (Ventron, France) in 10-fold excess of DTPA-mAb 19-9 was dissolved in saline (0.15 M NaCl) and added to the DTPA 16-mAb 19-9 conjugate. The pH was adjusted to 5 by addition of 1 M sodium acetate. After stirring for 1 hr at room temperature, free DTPA was added and the solution was purified through a Sephadex G-100 column (30 cm × 1 cm) and eluted with 0.15 M NaCl. In these studies more than 90% of the DTPA-mAb 19-9 was complexed to the gadolinium. The efficiency of the labeling was controlled by incubating the Gd·DTPA-mAb 19-9 solution with a carrier-free ^{111}In acetate solution. ^{111}In·DTPA-mAb 19-9 accounted for less than 10% of the total preparation.

Tumor Model. HRT 18 CRC cells, originated in W. A. Tompkins' laboratory (14), were injected (10^7 cells) subcutaneously in the right flank of 8- to 10-week-old nude mice. Twelve to 15 days after tumor transplantation, when tumor diameter was 8–15 mm, mice were injected in the tail vein with the contrast agent conjugate.

Pharmacokinetics. A solution of $^{153}GdCl_3$ (in 0.1 M hydrochloric acid, 0.05–5 mCi/mg, 0.6 mCi/ml; Amersham) was adjusted to pH 5 with 1 M sodium acetate. This solution was mixed with DTPA-mAb 19-9 conjugate (16 DTPa per mAb) and kept for 1 hr at room temperature. Unbound ^{153}Gd was removed by gel filtration on a Sephadex G-100 column in saline buffer. The chelated antibody labeled with ^{153}Gd was stable in these conditions. A labeling efficiency of 50% was found, and the specific activity of the resulting solution used for the study was 0.2–0.3 mCi/mg. Mice were injected with 20 μCi of ^{153}Gd-labeled compounds: ^{153}Gd·DTPA-mAb 19-9, ^{153}Gd·DTPA-unrelated IgG (anti-hepatitis mAb A_2C_6), or $^{153}GdCl_3$. Mice were killed by cervical dislocation 1, 2, or 5 days after injection. All organs (liver, spleen, kidneys, intestine, lung, skin, muscles, heart, brain, bone, blood, and tumor) were removed from each animal and immediately weighed. Tumor weight at the time of removal was 0.555–1.420 g. Radioactivity was measured by γ scintillation counting (LKB Compugamma) and results are expressed as percentage (mean ± SEM) of injected dose per gram of tissue for an average of five mice injected for each time interval. For imaging purposes, mice were anesthetized by intraperitoneal injection of ketamine, and scans were recorded with a γ camera equipped with a pinhole collimator (Searle Siemens FLOW).

Proton Relaxation Times. The effects of paramagnetic compounds on proton relaxation times were measured in aqueous solutions and in tumors *in vitro* at 90 MHz on a Bruker WH 90 spectrometer. All tumors were minced and analyzed at room temperature within 1 hr of sacrifice and placed in a 5-mm NMR tube. T_1 values and standard deviations were calculated by using a three-parameter exponential fit (15). At least 10 τ values were used for each T_1 determination.

RESULTS

Effect of Gd·DTPA-mAb 19-9 Solution on Water Relaxation Time. Fig. 1 shows the comparison of the effects of Gd·DTPA and Gd·DTPA-mAb 19-9 on water relaxation time T_1. In these experiments, the concentration of mAb 19-9 was kept constant (0.1 mM), and the Gd·DTPA concentration was adjusted by modifying the mean number of chelates per mAb 19-9 molecule. As previously shown (16), increases in the concentration of paramagnetic agents resulted in a decrease in T_1 relaxation time. However, Gd·DTPA-mAb 19-9 was more effective than Gd·DTPA in reducing relaxation times.

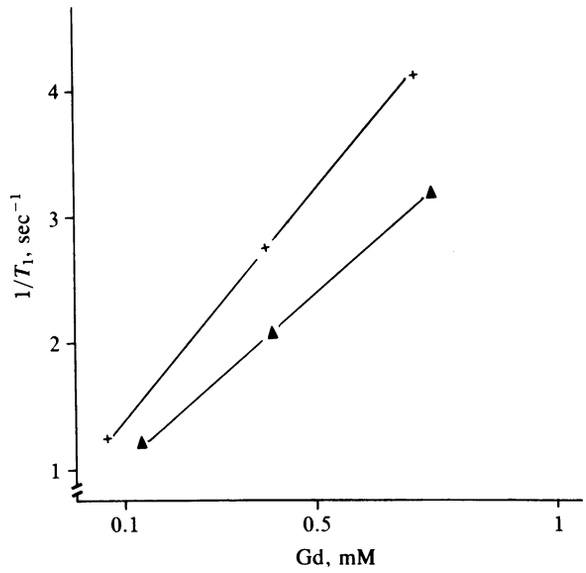

FIG. 1. Effect of gadolinium concentration in aqueous solution on T_1 (plotted as $1/T_1$) at 90 MHz and 25°C. ▲, Gd·DTPA; +, Gd·DTPA-mAb 19-9.

Pharmacokinetics. The biodistributions of radioactivity (% injected dose per gram of tissue) were compared in different tissues (tumor, blood, liver, kidney, spleen) at days 1 and 5 (Fig. 2). At day 1 (Fig. 2 *Upper*), radioactivity associated with ^{153}Gd·DTPA-mAb 19-9 in the tumor was significantly higher than that of ^{153}Gd·DTPA-unrelated IgG or of $^{153}GdCl_3$ (5.18 ± 0.94, 2.75 ± 0.56, and 0.99 ± 0.10 %/g, respectively). Radioactivity in blood was similar in groups infused with ^{153}Gd·DTPA-mAb 19-9 or ^{153}Gd·DTPA-unrelated IgG (5.56 ± 1.10 vs. 5.73 ± 0.89 %/g, respectively) but was much lower in the group infused with $^{153}GdCl_3$ (0.01 ± 0.01 %/g). Analysis at day 5 (Fig. 2 *Lower*) indicated approximately the same high tumor uptake for ^{153}Gd·DTPA-mAb 19-9 as compared with ^{153}Gd·DTPA-unrelated-IgG and $^{153}GdCl_3$ (4.99 ± 0.34, 1.92 ± 0.62, and 0.65 ± 0.06 %/g, respectively). These results reflect good localization of mAb 19-9 in the tumor sites. Clearance of radioactivity was faster in normal tissues (mainly blood, kidney, and spleen), except in liver, where uptake remained at high levels.

At day 5, the tumor was clearly visualized by planar scintigraphy on the right flank, with good contrast in the nude mice injected with ^{153}Gd·DTPA-mAb 19-9 (Fig. 3 *Upper*), whereas no tumor was visualized in the mice injected with unrelated immunoglobulin (Fig. 3 *Lower*). In both cases, the liver was also clearly visualized.

Spin Lattice Relaxation Time in Tumor *in Vitro*. Relaxation time T_1 was measured in normal colon tissue and in human tumor (HRT 18 cells grafted into nude mice) at 90 MHz, 24 hr after injection of mAb 19-9 alone, $GdCl_3$, Gd·DTPA, or Gd·DTPA-mAb 19-9 (Table 1). Consistent with previous reports (17), T_1 in tumor (1225 ± 13 msec) was more than double that in normal tissue (470 ± 45 msec). To detect the specific effect of Gd·DTPA-mAb 19-9, we measured T_1 of tumor grafts in nude mice 24 hr after injection, taking into account the results of the pharmacokinetic studies. For the control groups injected with mAb 19-9, $GdCl_3$, or Gd·DTPA, no significant variations in T_1 were detected, and mean T_1 value was 1225 ± 28 msec. However, T_1 of tumors in mice injected with Gd·DTPA-mAb 19-9 was 1046 ± 29 msec—i.e., a significant 15% decrease.

T_1 relaxation times were then measured on tumors *in vitro* obtained from several mice on day 3 after injection with

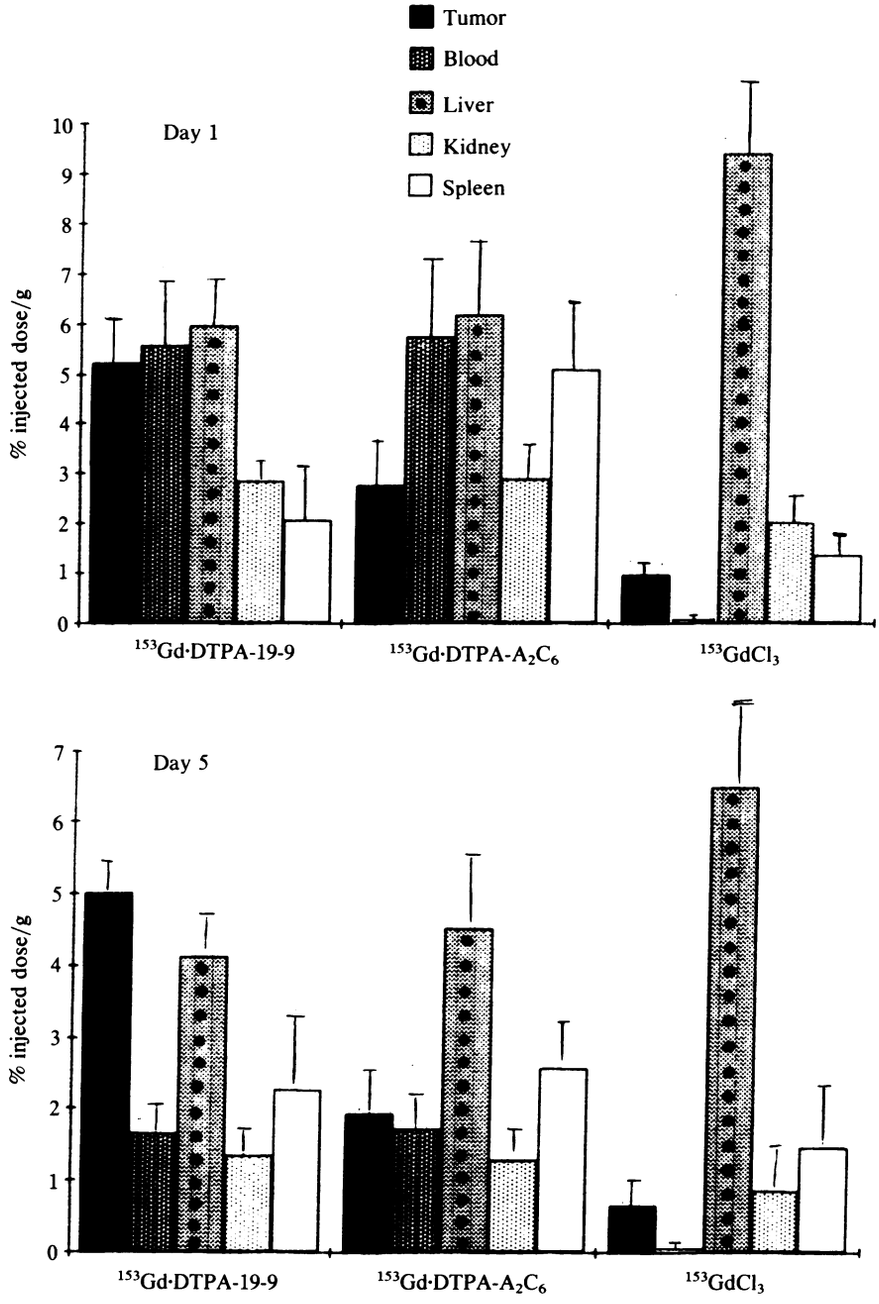

FIG. 2. Distribution in various organs of ^{153}Gd·DTPA-mAb 19-9 (20 μCi per mouse, specific activity 0.3 μCi/μg) in nude mice bearing human CRC HRT 18 compared with distributions in control groups infused with ^{153}Gd·DTPA-unrelated IgG (mAb A_2C_6) or with ^{153}GdCl$_3$. Results are expressed as percent of injected dose per gram of tissue; they are mean (±SEM) values obtained from an average of five mice. Mice were sacrificed at day 1 (*Upper*) or day 5 (*Lower*) after injection.

increasing amounts of Gd·DTPA-mAb 19-9 or Gd·DTPA (Fig. 4). Injection of increasing amounts of Gd·DTPA produced a slight decrease in T_1 that was not directly proportional to the paramagnetic ion concentration used, indicating a rapidly saturable effect. At the corresponding concentrations of Gd·DTPA-mAb 19-9, tumor relaxation time T_1 was reduced to a greater extent and was less sensitive to saturation in the injected concentration range used.

DISCUSSION

Before assessing the potential clinical value of Gd-coupled mAb 19-9 for magnetic resonance imaging of tumors, it was necessary to ensure that the antibody did not modify the paramagnetic properties of Gd in the complex. The studies performed on aqueous solutions of Gd·DTPA confirmed that T_1 is inversely proportional to the concentration of the paramagnetic agent, as previously shown (16). When Gd was conjugated with the antibody by means of the chelator DTPA, a linear relation was observed between the reversal of T_1 relaxation time and the Gd concentration. However, the effect of Gd on the relaxation time was greater when it was coupled with the antibody. In fact, Gd binding on this macromolecule caused an increase in the correlation time (τ_c) of the paramagnetic complex, thus leading to an increase in the speed of relaxation (17). This result also demonstrated that accessibility to water in the vicinity of the coupled paramagnetic agent remains sufficient to allow efficient induced T_1 relaxation.

Pharmacokinetic studies with ^{153}Gd·DTPA-mAb 19-9 were performed to verify that the antibody had retained its biological and immunological properties after labeling. The distribution of ^{153}Gd·DTPA-mAb 19-9 in the appropriate model was compared with that of an unrelated mAb of the same isotype labeled in the same conditions. The results clearly demonstrated selective tumor uptake of the specific antibody as compared to the unrelated antibody, whereas their distributions were similar in normal tissues. The distri-

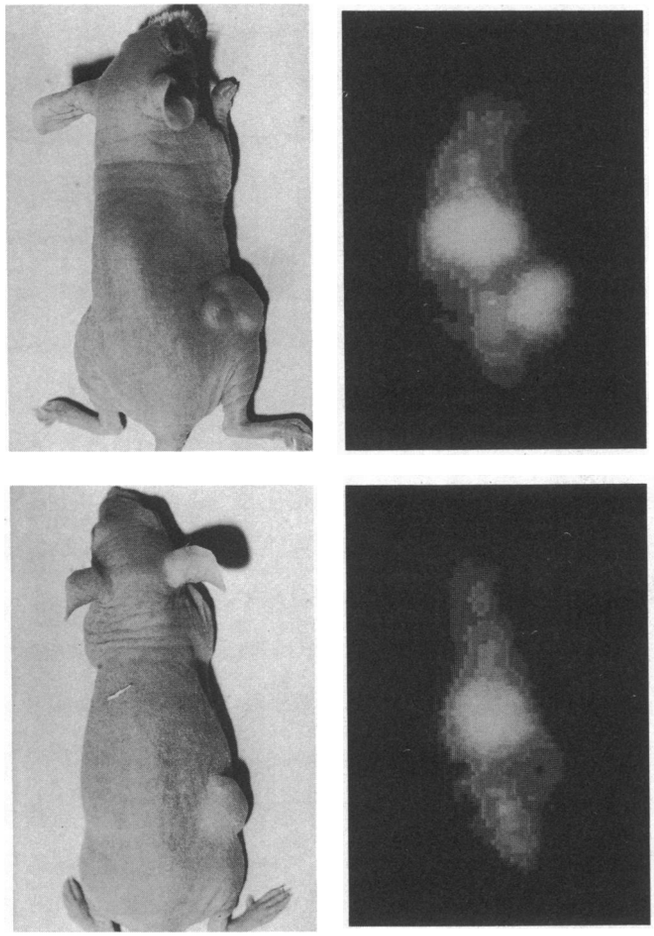

FIG. 3. Tumor-bearing nude mice (*Left*) were injected with 20 μCi of ^{153}Gd·DTPA-mAb 19-9 (*Upper*) or ^{153}Gd·DTPA-unrelated IgG (*Lower*) at a specific activity of 0.3 μCi/μg. Scanning (*Right*) was performed at day 5, using a γ camera equipped with a pinhole collimator.

bution of ^{153}Gd·DTPA-mAb 19-9 as compared with that of ^{131}I-labeled (18) or ^{111}In-labeled mAb 19-9 in an identical experimental model should be interpreted to take into account that the specific activity of ^{153}Gd is 1/10th that of those isotopes. This variation may induce a difference in tumor uptake and in distribution (19). In view of such methodological variations, the distribution of ^{153}Gd·DTPA-mAb 19-9 was rather close to that of ^{131}I-labeled mAb-19-9, even if the tumor uptake of the former was slightly greater at day 5 (4.99 ± 0.34 vs. 3.38 ± 1 %/g). However, the reverse was true for the liver (4.12 ± 0.29 vs. 1.04 ± 0.2 %/g). Distribution of ^{153}Gd·DTPA-

Table 1. NMR relaxation time T_1 of water protons in human tumors

Tissue	T_1, msec
Normal colon	470 ± 45
Colon carcinoma	
+ mAb 19-19	1228 ± 13
+ $GdCl_3$	1205 ± 3
+ Gd·DTPA	1239 ± 23
+ Gd·DTPA-mAb 19-9	1046 ± 29

T_1 was measured at 90 MHz and 25°C on normal colon tissue and CRC tumor grafted into nude mice injected with mAb 19-9 (0.1 mM), $GdCl_3$ (1 mM), Gd·DTPA (1 mM), or Gd·DTPA-mAb 19-9 (Gd 1 mM, 16 DTPA per mAb 19-9). Tumors were removed 24 hr after injection, minced, and placed in a 5-mm NMR tube before analysis. Data are mean ± SEM of values obtained from an average of three mice.

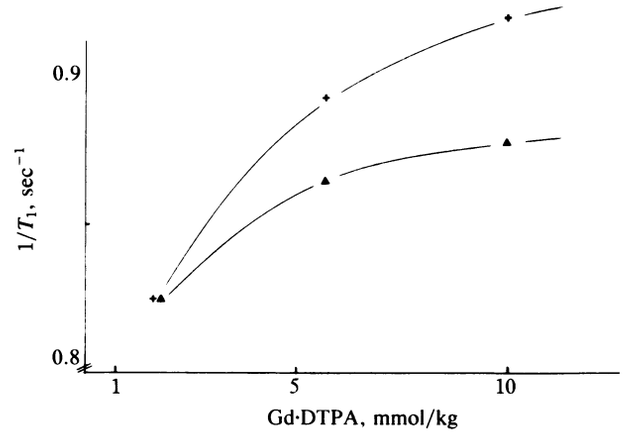

FIG. 4. Effect of gadolinium concentration on T_1 in tumor-bearing nude mice injected with Gd·DTPA (▲; Gd = 2, 6, 10 μmol/kg) or with Gd·DTPA-mAb 19-9 (+; Gd = 2, 6, 10 μmol/kg, 16 DTPA per mAb 19-9), determined at 90 MHz and 25°C.

mAb 19-9 is more directly comparable with that of ^{111}In·DTPA-mAb19-9 in light of their common coupling agent, DTPA (16 DTPA for ^{153}Gd, 2 DTPA for ^{111}In). The results showed a difference between the two conjugates in uptake by the tumor (4.99 ± 0.34 %/g for ^{153}Gd vs. 2.59 ± 0.3 %/g for ^{111}In), whereas the accumulation remained practically unchanged for the liver (unpublished data). Halpern and Hagan (19) have demonstrated that the behavior of antibody to carcinoembryonic antigen coupled with DTPA and labeled with ^{111}In is rather close to that of the same antibody labeled endogenously with [^{75}S]selenomethionine. Presumably, endogenous labeling preserves the immunological properties of the antibody. In these conditions, the behavior of the ^{153}Gd·DTPA-mAb 19-9 antibody probably reflects rather well that of the unlabeled 19-9 antibody and the same can be expected after nonradioactive Gd coupling for magnetic resonance imaging applications.

A specific and significant (15%) modification in T_1 relaxation time of the water in human colon tumors grafted into nude mice was revealed after injection of Gd·DTPA-mAb 19-9 (Table 1). The maximum dose used (0.2 ml of 0.8 mM solution, 6 Gd·DTPA per antibody molecule) corresponded to a maximum amount of Gd·DTPA injected per mouse of 8 μmol/kg, which is considerably less than the doses of Gd·DTPA commonly used when this reagent is employed as a contrast agent in magnetic resonance imaging (0.01–0.1 mmol/kg) (16, 20, 21). The pharmacokinetic studies revealed 5% of the injected dose still present in the tumor on the 5th day after injection of Gd·DTPA-mAb 19-9. The maximal concentration of Gd·DTPA in the tumor can be estimated at 0.04 mM. At this concentration in aqueous solution, no significant variation in T_1 has been detected (16). Antibody uptake in the tumor, causing a local concentration of the paramagnetic reagent, would thus seem to enhance the surrounding water-relaxation mechanism and permit a variation in relaxation time detectable at doses of less than 10^{-4} M.

Previous studies have been performed at 90 MHz—i.e., at a higher proton frequency than that currently used in NMR imaging. Curves of variation in T_1 relaxation induced by Gd^{3+} as a function of frequency of observation (8) indicate a maximum of relaxativity for frequencies between 20 and 60 MHz. If the present results *in vitro* still do not lead to sufficient contrast in NMR imaging, this type of contrast agent used at 20 or 40 MHz might still be promising.

Several approaches might be considered to increase Gd concentration in the tumor. It is possible to increase the amount of DTPA coupling per molecule of mAb 19-9,

although with a corresponding loss of immunoreactivity. It is also possible to increase the amount of antibody injected into each mouse, within the limits compatible with human extrapolation. Finally, one can select an antibody with a higher affinity for a greater number of antigenic sites per tumor cell. Among the antibodies with a relative specificity for colorectal carcinomas, 17-1A recognizes approximately 8×10^4 sites per SW 948 CRC cell. Another antibody, GA 73-3, recognizes 10^6 sites per cell in the same CRC line (22). Such antibodies might also be good candidates for magnetic resonance imaging applications.

This study demonstrates that the Gd^{3+} paramagnetic ion coupled with a mAb leads to a specific decrease in the T_1 relaxation rate of water in a tumor at much lower doses (0.008 mmol/kg) than those currently used (0.01–0.1 mmol/kg) with contrast agents such as Gd·DTPA. The use of such reagents has the double advantage of specificity and efficiency that enables their use at concentrations well below the toxicity threshold, suggesting that they are of interest as contrast agents in NMR imaging. Our studies using mAb 19-9 and a colorectal tumor model grafted into nude mice indicate that this approach is feasible.

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