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Monoclonal Antibodies Labeled with Rhenium-186 Using the MAG3 Chelate: Relationship between the Number of Chelated Groups and Biodistribution Characteristics

Frank B. van Gog, Gerard W.M. Visser, Rob Klok, Roel van der Schors, Gordon B. Snow and Guus A.M.S. van Dongen
Departments of Otolaryngology/Head and Neck Surgery, Free University Hospital; Radionuclide Center; and Research Institute Neurosciences, Free University, Amsterdam, The Netherlands

Our previous studies on the preparation of ^{186}Re-MAb conjugates for clinical radioimmunotherapy (RIT) were extended with the aim to derive conjugates which have a high Re:MAb molar ratio, are stable in vitro and in vivo, have favorable biodistribution characteristics and can be used together with ^{99m}Tc-MAb conjugates as a matched pair in combined radioimmunoscintigraphy/RIT studies. **Methods:** Rhenium and ^{99m}Tc-conjugates of intact MAb E48 were prepared according to our previously described multistep procedure using the MAG3 chelate and analyzed by protein mass spectrometry for the number of chelate molecules coupled to the MAb. For biodistribution analysis, tumor-free nude mice were simultaneously injected with ^{186}Re-, ^{99m}Tc/^{99}Tc- and/or ^{125}I-labeled E48 IgG and dissected 1–48 hr postinjection. **Results:** Rhenium-186-MAb conjugates with up to 20 Re-MAG3 groups per MAb molecule were prepared with an overall radiochemical yield of 40%–60%. The conjugates did not contain empty MAG3 groups and no aggregates were formed. Only conjugates with a ^{186}Re-MAG3:MAb molar ratio higher than 12 demonstrated slightly impaired immunoreactivity to a maximum of 15% decrease at the 20:1 molar ratio. Biodistribution experiments revealed that a proportion of the conjugate became rapidly eliminated from the blood for conjugates with a Re-MAG3:MAb molar ratio higher than 8. In this case, an increased uptake of activity was observed in the liver and intestines. The ^{99m}Tc/^{99}Tc-MAb conjugates showed a similar enhanced blood clearance when containing more than eight Tc-MAG3 groups, while dual labeling of MAbs revealed that the in vivo stability of the conjugated Re-MAG3 complex itself does not differ from the corresponding Tc-MAG3 complex. **Conclusion:** With the method described in this study, it is possible to prepare ^{186}Re-MAG3-MAb conjugates that fulfil all the aforementioned criteria for use in clinical RIT. Coupling of too many metal-MAG3 groups to MAbs results in rapid blood clearance. At the same metal-MAG3:MAb molar ratio, ^{99m}Tc/^{99}Tc-MAb conjugates show a similar pharmacokinetic behavior as ^{186}Re-MAb conjugates and can thus be used to predict the localization of ^{186}Re-labeled MAbs and make dosimetric predictions in individual patients.

Key Words: radioimmunotherapy; radioimmunoscintigraphy; rhenium-186-labeled monoclonal antibody; technetium-labeled monoclonal antibody; head and neck cancer

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The world wide incidence of squamous-cell carcinoma of the head and neck (HNSCC) is estimated at 500,000 cases a year (*1*). Patients with early stage disease (Stages I and II) are usually treated with surgery and radiotherapy and have a relatively good prognosis. Patients with Stages III and IV usually undergo combined surgery and radiotherapy but with limited success. In 50%–60% of these patients, locoregional recurrences occur after locoregional therapy, whereas 15%–25% develop distant metastases. Therefore, at least 30% of the HNSCC patients would benefit from the availability of an effective systemic adjuvant therapy. With respect to the application of (neo)adjuvant chemotherapy almost all studies, unfortunately, have failed to show any improvement in survival (*2,3*). One of the approaches to improve adjuvant therapy for patients is the use of monoclonal antibodies (MAbs) (*4,5*). Radiolabeled MAbs may be particularly suitable for the treatment of HNSCC due to the intrinsic radiosensitivity of this tumor type (*6*). To this end, we recently tested a panel of MAbs for targeting HNSCC in preclinical and clinical studies (*7–13*).

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For correspondence or reprints contact: Guus A.M.S. van Dongen, PhD, Department of Otolaryngology/Head and Neck Surgery, Free University Hospital; De Boelelaan 1117, 1081HV Amsterdam, The Netherlands.

Radionuclide scans and biopsies obtained from surgical specimens revealed high and selective accumulation of ^{99m}Tc-labeled MAb E48 and MAb U36 in primary and metastatic HNSCC (*10–13*). These data justify further development of these MAbs for adjuvant radioimmunotherapy (RIT).

For effective adjuvant therapy, it can be anticipated that all tumor deposits, predominantly small tumor nodules, should be efficiently targeted. Rhenium-186 seems to be a suitable radionuclide for adjuvant RIT because of its appropriate physical half-life of 3.7 days and high abundance of medium energy beta emission (71% of 1.07 MeV and 21% of 0.94 MeV). Furthermore, ^{186}Re has a 9% of gamma emission with an energy of 137 keV that is ideal for gamma imaging (*14*).

Rhenium has chemical properties similar to ^{99m}Tc, the radionuclide of choice for radioimmunoscintigraphy (RIS). Therefore, at our institution, effort has been put on developing analogous ^{99m}Tc- and ^{186}Re-labeling chemistry for MAbs directed to HNSCC, with the clinical option in mind to use ^{99m}Tc and ^{186}Re as a matched pair for imaging, dosimetry calculations and therapy. In this concept, it is clear that the pharmacokinetic behavior of the MAb-conjugate should be the same whether it is labeled with ^{99m}Tc or ^{186}Re.

Several methods for coupling ^{99m}Tc to MAbs have been described (*15–19*). Besides methods for indirect coupling, methods for direct coupling are also used successfully in clinical RIS studies. The development of ^{186}Re-MAb conjugates suitable for clinical application appears to be more difficult. Basically, three kinds of limitations are observed with the methods for direct or indirect coupling of ^{186}Re to MAbs currently available: (a) conjugates are instable, (b) Re:MAb molar ratios are too low and (c) conjugates accumulate at nontumor sites.

In direct labeling methods, ^{186}Re is bound to free thiol groups after reduction of the antibody (*20–22*). Besides the in vivo instability, another limitation of these methods is the low Re:MAb molar ratio obtained, which is typically 1.5:1 (*20,21*). On the basis of this molar ratio, one can calculate that for administration of 300 mCi ^{186}Re, as was done in initial clinical RIT trials (*23*) with commercially available ^{186}Re, about 125 mg MAb are required. Administration of such a high MAb dose in adjuvant therapy may result in saturation of the target antigens, whereas other drawbacks are related to costs and immunogenicity.

Also, several methods for indirect ^{186}Re-labeling have been reported Najafi et al. (*24*) described a method for ^{186}Re labeling using a N_2S_4 chelate precoupled to the protein through a disulphide bond. The Re:MAb molar ratios obtained in their studies did not exceed 1:1, although instability of the disulphide bond might be another limitation. Ram and Buchsbaum (*25*) described a method in which a N_3S bifunctional chelate was used for coupling of ^{186}Re to MAbs. The radiochemical yield with this method was 13%, despite the fact that, during the conjugation step, an extremely high chelate:antibody molar ratio of 281:1 was used. Goldrosen et al. (*26*) described the preparation of ^{186}Re-MAb conjugates by a pre-ester method using N_2S_2 and N_3S chelates. These conjugates have been evaluated in animal RIT studies in tumor-bearing nude mice (*26,27*), as well as in clinical RIT trials (*23,28,29*). The use of conjugates containing two to three Re-MAG2-GABA chelates per MAb resulted in extensive accumulation of activity in the liver and intestines, which is indicative for blood clearance and catabolism of the conjugate by the liver (*27,28*). As a result, hepatic enzyme elevation was seen in the majority of patients.

The aforementioned data indicate that current methods for ^{186}Re-labeling of MAbs leave room for improvement. Therefore, we recently reported on a technical protocol for the reproducible and aseptical production of stable ^{99m}Tc-MAb and ^{186}Re-MAb conjugates using the MAG3 chelate and on the tumor targeting capacity of these conjugates in HNSCC xenograft-bearing nude mice (*30*). MAbs E48 and U36 labeled with ^{99m}Tc according to this method have been evaluated in radioimmunoscintigraphy and biodistribution studies in 50 head and neck cancer patients (*8,10,13*). These MAbs accumulated selectively in the tumor, but their pharmacokinetic behavior was not influenced by the coupling of ^{99m}Tc (*12,30*).

Whether these MAbs exhibit a similar optimal biodistribution when labeled with ^{186}Re according to the same procedure is subject of the present preclinical study. Because the specific activity of ^{186}Re is much lower than of ^{99m}Tc (the largest proportion of a ^{186}Re preparation consists of ^{185}Re), many more Re-MAG3 groups have to be coupled to a MAb molecule to obtain conjugates suitable for clinical RIT, and this might influence the biological behavior of the MAb. Besides that, the ^{186}Re-MAG3 complex and the ^{99m}Tc-MAG3 complex might show a difference in stability in the in vivo situation.

In the present study, we describe the preparation of ^{186}Re-MAb conjugates labeled at a high Re-MAG3:MAb molar ratio, as is needed for clinical RIT. The biodistribution characteristics of ^{186}Re-MAb conjugates have been related to aspects of conjugate stability and chelate:MAb molar ratios and compared to those of ^{99m}Tc-MAb conjugates. These insights have resulted in the synthesis of ^{99m}Tc/^{99}Tc-MAb conjugates, which are better suited for use in a "matched pair" approach with ^{186}Re-MAb conjugates than ^{99m}Tc-MAb conjugates. Reasons for ^{186}Re uptake at nontumor sites as observed with other ^{186}Re labeling methods will be indicated.

METHODS

Preparation of Rhenium-186-MAG3-MAb Conjugates

Rhenium-186-MAb IgG conjugates were prepared following a multistep procedure using the chelate S-benzoylmercaptoacetyltriglycine (S-benzoyl-MAG3) as previously described and illustrated by Figure 1 (*30*). In this procedure, a solid-phase synthesis for preparation of ^{186}Re-MAG3 is followed by esterification and conjugation to the MAb. In short: To a solution containing 750 nmole [^{186}Re]ReO_4^-, amount and specific activity known at calibration time, 150 μl Na_2CO_3 (1 *M*), 150 μl Na_2SO_3 (100 mg · ml^{-1}), 1725 nmole S-benzoyl-MAG3 (1 mg · ml^{-1} MeCN/H_2O 9:1) and 6000 nmole $SnCl_2$ (1 mg · ml^{-1}) were added. In this reduction step, the amount of [^{186}Re]ReO_4^- can be varied, if the amounts of MAG3 and Sn^{2+} are adjusted accordingly. The Re:MAG3 molar ratio should be 1:2.3 and the Re:Sn^{2+} molar ratio should be 1:8. The solvent was evaporated at 100°C under N_2 until dry and the mixture was heated for an extra 15 min. After cooling of the mixture at 0°C, 500 μl water, 480 μl 1N H_2SO_4, 200 μl 2,3,5,6-Tetrafluorophenol (100 mg · ml^{-1} MeCN/H_2O 9:1) and 100 mg 1-ethyl-3-(3-dimethylaminopropyl)-carbidiimide (EDC) were added and the pH was adjusted to 6 with 1 N H_2SO_4. The reaction mixture was incubated at room temperature for 30 min. The ^{186}Re-MAG3-TFP ester was diluted with water to a volume of 8 ml and purified on two conditioned Sep-pak cartridges (Waters, Millipore, MA). For washing, 20 ml water for injection, 30 ml 20% (vol/vol) EtOH/0.01 *M* sodium phosphate (pH 7.0), 10 ml water and 0.5 ml diethylether were used. The active ester was eluted with 2.5 ml MeCN. The purified ester was dried at 30°C under N_2-flow and dissolved in 500 μl 0.9% NaCl. The conjugation reaction of the MAb and the ester was carried out in 0.9% NaCl at pH 9.5

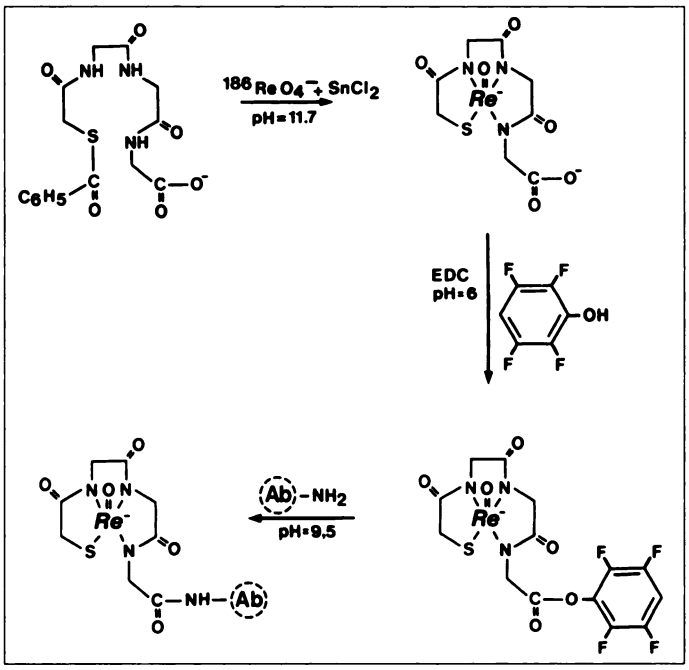

FIGURE 1. Schematic representation of the synthesis of ^{186}Re-MAG3, its esterification and conjugation to MAbs.

(adjusted with 50 m*M* Na_2CO_3) at room temperature for 30–45 min. The conjugate was purified on a PD10-column (Pharmacia-Biotech, Woerden, The Netherlands) with 0.9% NaCl as eluant. Conjugate-containing fractions were collected in tubes containing 175 μl of a 100 mg · ml^{-1} ascorbic acid solution that was adjusted to pH 5.0 with 50 m*M* Na_2CO_3 (final concentration 5 mg · ml^{-1}). Thin-layer chromatography (TLC) of the labeled MAbs was performed as described previously (*30*).

Quantitative measurement of the ^{186}Re activities in solutions was performed in glass vessels with an internal diameter of 13.5 mm in the ^{99m}Tc 140 keV channel of a dose calibrator. It was found that the amount of radioactivity needed to be multiplied with a factor of 2.5 to obtain the ^{186}Re activity (*30*).

Preparation of No-Carrier-Added Technetium-99m-MAG3-MAb Conjugates

The ^{99m}Tc-conjugates were prepared as described for ^{186}Re-conjugates, but 25 μl (68 nmole) S-benzoyl-MAG3 (1 mg · ml^{-1} MeCN/H_2O 9:1) and 442 nmole $SnCl_2$ (1 mg · ml^{-1}) were used in the reduction step; the synthesis of ^{99m}Tc-MAG3 was conducted in a volume of 5 ml by heating the solution at 100°C for 10 min (no solid-state synthesis). After reaction, addition of 500 μl water is not necessary, but 250 μl 1 N H_2SO_4 is enough to bring the pH to 6.

Preparation of Technetium-99m/Technetium-99-MAG3-MAb Conjugates

Technetium-99 with a specific activity of 1.635 mCi · mmole^{-1} was obtained as [^{99}Tc]TcO_4^- and sonificated for 15 min at room temperature before use. The ^{99m}Tc/^{99}Tc-conjugates were prepared essentially as described above for ^{99m}Tc-conjugates with slight modifications in the synthesis of ^{99m}Tc/^{99}Tc-MAG3: In short: 150 μl Na_2CO_3 (1 *M*) were added to 750 nmole $^{99}TcO_4^-$. Hereafter, $^{99m}TcO_4^-$, 150 μl Na_2SO_3 (100 mg · ml^{-1}), and 1725 nmole S-benzoyl-MAG3 (1 mg · ml^{-1} MeCN/H_2O 9:1) were added and the mixture was sonificated for 5 min. After addition of 2625 nmole $SnCl_2$ (1 mg · ml^{-1}) and adjustment of the total volume to 5 ml, the reaction mixture was heated at 100°C for 10 min (no solid-state synthesis), after which standard procedures for esterification and conjugation were used as described before for preparation of ^{186}Re-MAG3-MAb conjugates. In the reduction step, the amount of ^{99}Tc can be varied if the amounts of MAG3 and Sn^{2+} are adjusted accordingly. The optimal Tc:MAG3 molar ratio is 1:2.3 and the optimal Tc:Sn^{2+} molar ratio is 1:3.5.

Preparation of Dual-Labeled MAb Containing Rhenium-186/Rhenium-185-MAG3 and Technetium-99m/Technetium-99-MAG3

The ^{186}Re/^{185}Re-MAG3-TFP and ^{99m}Tc/^{99}Tc-MAG3-TFP esters were prepared and purified in parallel procedures. After removal of MeCN and dissolution in 0.9% NaCl, the obtained ester solutions were mixed in the appropriate amounts and the conjugation to the MAb was performed as previously described.

Quality Control: HPLC Analysis

The chemical purity of ^{186}Re-, ^{99m}Tc- and ^{99m}Tc/^{99}Tc-labeled MAG3 and their corresponding MAG3-TFP-esters was determined by HPLC analysis using a LKB 2150 HPLC-pump (Pharmacia Biotech), a LKB 2152 LC controller (Pharmacia Biotech) and a 25-cm Lichrosorb 10 RP 18 column (Chrompack, Middelburg, The Netherlands). For analysis of the radiolabeled MAG3 complex, the eluant consisted of a 5:95 mixture of EtOH and a 0.01 *M* sodium phosphate buffer plus 0.015 *M* sodium azide (pH 6) solution (eluant A). For analysis of the radiolabeled MAG3-TFP-esters, a gradient was used in which eluant A was gradually replaced by a 9:1 mixture of MeOH and H_2O (eluant B). The gradient (flow rate 1 ml · min^{-1}) was as follows: 10 min 100% eluant A; linear increase of eluant B to 100% during 10 min; 10 min 100% eluant B.

For HPLC analysis of ^{186}Re-, ^{99m}Tc- and ^{99m}Tc/^{99}Tc-labeled MAb a LKB 2150 HPLC-pump, a LKB 2152 LC controller and a 8.0 × 300 mm Waters 300 SW column were used. The eluant consisted of 0.1 *M* sodium phosphate/0.1 *M* potassium sulphate plus 0.05% sodium azide (pH 6.5) and the flow was set at 0.4 ml · min^{-1}.

A Pharmacia LKB VWM 2141 UV detector was used while radioactivity was detected continuously by a single-channel analyzer connected to a data collector and 1-ml fractions were collected.

Comparison of the injection standard with the total effluent from the HPLC columns showed a quantitative recovery of the activity (>98%) from the HPLC column in all cases. The HPLC retention times on the 25-cm Lichrosorb 10 RP 18 column were 3.0 min (most probably low valent oxo-^{186}Re species), 3.6 min ($^{186}ReO_4^-$), 6.5 min (^{186}Re-MAG3), 23.0 min (^{186}Re-MAG3-TFP), 3.1 min (most probably a low valent oxo-^{99m}Tc species), 3.9 min ($^{99m}TcO_4^-$), 9.4 min (^{99m}Tc-MAG3) and 23.4 min (^{99m}Tc-MAG3-TFP); on the 8.0 × 300 mm Waters 300 SW column they were 21.5 min for ^{186}Re-MAb IgG, ^{99m}Tc-MAb IgG and ^{99m}Tc/^{99}Tc-MAb IgG and 35 min for $^{186}ReO_4^-$ and $^{99m}TcO_4^-$.

Monoclonal Antibodies

Production and selection of MAb E48 has been described previously (*8*). MAb E48 detects a 22-kDa surface antigen which in normal tissue is present only in stratified and transitional epithelium. As tested so far, MAb E48 was shown to be reactive with 89% of the primary head and neck tumors (n = 195) and with the majority of cells within these tumors. A comparable reactivity pattern was observed in 31 tumor-infiltrated lymph nodes from neck dissection specimens (*9*). MAbs K928 and SF-25, which were used to evaluate the generality of accelerated conjugate blood clearance at increased Re:MAb and Tc:MAb molar ratios, have been described in detail elsewhere (*31,32*). Also, chimeric MAbs

(cMAbs) E48 and SF-25 were used for this purpose. These cMAbs contain variable regions of the light and heavy chains derived from the respective murine MAbs, which are joined to the constant regions of human kappa and gamma-1 chains. Production and characterization of these cMAbs has been described previously (*33,34*). MAbs E48 and SF-25 are of the IgG_1 isotype, whereas K928 has the IgG_{2b} form.

Protein-Mass Spectrometry

Mass spectra of the conjugates were recorded using a triple quadropole mass spectrometer equipped with an electrospray atmospheric pressure ionization source (*35*). The conjugates were prepared according to standard procedures. Elution of the conjugates from the PD10-column, however, was performed with water for injection instead of 0.9% NaCl. Samples were diluted to a concentration of 1 $mg \cdot ml^{-1}$ and passed through a 0.45 μm sterilization filter. To the samples, 10% of 1 N acetic acid was added and 10 μl were injected into the electrospray source (flow rate: 5 $\mu l \cdot min^{-1}$, mobile phase: 50:50 $MeCN/H_2O$). Data were acquired over a suitable mass range and several 10-sec continuum scans were accumulated.

Isoelectric Focusing

For isoelectric point (pI) measurement of MAbs before or after $^{99m}Tc/^{99}Tc$-MAG3 conjugation, the PhastSystem™ (Pharmacia-LKB) was used. MAb samples were dissolved in 0.9% NaCl and 1 μg in 1 μl was applied to the gel. For pI calibration, a broad pI calibration kit was used according to the manufacturer's instructions. The gel was prefocused for 10 min at 2000 V, the samples applied at 200 V, while the focusing step took 20 min at 2000 V. Proteins were stained with Coomassie R 350 dye. Plotting of the pI values of the calibration proteins versus distance to a reference point showed a linear relationship. Since coupling of $^{99m}Tc/^{99}Tc$-MAG3 groups to MAbs resulted in broader protein bands on gel, the center of the bands was taken for pI assessment.

Immunoreactivity Assay

In vitro binding characteristics of MAb E48 IgG labeled with ^{186}Re, ^{99m}Tc, $^{99m}Tc/^{99}Tc$ or ^{125}I were determined in an immunoreactivity assay as described previously using UM-SCC-22B cells as target cells (*36*).

Biodistribution Studies

The influence of the number of Re-MAG3 or Tc-MAG3 groups coupled to the MAb on the biodistribution characteristics of a conjugate was determined in tumor-free nude mice; when necessary ^{125}I-labeled MAb was coinjected as a standard (*36*). Tumor-free nude mice were used to avoid a differential influence of tumors on the biodistribution characteristics of the conjugates in normal tissues. Female mice (Hsd: Athymic nu/nu, 25–32 g) were 8–10 wk old at the time of the experiments. Conjugates were intravenously injected in 0.9% NaCl. Injection volumes were 100 μl, while the total immunoglobulin dose ranged from 20–90 μg. At indicated time points postinjection, mice were anesthetized, bled, killed and dissected. The urine was collected and the organs were removed. After weighing, radioactivity in organs, organ contents, blood and urine was counted in a LKB-Wallac Compugamma. Radioactivity uptake in the tissues was expressed as the percentage of the injected dose per gram of tissue (%ID $\cdot g^{-1}$).

Counting Simultaneously Injected Technetium-99m, Rhenium-186 and Iodine-125

Making use of the differences in half-life, amounts of ^{99m}Tc ($t_{1/2}$: 6.02 hr), ^{186}Re ($t_{1/2}$: 3.7 days) and ^{125}I ($t_{1/2}$: 60.0 days) were measured by repeated counting of samples over a time period of 30 days in a LKB Compugamma. For simultaneous measurements of ^{99m}Tc and ^{186}Re, differential counting methods cannot be used due to their similar gamma energies (^{99m}Tc γ-140 keV; ^{186}Re γ-137 keV). To obtain accurate values, ^{99m}Tc plus ^{186}Re and ^{125}I were measured in the corresponding window settings repeatedly for at least 16 cycles during 48 hr (dual-isotope measurements, 10 sec per sample per cycle). After complete decay of ^{99m}Tc (>90 hr postinjection), ^{186}Re and ^{125}I were counted simultaneously. As a final check, after 30 days (after decay of ^{99m}Tc as well as ^{186}Re) ^{125}I was counted once more.

Using the ^{186}Re values obtained at t = 90 hr, separate ^{99m}Tc and ^{186}Re values could be calculated for the initial 48 hr of the counting period. As a result, by using the half-life times of all three radionuclides, the repeated countings could be transformed to values at the time of obduction and as such be used to calculate the %ID $\cdot g^{-1}$.

RESULTS

Biodistribution of Rhenium-186-MAb Conjugates Labeled at High Re-MAG3:MAb Molar Ratios

We recently described a flexible multistep procedure for coupling ^{99m}Tc and ^{186}Re to MAbs by the MAG3 chelate (*30*). It was shown that once the radiolytic decomposition was suppressed by the use of the antioxidant ascorbic acid, the in vitro stability of the ^{186}Re-MAb conjugate was similar to that of the analogous ^{99m}Tc-MAb compound. By slightly modifying the protocol by reducing the volume during conjugation, it appeared possible to produce ^{186}Re-MAb conjugates with Re-MAG3:MAb molar ratios up to 20; this molar ratio was achieved when the active ester was conjugated to 1 mg E48 IgG in a volume of 0.5 ml. After purification, ^{186}Re-conjugates produced in this way never contained more than 5% of unbound ^{186}Re as analyzed by TLC and HPLC, irrespective the metal-MAG3:MAb molar ratio achieved. No aggregates were formed as analyzed by HPLC. Only conjugates with a molar ratio higher than 12 demonstrated a slightly decreased immunoreactivity. For a conjugate with a molar ratio of 20:1, this was approximately 85% of the reactivity at a molar ratio of 2:1 and of the reactivity of the corresponding ^{125}I-labeled MAb conjugate.

These analyses indicated that the integrity of MAb E48 remained mostly intact upon coupling of up to 20 Re-MAG3 groups. To evaluate whether the MAb loaded with a high number of Re-MAG3 groups had retained its pharmacokinetic characteristics, biodistribution studies were performed with conjugates labeled at various Re-MAG3:E48 IgG molar ratios in tumor-free nude mice. Twenty μg ^{186}Re-E48 IgG labeled at Re-MAG3:MAb molar ratios of 2.9:1, 9.5:1 or 12.8:1, respectively, were injected (four mice per molar ratio). The mice were killed 40 hr postinjection and the biodistribution was determined. For the conjugate with the Re-MAG3:MAb molar ratio of 2.9:1, at that time point 17.6% ID $\cdot g^{-1}$ was localized in the blood (Fig. 2). For those with a molar ratio of 9.5:1 and 12.8:1, a remarkable lower level was found in the blood: 9.6% and 3.5% ID $\cdot g^{-1}$, respectively. For these latter two ^{186}Re-E48 IgG conjugates, the distinctly lower blood level corresponded with lower levels in other tissues.

Analysis of Rhenium-186-MAb E48 by Protein Mass Spectrometry

As a first step in finding an explanation for the rapid blood clearance of ^{186}Re-MAb conjugates labeled at high Re-MAG3:MAb molar ratios, we investigated the possibility that artifacts might have been introduced in the labeling procedure during our attempt to enhance the Re-MAG3:MAb molar ratio. One realistic possibility was that upon increasing the amount of chemicals a certain percentage of empty MAG3-ester was not eliminated during the Sep-pak procedure due to overloading. It

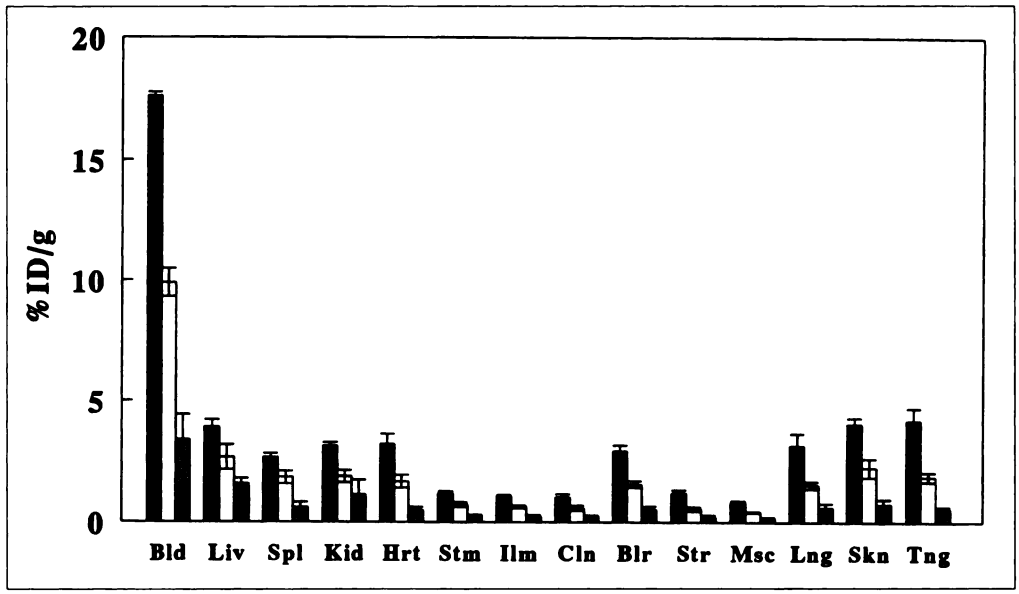

FIGURE 2. Biodistribution of ^{186}Re-E48 IgG labeled at various Re-MAG3:MAb molar ratios. At 40 hr postintravenous injection of 20 μg (8.5 μCi) ^{186}Re-E48 IgG containing 2.9 Re-MAG3 groups per IgG molecule (black bars), 20 μg (27.7 μCi) ^{186}Re-E48 IgG containing 9.5 Re-MAG3 groups (open bars), and 20 μg (37.3 μCi) ^{186}Re-E48 IgG containing 12.8 Re-MAG3 groups (hatched bars), mice were bled, killed, dissected and the %ID · g^{-1} was calculated. Bld = blood; Liv = liver; Spl = spleen; Kid = kidney; Hrt = heart; Stm = stomach; Ilm = ileum; Cln = colon; Blr = bladder; Str = sternum; Msc = muscle; Lng = lung; Skn = skin; Tng = tongue. Number of mice: four per ^{186}Re-MAb conjugate.

can be anticipated that under such conditions empty MAG3 molecules were also coupled to the MAb. Protein-mass spectrometric analysis was used to investigate this possibility. For a ^{186}Re-E48 IgG conjugate prepared in the presence of a high excess of MAG3 and calculated to contain 7 Re-MAG3 groups per MAb E48 molecule on the basis of radioactivity measurement, protein-mass spectrometric analysis revealed that upon rhenium coupling the molecular mass of the MAb had increased from 149,214 to 152,335 Da (increase 3121). Based on these results, it can be concluded that besides the seven Re-MAG3 molecules (total mass 7 × 442 = 3094), no other groups were coupled to the MAb. The same conclusion was drawn after analysis of conjugates with a Re-MAG3:MAb molar ratio of 2:1 and 11:1, respectively.

A high number of Re-MAG3 groups might make a conjugate susceptible to catabolic processes in the in vivo situation resulting in the observed elimination of ^{186}Re from the blood (Fig. 2). In vivo instability of the ^{186}Re-MAG3 complex might be another explanation for rapid elimination. As a tool to differentiate between these two possibilities, we decided to develop Tc-MAG3-E48 IgG conjugates labeled to various Tc-MAG3:MAb molar ratios. If the number of metal-MAG3 groups is the driving force behind rapid blood elimination, one may expect Tc-MAG3-MAb conjugates labeled at a high Tc-MAG3:MAb molar ratio to behave in the same way. If the Re-MAG3 complex itself has a different in vivo stability, one can measure this aspect in biodistribution experiments using dual-labeled MAbs containing both Tc-MAG3 groups and Re-MAG3 groups on each MAb molecule.

Preparation of Technetium-99m/Technetium-99-MAG3-MAb Conjugates

Coupling of ^{99m}Tc to E48 IgG, resulted in a conjugate mass of 149,160 Da, which is similar to that of unconjugated E48 IgG. This is in accordance to the expectations since ^{99m}Tc obtained from a ^{99}Mo generator is almost carrier-free and only a small proportion of the MAG3 molecules will be filled with ^{99m}Tc upon reduction. To obtain Tc-MAb conjugates at desired Tc-MAG3:MAb molar ratios up to 20:1, as is possible for the preparation of ^{186}Re-MAb conjugates, we used $^{99}TcO_4^-$ with $^{99m}TcO_4^-$ as the tracer. At one step, the preparation of ^{99m}Tc/^{99}Tc-MAb conjugates differed from the procedures we previously described for preparing ^{99m}Tc-MAb conjugates: the ^{99m}Tc/^{99}Tc-reduction required more $SnCl_2$ than ^{99m}Tc. When a mixture of ^{99m}Tc/^{99}Tc was used for labeling, the efficiency of incorporation of ^{99m}Tc into MAG3 was more than 95% as assessed by counting of the gamma emission of ^{99m}Tc in TLC or HPLC analyses. The efficiency of the esterification was assessed in the same way and appeared to be 80%–90%, while conjugation of the ester to 1 mg of MAb E48 IgG was reproducible with an efficiency of 60%. When ^{99m}Tc/^{99}Tc-labeled active ester and MAb conjugate were stored till decay of ^{99m}Tc and counted for beta emission, it appeared that ^{99}Tc had been incorporated exactly as efficiently as ^{99m}Tc, which confirmed that ^{99m}Tc and ^{99}Tc behave the same chemically during the labeling procedure. Therefore, on the basis of ^{99m}Tc counting, the ^{99m}Tc/^{99}Tc-MAG3:MAb molar ratio can be determined. The fact that Tc-MAG3:MAb molar ratios found in this way corresponded to that assessed by protein-mass spectrometry confirmed this once more. For example, conjugates determined to contain 12 Tc-MAG3 groups per MAb gave a mass of 153,480 Da, which is in accordance to the mass corresponding to E48 IgG (149,214 Da) plus the mass of 12 Tc-MAG3 groups (4,272). These data demonstrate again that the conjugates do not contain any empty MAG3 (mol wt = 242) or other groups.

With this labeling procedure, it became possible to produce ^{99m}Tc/^{99}Tc-E48 IgG conjugates with each desired ^{99m}Tc/^{99}Tc-MAG3:MAb molar ratio up to 20:1. After purification, conjugates never contained more than 6.0% of unbound ^{99m}Tc/^{99}Tc, while the immunoreactivity was not affected for conjugates containing less than 12 Tc-MAG3 groups per MAb molecule. From these results, it can be concluded that it is possible to prepare, in a reproducible and controlled way, uniform radioimmunoconjugates containing either rhenium or technetium.

Biodistribution of Dual-Labeled MAbs Containing Rhenium-186-MAG3 and Technetium-99m/Technetium-99-MAG3.

In this experiment, the in vivo stability of the ^{186}Re-MAG3 complex was compared to that of the ^{99m}Tc/^{99}Tc-MAG3 complex. To this end, E48 IgG was double labeled with 7.7 metal-MAG3 groups (4.5 Re-MAG3 groups and 3.2 Tc-MAG3 groups) and 60 μg of the conjugate were injected per mouse (four mice per molar ratio). The biodistribution of the ^{186}Re and ^{99m}Tc radionuclides was assessed at 1, 6, and 24 hr p.i.. Tissue uptake of ^{186}Re and ^{99m}Tc as percentage of injected dose was

TABLE 1
Biodistribution of Dual-Labeled $^{186}Re/^{185}Re/^{99m}Tc/^{99}Tc$-E48-IgG

Organ	%ID/g					
	1 hr		6 hr		24 hr	
	^{186}Re	^{99m}Tc	^{186}Re	^{99m}Tc	^{186}Re	^{99m}Tc
Blood	35.3 ± 1.0	36.3 ± 1.1	20.4 ± 0.1	21.0 ± 0.1	16.2 ± 1.3	16.3 ± 1.3
Liver	8.40 ± 0.29	8.84 ± 0.26	4.93 ± 0.20	5.02 ± 0.24	3.86 ± 0.17	3.85 ± 0.16
Spleen	4.70 ± 0.27	4.86 ± 0.32	2.72 ± 0.09	2.95 ± 0.12	2.34 ± 0.29	2.32 ± 0.29
Kidney	6.73 ± 0.40	7.02 ± 0.49	4.09 ± 0.26	4.45 ± 0.34	2.84 ± 0.43	3.13 ± 0.38
Heart	4.57 ± 0.44	4.79 ± 0.91	4.34 ± 0.23	4.51 ± 0.27	2.63 ± 0.40	2.69 ± 0.40
Stomach	1.58 ± 0.06	1.40 ± 0.05	1.72 ± 0.12	1.85 ± 0.13	1.13 ± 0.08	1.05 ± 0.09
Ileum	2.16 ± 0.05	2.23 ± 0.03	1.51 ± 0.06	1.55 ± 0.05	0.97 ± 0.11	0.99 ± 0.11
Colon	0.87 ± 0.02	0.90 ± 0.05	1.36 ± 0.11	1.43 ± 0.10	0.97 ± 0.15	1.01 ± 0.13
Bladder	0.59 ± 0.08	0.59 ± 0.03	1.30 ± 0.22	1.51 ± 0.27	2.22 ± 0.17	2.29 ± 0.22
Sternum	1.12 ± 0.09	1.16 ± 0.09	0.99 ± 0.07	1.02 ± 0.07	0.85 ± 0.07	0.86 ± 0.09
Muscle	0.42 ± 0.05	0.44 ± 0.04	0.47 ± 0.06	0.52 ± 0.09	0.57 ± 0.02	0.60 ± 0.04
Lung	3.72 ± 0.50	3.75 ± 0.47	2.65 ± 0.19	2.70 ± 0.24	2.35 ± 0.21	2.08 ± 0.31
Skin	1.07 ± 0.03	1.04 ± 0.03	2.79 ± 0.02	2.69 ± 0.06	3.25 ± 0.16	3.34 ± 0.16
Tongue	1.99 ± 0.15	2.10 ± 0.18	3.20 ± 0.20	3.25 ± 0.14	3.68 ± 0.14	3.71 ± 0.21

Intervals given are s.e.m.

the same (Table 1). These data show that the in vivo stability of the Re-MAG3 and the Tc-MAG3 complex is the same.

Co-injection of Technetium-99m/99 and Rhenium-186-Labeled MAbs

Based on the data described in previous paragraphs, it seemed that the number of metal-MAG3 groups is the driving force behind rapid blood clearance of ^{186}Re-MAb conjugates labeled at a high Re-MAG3:MAb molar ratio. When this is true, one may expect that Tc-MAG3-MAb and Re-MAG3-MAb molecules will show similar biodistribution characteristics when labeled to a same metal-MAG3:MAb molar ratio, a prerequisite for use as matched pair in RIS and RIT.

To evaluate whether Tc- and Re-MAb conjugates prepared according to our method fulfill this requirement, we performed biodistribution experiments with simultaneous injection of $^{99m}Tc/^{99}Tc$-MAb conjugates and ^{186}Re-MAb conjugates prepared at a similar metal-MAG3:E48 IgG molar ratio. The first group of mice received a $^{99m}Tc/^{99}Tc$-MAb conjugate with a metal-MAG3:MAb molar ratio of 6.0:1 as well as a ^{186}Re-MAb conjugate with a molar ratio of 6.3:1. For the second and third groups of mice, these molar ratios were 8.2:1 and 8.6:1, respectively, and 9.4:1 and 9.4:1. All mice were simultaneously injected with ^{125}I E48 IgG as a reference and the biodistribution of the three labels was determined at 24 hr postinjection. To obtain insight as to the route of activity excretion, samples of the stomach, ileum and colon contents as well as urine were collected in this experiment.

The biodistribution of technetium and Re-MAb conjugates co-injected into the Group 1 mice (metal-MAG3:MAb molar ratio 6.0:1 and 6.3:1, respectively) was the same (Fig. 3A). The ^{125}I-E48 IgG reference showed a higher accumulation of activity in the stomach contents, which is indicative for free ^{125}I, and a lower accumulation in both the contents of the ileum and the colon; for blood and other organs no difference was observed. A comparable pattern was obtained for the biodistribution of Tc-, Re- and ^{125}I-MAb conjugates coinjected into the Group 2 mice (Tc-MAG3:MAb molar ratio 8.2:1; Re-MAG3:MAb molar ratio 8.6:1) (Fig. 3B). A subtle increase in the urine and ileum and colon content levels of ^{99m}Tc and ^{186}Re was observed in comparison to the ^{125}I levels and those of the Group 1 mice. The two conjugates with the 9.4:1 metal-MAG3:MAb molar ratio showed the same blood level (Fig. 3C), but the level was markedly lower than the level of ^{125}I-MAb reference and also much lower than the blood levels of the other Tc-MAb and Re-MAb conjugates evaluated in Figure 3A, B. The conjugates with the 9.4:1 metal-MAG3:MAb molar ratio also showed the highest activity accumulation in colon contents and urine. These data indicate that conjugates with too many metal-MAG3 groups coupled to the MAb are increasingly susceptible to catabolic processes in in vivo situations which result in excretion of activity in the urine and feces.

Figure 4 shows that ^{186}Re-E48 IgG and $^{99m}Tc/^{99}Tc$-E48 IgG have a similar pharmacokinetic behavior when labeled to a same metal-MAG3:MAb molar ratio. In Figure 4, data of four independent experiments are combined in which the biodistribution at 40–48 hr postinjection of ^{186}Re-E48 IgG and/or $^{99m}Tc/^{99}Tc$-E48 IgG was compared with the biodistribution of ^{125}I-E48 IgG. At low metal-chelate:MAb molar ratios, these levels were similar to that of ^{125}I-E48 IgG, whereas for molar ratios ranging from about 8 up to 17, a gradual decrease of technetium as well as rhenium blood levels was found in comparison to ^{125}I blood levels.

To obtain additional insight in the kinetics of blood clearance and the route of excretion, the biodistribution of conjugates with different Re-MAG3:MAb molar ratio was assessed at various time points after injection. Therefore, mice were killed 1, 6, 24 or 40 hr after injection of conjugates with a Re-MAG3:E48 IgG molar ratio of 2:1, 7:1 or 10:1. As a reference, ^{125}I-labeled MAb E48 was administered to each group of mice. Figure 5 shows the ^{186}Re concentration and uptake of the ^{186}Re-MAb conjugates in the blood, liver and ileum and colon contents relative to the ^{125}I concentration and uptake. Figure 5A shows a gradual decrease of ^{186}Re blood levels in comparison to ^{125}I blood levels for the conjugate with the highest Re-MAG3:E48 IgG molar ratio of 10:1. This molar ratio, however, remains constant between 24 and 40 hr after injection. Disappearance of the conjugate with the Re-MAG3:MAb molar ratio of 10:1 from the blood is accompanied by a marked increase of ^{186}Re levels in the liver (Fig. 5B), ileum contents (Fig. 5C) and colon contents (Fig. 5D). Activity levels in blood and liver are similar for the ^{125}I-MAb conjugate and the conjugates with a Re-MAG3 molar ratio of 2:1 and 7:1, but the ^{186}Re levels of the latter two

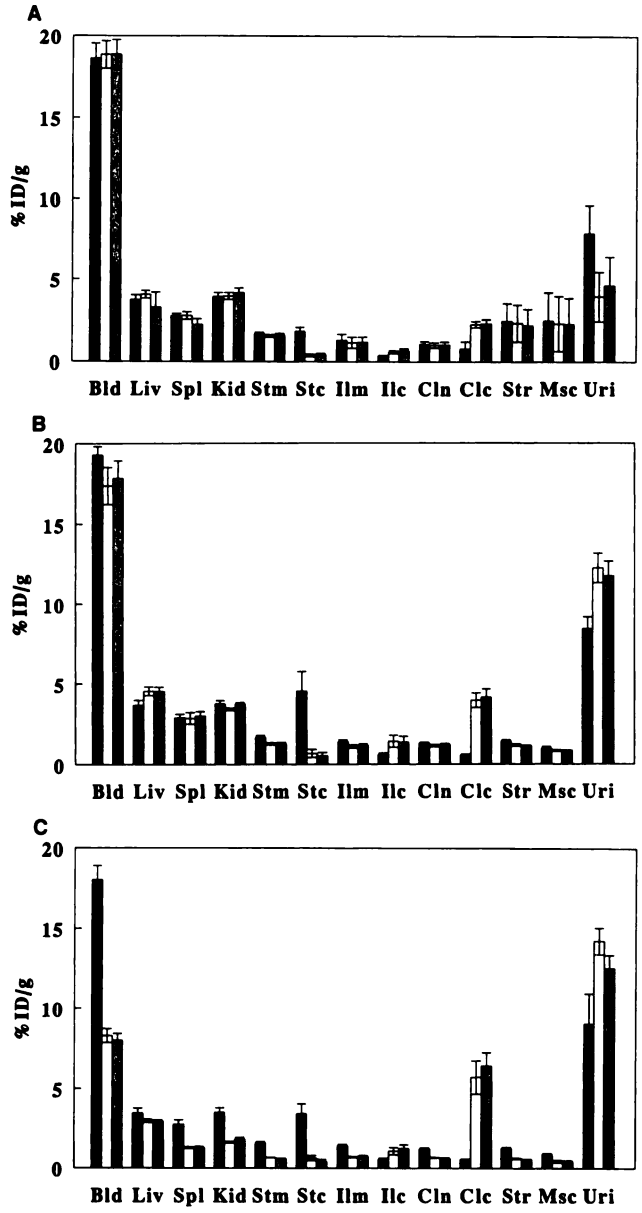

FIGURE 3. Comparison of the biodistribution of ^{186}Re-E48 IgG and ^{99m}Tc/^{99}Tc-E48 IgG labeled at various metal-MAG3:MAb molar ratios 24 hr following intravenous injection. Iodine-125-labeled E48 IgG was co-injected as a reference. Three groups of mice simultaneously received: (A) 30 μg (4.4 μCi) ^{125}I-E48 IgG (black bars), 30 μg (77 μCi) ^{99m}Tc/^{99}Tc-E48 IgG containing 6.0 Tc-MAG3 groups per MAb (open bars) and 30 μg (8.0 μCi) ^{186}Re-E48 IgG containing 6.3 Re-MAG3 groups per MAb (hatched bars), (B) 30 μg (4.4 μCi) ^{125}I-E48 IgG (black bars), 30 μg (103 μCi) ^{99m}Tc/^{99}Tc-E48 IgG containing 8.2 Tc-MAG3 groups per MAb (open bars) and 30 μg (10.8 μCi) ^{186}Re-E48 IgG containing 8.6 Re-MAG3 groups per MAb (hatched bars) or (C) 30 μg (4.4 μCi) ^{125}I-E48 IgG (black bars), 30 μg (118 μCi) ^{99m}Tc/^{99}Tc-E48 IgG containing 9.4 Tc-MAG3 groups per MAb (open bars), and 30 μg (11.9 μCi) ^{186}Re-E48 IgG containing 9.4 Re-MAG3 groups per MAb (hatched bars). At 24 hr postinjection, the mice were bled, killed and dissected and the %ID · g^{-1} was calculated. Abbreviations are the same as in Figure 2. Number of mice: four per group.

conjugates in the ileum and colon contents are higher than the ^{125}I levels. From these experiments, it can be concluded that conjugates with too many ^{186}Re-MAG3 groups coupled to the MAb become trapped in the liver within 1 hr after injection (Fig. 5B). Radioactivity is subsequently released from the liver and excreted through the feces (Figs. 3 and 5C, D) and urine (Fig. 3) within 24 hr after injection.

Biodistribution of Various MAbs Labeled with Technetium-99m/Technetium-99 by MAG3

To test whether the rapid blood clearance of radioimmunoconjugates at increased metal-MAG3:MAb molar ratios as observed with murine MAb E48 IgG is a general phenomenon, the biodistribution of three other MAbs labeled at various ^{99m}Tc/^{99}Tc-MAG3:MAb molar ratios was assessed in tumor-free nude mice. Murine MAb K928 was labeled with a ^{99m}Tc/^{99}Tc-MAG3:MAb molar ratio of 2.3:1, 6.4:1 and 12.1:1, while the chimeric mouse/human MAbs E48 and SF-25 were labeled with molar ratios of 2.4:1, 6.6:1, 12.1:1 and 2.2:1, 6.2:1, 12.0:1, 15.3:1, respectively. The ^{99m}Tc/^{99}Tc-labeled MAbs were co-injected with their corresponding ^{125}I-labeled MAbs, their biodistribution assessed at 24 hr postinjection and the level of ^{99m}Tc/^{99}Tc activity in the blood expressed relative to the level of ^{125}I activity. For all conjugates with six or fewer ^{99m}Tc/^{99}Tc-MAG3 groups, the %ID ^{99m}Tc:%ID ^{125}I ratio was about 1, indicating that the pharmacokinetic behavior of the four MAbs does not become impaired by coupling of up to six ^{99m}Tc/^{99}Tc-MAG3 groups (Table 2). This percentage was less than 1 when 12 such groups were coupled to a MAb, indicating a more rapid blood clearance of the ^{99m}Tc/^{99}Tc-conjugates than of the corresponding ^{125}I-conjugates. Moreover, the ratio was higher for the two cMAbs E48 and SF-25 (0.68 and 0.79, respectively) than for the two mMAbs (0.39 and 0.44, respectively), which means that the two murine MAbs become more readily cleared from the blood than the two chimeric MAbs.

DISCUSSION

We used the MAG3 chelate to develop a ^{186}Re labeling procedure which ensures that the low specific activity of ^{186}Re is no longer a restrictive factor. In the present study, we investigated the chemical scope of this labeling method and the possible pharmacokinetic consequences if applied to high-dose ^{186}Re labeling for clinical RIT studies. We found that one should not only be able to couple a large number of Re-MAG3 groups to a MAb, but these conjugates should also be fully immunoreactive, be stable in vitro as well as in vivo and not result in accumulation of the conjugate at nontarget sites.

We were able to couple 20 Re-MAG3 groups to one MAb molecule. For direct methods, the Re:MAb molar ratio obtained is about 1.5:1; for indirect methods it is slightly higher, 2–3:1. The overall radiochemical yield with our method was 40%–60%. HPLC analysis indicated that there was no aggregate formation. Conjugates with a Re-MAG3:MAb molar ratio up to 12 remained fully immunoreactive. For conjugates with a higher molar ratio, the immunoreactivity decreased slightly. For a conjugate with a molar ratio of 20:1, the immunoreactivity was about 85% of the maximum.

Although these data suggest that the integrity of MAbs remained nearly intact upon coupling of up to 20 Re-MAG3 groups, there was a serious alteration of the pharmacokinetic behavior of conjugates containing more than eight Re-MAG3 groups on average. For the conjugate with a Re-MAG3:MAb molar ratio of 12.8:1, for example, the level of radioactivity in the blood at 40 hr postinjection was just 20% of that of a conjugate with a molar ratio of 2.9:1 (Fig. 2). In the present study, we show a strong correlation between the Re-MAG3:MAb E48 molar ratio and blood clearance. To arrive at this conclusion, we first had to prove that no other chemical groups than Re-MAG3 were coupled to the MAb during the labeling procedure. Thereafter, through dual labeling of Re-MAG3 and Tc-MAG3 groups to the same MAb molecule, we found that the in vivo stability of the Re-MAG3 complex was comparable to its corresponding Tc-MAG3 complex (Table 1). In our previous

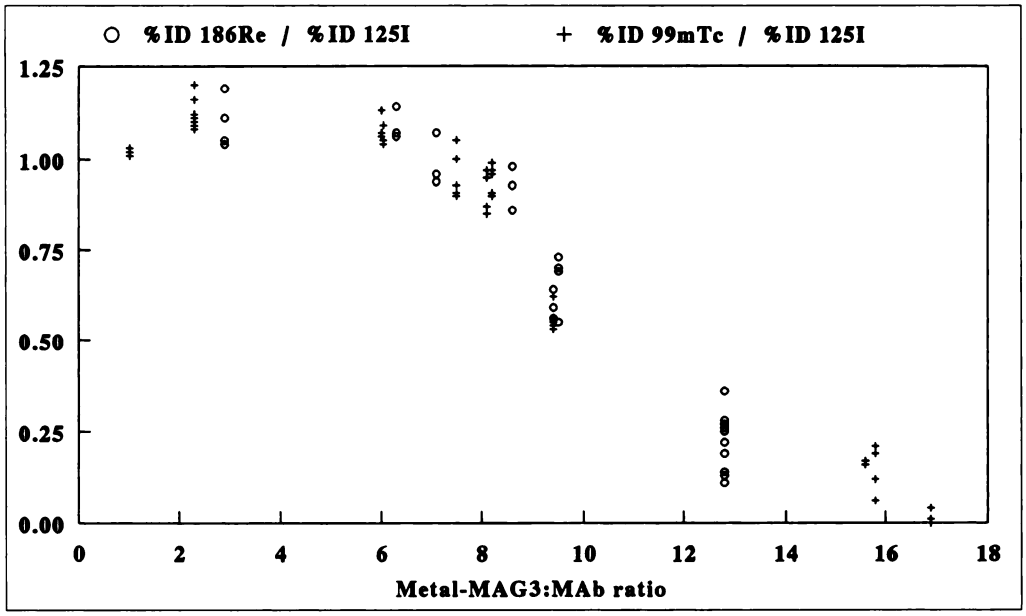

FIGURE 4. Comparison of the blood levels of ^{186}Re-E48 IgG and ^{99m}Tc/^{99}Tc-E48 IgG labeled at various metal-MAG3:MAb molar ratios 40–48 hr following intravenous injection. Rhenium-186-E48-IgG (circles) and ^{99m}Tc-E48-IgG (crosses) blood levels relative to the levels of coinjected reference ^{125}I-E48-IgG for various metal-MAG3:MAb molar ratios. Data given are the combined results of four independent experiments (number of mice = 73). Antibody dose was 40–90 μg.

paper, we demonstrated that once the radiolytic decomposition was suppressed, the ^{186}Re-MAG3 complex was a normal stable compound in vitro. This dual-labeling experiment implies that no detectable difference exists between rhenium and technetium with respect to the ease of reoxidation in vivo. Finally, we observed similar biodistribution characteristics of ^{186}Re-E48 IgG conjugates and ^{99m}Tc/^{99}Tc-E48 IgG conjugates labeled at a same metal-MAG3:MAb molar ratio (Figs. 3 and 4).

When the blood clearance experiments on MAb E48 are evaluated together (Fig. 4), a consistent pattern becomes clear. Assuming a Poisson distribution of the number of Re-MAG3 groups bound to a MAb after conjugation, a critical molar ratio of 10 could be calculated from these data, which means that Re-MAG3-MAb molecules with a molar ratio of about 10 or more are removed from the blood and taken up by the liver shortly after injection. Based on this Poisson distribution, it could be calculated that about 50% of the MAb molecules will contain 10 or more metal-MAG3 groups if the conjugate has a mean metal-MAG3:MAb molar ratio of 10. We found that about 50% of the conjugate was cleared from the blood shortly after injection. The remaining 50% appeared to exhibit a pharmacokinetic profile similar to that of ^{125}I-labeled MAb or MAb conjugated with seven or less metal-chelate groups (Fig. 5). Although for MAb E48 a Re-MAG3:MAb molar ratio of about 10 seems to be critical for the pharmacokinetic behavior of the conjugate, this critical molar ratio appeared to be slightly higher for the cMAbs E48 and SF-25.

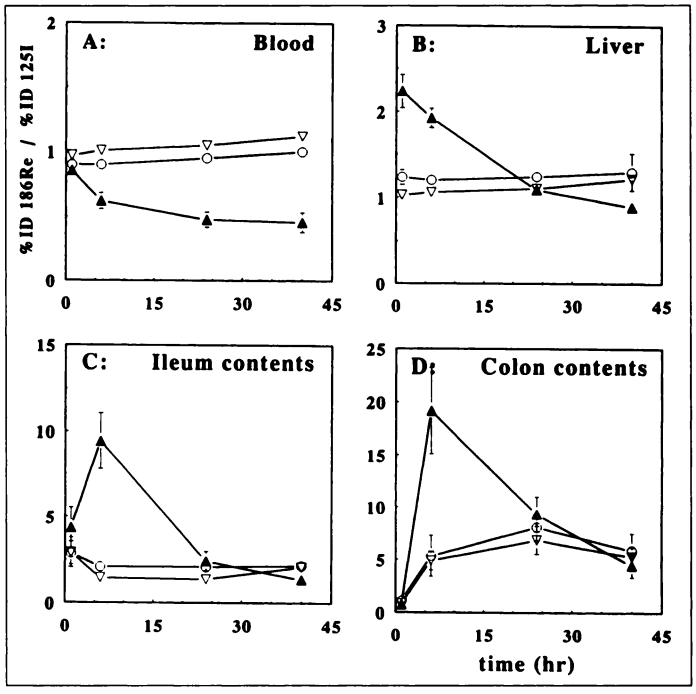

FIGURE 5. Biodistribution of ^{186}Re-E48 IgG labeled at various Re-MAG3:MAb molar ratios at various time intervals after injection. Iodine-125-E48 IgG was co-injected as a reference. Rhenium-186-E48-IgG conjugates with a Re-MAG3:E48 IgG molar ratio of 2:1 (▽)(5.1 μg, 30 μCi), 7:1 (○)(15.9 μg/30 μCi) or 10:1 (▲)(23.4 μg/30 μCi) and ^{125}I-E48 IgG (5.1 μg/30 μCi) were injected into tumor-free mice and ^{186}Re activity levels relative to ^{125}I levels were assessed in blood (A), liver (B) and ileum (C) and colon contents (D) 1, 6, 24 and 40 hr postinjection. Number of mice: four per time point.

Rapid blood clearance and extensive liver accumulation has also been observed for MAbs coupled with other chemical groups to their lysine residues (*37–39*). Like MAbs labeled with ^{153}Sm through DTPA, rapid blood clearance and liver accumulation was observed in rats when 20 chelate groups were coupled per MAb (*37*). A similar phenomenon was observed for MAbs labeled with ^{67}Cu through the 14N4 macrocycle in mice (*38*). Pelegrin et al. (*39*) evaluated the immunoreactivity and biodistribution in mice of MAbs coupled with 4–19 fluorescein groups. Coupling of more than 10 groups per MAb resulted in impaired immunoreactivity and enhanced blood clearance. Conjugates with more than 14 fluorescein molecules had dramatically shorter whole-body half-lives.

In our method for labeling MAbs with ^{186}Re, the purification of the active ester by a Sep-Pak procedure prevented the coupling of empty MAG3 groups to the MAb, thus allowing the coupling of a relatively high number of Re-MAG3 molecules before impairment of conjugate immunoreactivity and biodistribution occurred. Fritzberg et al. (*26,40*) recently reported on a pre-ester method for coupling of ^{186}Re to MAbs with N_2S_2 and N_3S chelates: the pre-ester method implies a one-pot reduction, transchelation and conjugation. Using the N_3S butyrate chelate, ^{186}Re-MAb conjugates prepared by this latter method have been extensively evaluated in clinical RIT trials (*23,28,29*). Despite the fact that only two to three Re-MAG2-GABA groups were coupled per MAb (*41*), extensive accumulation of activity was observed in the liver and intestines, resulting in hepatic enzyme elevation in the majority of patients

TABLE 2
Blood Levels of Various Technetium-99m/Technetium-99-MAbs Relative to Co-injected Iodine-125-MAb

mMAb E48		mMAb K928		cMAb E48		cMAb SF-25	
	%ID ^{99m}Tc		%ID ^{99m}Tc		%ID ^{99m}Tc		%ID ^{99m}Tc
Ratio	%ID ^{125}I	Ratio	%ID ^{125}I	Ratio	%ID ^{125}I	Ratio	%ID ^{125}I
2.0	1.04 ± 0.02	2.3	1.11 ± 0.01	2.4	1.14 ± 0.05	2.2	1.05 ± 0.06
6.0	0.97 ± 0.05	6.4	0.99 ± 0.01	6.6	1.01 ± 0.03	6.2	0.93 ± 0.05
12.0	0.39 ± 0.06	12.1	0.44 ± 0.01	12.1	0.68 ± 0.04	12.0	0.79 ± 0.03
15.6	0.17 ± 0.01		nd		nd	15.3	0.49 ± 0.01

nd = not determined.

(*29*). Therefore, liver accumulation was observed at a much lower Re-chelate:MAb molar ratio than we found in mice. Since the conjugate is formed in one vial without a preceding purification step in the pre-ester method, however, it might be that besides Re-MAG2-GABA molecules also empty MAG2-GABA molecules that become coupled to the MAb. Empty chelate molecules may contribute to the liver and intestine accumulation of ^{186}Re. This aspect also has to be considered when using postlabeling methods. In these latter methods, the number of chelates may alter the pharmacokinetic behavior of the MAb. When this is combined with potentially in vivo labile chelating groups, nonspecific binding of the radiolabel to the MAb and the presence of aggregates, the reasons for increased liver uptake can become manifold. John et al. (*42*), for example, used a N_2S_4 chelate in a postlabeling method to couple ^{99m}Tc to MAbs and found indications for partial amino-group mediated nonspecific binding of ^{99m}Tc (*42*). Liver uptake was much higher for ^{99m}Tc-MAb conjugates than for the corresponding ^{125}I-MAb conjugates. For ^{186}Re-MAb conjugates prepared with the same method, they found a similar high liver uptake (*22*). The lower ^{125}I levels in the liver were postulated by the authors to be due to dehalogenation and rapid elimination of ^{125}I from this tissue. This dehalogenation hypothesis does not agree with our experiments, in which we demonstrate similar activity levels in the liver for ^{125}I-E48 IgG and ^{186}Re-E48 IgG labeled at a Re-MAG3:MAb molar ratio of 2:1 and 7:1 (Fig. 5). The data seem to indicate that in order to avoid chemical artifacts and nonspecific binding of radiolabel, purification of the radionuclide-chelate complex before conjugation to the MAb, as performed in our protocol, is the best way for coupling of a high dose of ^{186}Re without influencing the integrity of the MAb.

The mechanism behind the rapid blood clearance of MAbs containing too many metal-MAG3 groups is not yet known. Interestingly, cMAbs E48 and SF-25 can carry more metal-MAG3 groups before clearing from the blood than mMAbs E48 and K928. One possibility might be that conjugate clearance is related to its charge. Therefore, we assessed the isoelectric point of the four MAbs before and after conjugation of various ^{99m}Tc/^{99}Tc-MAG3 groups by isoelectric focusing electrophoresis. The pI of MAb E48 changed from pI 7.1 in its unconjugated form to pI 6.5 upon coupling of two ^{99m}Tc/^{99}Tc-MAG3 groups (Fig. 6). Upon coupling of 8 or 12 groups, the pI became 5.2 and 4.9, respectively. The pI of MAb K928 appeared to be 6.8, which is similar to that of MAb E48. The pI of cMAbs E48 and SF-25 appeared to be higher, 9.5 and 8.5 respectively, and also decreased upon coupling of ^{99m}Tc/^{99}Tc-MAG3 (to 6.0 and 6.2, respectively, upon coupling of 12 ^{99m}Tc/^{99}Tc-MAG3 groups). Based on these results, we postulate that a metal-MAG3-MAb molecule becomes rapidly cleared in vivo when its pI is too low. When this is true, the higher pI value of the cMAbs allows more metal-MAG3 molecules to be coupled per MAb before the critical molar ratio for rapid blood clearance is reached. As mentioned above, however, besides the total charge of the conjugate, it might also be that one simply cannot unlimitedly change a cationic lysine-group into an anionic or neutral one without affecting the pharmacokinetic behavior.

We found that no-carrier-added ^{99m}Tc-MAG3-MAb forms a matched pair with ^{186}Re-MAG3-MAb to a limited extent, while ^{99m}Tc/^{99}Tc-MAb conjugates always showed a similar biodistribution to ^{186}Re-MAb conjugates at the same metal-MAG3:MAb molar ratio. The ^{99m}Tc/^{99}Tc-MAG3-MAb conjugates are ideal for use in RIS and can be safely used in patients. As for the ethical concerns of using ^{99}Tc, one has to realize that this will result in just minimal extra radiation exposure for the patients. The yearly intake of cosmogenic ^{14}C by air is on the same order of magnitude (0.6 μCi) and has a similar energy (E_β 200 keV). With respect to its effective half-life, we can compare $^{99}TcO_4^-$ with ^{14}C. The $t1/2_{eff.}$ of $^{99}TcO_4^-$ for the total body is 1 day and 25 days for bone; for ^{14}C, these values are 10 and 40 days, respectively. Moreover, possibly formed ^{99}Tc-MAG3 is rapidly excreted by the kidneys.

Griffiths et al. (*21*) described a uniform method for direct labeling of MAbs with ^{99m}Tc, ^{186}Re and ^{188}Re (*21*), but the ^{99m}Tc-MAb and ^{188}Re-MAb conjugates prepared in this way showed a different biodistribution in tumor-bearing nude mice. Breitz et al. (*28*) evaluated the feasibility of using ^{99m}Tc and ^{186}Re as a matched pair for clinical imaging, dosimetry calculations and therapy (*28*). In a Phase I trial in patients with refractory metastatic epithelial carcinomas, a group of patients were imaged with ^{99m}Tc-labeled NR-LU-10 Fab followed by RIT with ^{186}Re-labeled NR-LU-10 IgG; another group received ^{99m}Tc-labeled NR-CO-02 $F(ab')_2$ for RIS followed by ^{186}Re-labeled NR-CO-02 $F(ab')_2$ for RIT. In the latter group, the ^{99m}Tc-immunoconjugates were able to predict localization of

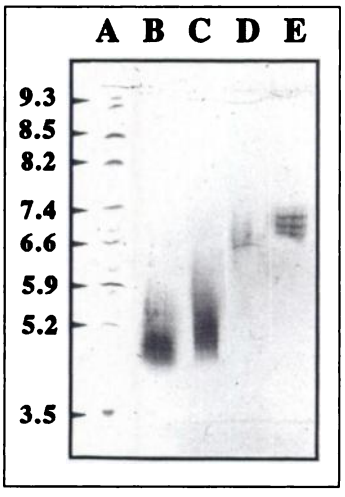

FIGURE 6. Analysis of E48 IgG labeled with 12 (lane B), 8 (lane C) and 2 (lane D) ^{99m}Tc/^{99}Tc-MAG3 groups per IgG by isoelectric focusing. Lane E is unconjugated E48 IgG and Lane A is pI calibration markers.

the ^{186}Re-labeled MAb. The pharmacokinetics and mean absorbed doses predicted from the ^{99m}Tc studies and calculated from the ^{186}Re studies compared favorably, but there was considerable patient variation, resulting in limited value of dosimetry predictions for any one patient. An explanation provided by the authors was that different antibody doses were used for imaging and therapy. In these studies, the previously described pre-ester method with MAG2-GABA as chelate was used to couple ^{99m}Tc and ^{186}Re. In view of our results, it might be that the ^{99m}Tc-conjugates and the ^{186}Re-conjugates used in these studies were not exactly the same chemically.

Recently, we tested ^{186}Re-E48-IgG in RIT studies in HNSCC xenograft bearing nude mice (*43,44*). A single bolus injection of 500 μCi ^{186}Re-labeled MAb E48 in mice bearing tumors of 140 $\pm$ 60 mm^3 resulted in 50% complete remissions (6 of 12). Treatment of mice with smaller tumors (75 $\pm$ 17 mm^3) with 600 μCi ^{186}Re-E48-IgG resulted in complete ablation of all tumors (n = 12). In this animal model, ^{186}Re-labeled MAb E48 appeared to be more effective than ^{131}I-labeled MAb E48, despite the fact that tumor uptake levels of the former conjugate were slightly lower as a result of the more rapid blood clearance (*36*). We can now interpret this latter observation by the relatively high Re-MAG3:MAb molar ratio of the conjugate used in these studies (this molar ratio was 8.6).

CONCLUSION

We have developed a method for efficient and reproducible production of stable ^{186}Re-MAb conjugates. Due to the high Re:MAb molar ratios obtained and the favorable biodistribution characteristics of the conjugates, this method may be better suited for clinical RIT than other available methods. In addition, we have automated the coupling procedure for high doses of ^{186}Re to MAbs (Fig. 1), which will limit the radiation exposure to the operator. Our results suggest that RIT studies with ^{186}Re-labeled MAb E48 and/or U36 in head and neck cancer patients will be initiated in the near future. As a bridging study to RIT, however, we will first perform RIS studies using ^{99m}Tc/^{99}Tc-MAb conjugates with increasing ^{99m}Tc/^{99}Tc-MAG3:MAb molar ratios. Images will be used to estimate activity in the tumor and sites at risk for RIT. By using ^{99m}Tc/^{99}Tc instead of ^{186}Re, the optimal metal-MAG3:MAb molar ratio can be assessed safely. This optimal molar ratio will subsequently be used in ^{186}Re-MAb therapy. It can be anticipated that the metal-MAG3:MAb molar ratio giving optimal biodistribution in mice may be different in humans. In humans, however, individual differences may also occur. Our preclinical data indicate that the use of ^{99m}Tc/^{99}Tc-MAb imaging to select candidates for ^{186}Re therapy may become feasible.

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Bifunctional NHS-BAT Ester for Antibody Conjugation and Stable Technetium-99m Labeling: Conjugation Chemistry, Immunoreactivity and Kit Formulation

M. Eisenhut, W.D. Lehmann, W. Becker, T. Behr, H. Elser, W. Strittmatter, A. Steinsträsser, R.P. Baum, T. Valerius, R. Repp and Y. Deo

Department of Nuclear Medicine, University of Heidelberg; German Cancer Research Center, Heidelberg; Department of Nuclear Medicine, University of Erlangen-Nürnberg; E. Merck, Darmstadt; Hoechst AG, Frankfurt; Department of Nuclear Medicine, University of Frankfurt; Department of Medicine III, University of Erlangen-Nürnberg, Germany; and MEDAREX, Inc., Annandale, New Jersey

Conjugation chemistry and kit formulated binding of the NHS ester of 6-(4′-(4″-carboxyphenoxy)butyl)-2, 10-dimercapto-2,10-dimethyl-4,8-diazaundecane (NHS-BAT ester) to monoclonal antibodies (MAbs) was investigated. The functionalities of the resulting BAT conjugated and ^{99m}Tc-labeled MAbs BW 431/26, MAb 425 and bispecific MDX210 (fragment construct) were tested by immunoreactivity and immunoscintigraphy. **Methods:** The kinetics and chemistry of the conjugation reaction were monitored by high-performance liquid chromatography, size-exclusion chromatography and positive fast-atom-bombardment mass spectra (FAB-MS). The ^{99m}Tc BAT-MAbs were tested with various immunoreactivity assays. The biodistribution of ^{99m}Tc-BAT-BW 431/26 in rats was compared with directly labeled BW 431/26. **Results:** At pH 8.5 and 25°C, the reactivity of the NHS-BAT ester was high with 90% completion after 30 min. The conjugation yield of 19 μM MAb and 228 μM NHS-BAT ester amounted to 30%. Higher NHS-BAT ester concentrations afforded higher BAT-to-MAb ratios. According to FAB-MS, the conjugation competing hydrolysis surprisingly occurred at the NHS ring. Almost quantitative ^{99m}Tc labeling was achieved after 5 min at 25°C. Immunoreactivity of the ^{99m}Tc-BAT antibodies showed >90% recovery and proved to be insensitive to BAT-to-MAb ratios of up to 10. The ^{99m}Tc-BAT-BW 431/26 showed similar organ distribution but revealed less urinary excretion compared with the directly labeled BW 431/26. Immunoscintigraphy with ^{99m}Tc-labeled and BAT-BW 431/26 and BAT-MAb 425 showed the respective biological function in vivo. **Conclusion:** According to straightforward conjugation chemistry, the ease of ^{99m}Tc labeling and the application of a simple ultrafiltration technique, the NHS-BAT ester represents a nondestructive, universally applicable bifunctional ligand to introduce stable ^{99m}Tc protein binding sites. Kit formulated conjugation/labeling can be performed with little time requirements and laboratory experience.

Key Words: bifunctional ligand; NHS-BAT ester; technetium-99m; radioimmunoscintigraphy; BW 431/26; MAb 425; MDX210

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The efforts that have been undertaken to improve the binding of complexed ^{99m}Tc to antibodies or antibody fragments resulted in a series of experimental approaches. The techniques that have been described are commonly subdivided into two categories: direct labeling methods in which the metal is complexed by preformed sulfhydryl groups, and indirect methods in which chelating groups are introduced into proteins and used thereafter for complexation with ^{99m}Tc (preconjugation route). Additionally, the latter route was modified by complexing the activated ligand before conjugation (precomplexation route). The current state of research was recently reviewed (*1–4*).

Because of the favorable complex chemistry of multidentate chelators, the emphasis of our work was focused on indirect methods. The design of heterobifunctional ligands which have the chelating moiety on one side of the molecule and the activated ester on the other is hampered, however, by the intrinsic problem that nucleophilic heteroatoms of the ligand potentially interact with the electrophilic center at the location of the leaving group. In early experiments, this problem was bypassed by applying protection groups at the sulfhydryls and by using amide nitrogens on the chelate, as realized with the activated esters of the S protected N_2S_2-pentanoate (*5–8*). At ambient temperature and neutral pH, the complexation yields of the respective antibody conjugates proved to be insufficient. The binding of ^{99m}Tc(V) needed elevated temperatures or high

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For correspondence contact: Michael Eisenhut, PhD, Department of Nuclear Medicine, University of Heidelberg, Im Neuenheimer Feld 400, D-69120 Heidelberg, Germany.