Labeling of Monoclonal Antibodies with Rhenium-186 Using the MAG3 Chelate for Radioimmunotherapy of Cancer: A Technical Protocol

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A detailed technical protocol is provided for reproducible and aseptical production of stable ^{186}Re-monoclonal antibody conjugates. Labeled Mab E48 IgG and its F(ab')$_2$ fragment which are promising candidates for radioimmunotherapy of squamous cell carcinoma of the head and neck were used for evaluation. S-benzoylmercaptoacetyltriglycine (S-benzoyl-MAG3) was used as a precursor. Rhenium-186-MAG3 was prepared via a unique solid-phase synthesis, after which known strategies for esterification and conjugation to Mab IgG/F(ab')$_2$ were applied. The biodistribution of ^{186}Re-E48 F(ab')$_2$ in tumor-bearing nude mice was found to be comparable to that of analogously labeled ^{99m}Tc-E48 F(ab')$_2$ or ^{131}I-E48 F(ab')$_2$, indicating that the intrinsic behavior of the antibody remains preserved when using this labeling technique. Radiolytic decomposition of ^{186}Re-E48 IgG/F(ab')$_2$ solutions of 10 mCi · ml^{-1} was effectively reduced by the antioxidant ascorbic acid. Upon increase of the Re-MAG3 molar amount, a conjugation of seven to eight Re-MAG3 molecules per Mab molecule was generally the maximum ratio that could chemically be obtained. Such a ratio did not impair the immunoreactivity or alter the in vivo biodistribution characteristics of the immunoconjugate, making this labeling procedure suitable for general clinical application.

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Tumor targeting with radiolabeled monoclonal antibodies (Mabs) might be a valuable approach for diagnosis and therapy of squamous cell carcinoma of the head and neck (HNSCC) which represents the vast majority of all malignant tumors of the head and neck. HNSCC has a proclivity to metastasize to regional lymph nodes in the neck rather than to spread hematogeneously. The status of the cervical nodes has been recognized as the single most important prognostic factor in HNSCC.

Most early stage HNSCC (Stage I and II) can be safely cured with surgery and/or radiation therapy. Less effective, however, is the treatment of Stage III and IV disease: 50%–60% of patients with resectable tumors develop locoregional recurrence, while 15%–25% develop distant metastases. The role of chemotherapy in these patients is limited. Responses are often seen but enhancement of survival is minimal.

Radioimmunoconjugates may be beneficial in assessment of tumor involvement in the lymph nodes. It can be expected that the relative superficial localization of the neck nodes allows accurate radioimmunodetection with a gamma camera. In addition, radioimmunoconjugates may also be of value for early detection and therapy of recurrent disease and distant metastases. A factor in favor of radioimmunotherapy is the intrinsic radiosensitivity of HNSCC.

We recently tested a panel of Mabs for targeting HNSCC in preclinical and clinical studies. The most promising antibody, Mab E48, recognizes a 22 kDa surface antigen. This antigen is probably involved in cell-cell adhesion and is exclusively expressed by squamous and transitional epithelia and their malignant counterparts (*1,2*). The capacity of Mab E48 IgG as well as F(ab')$_2$ for highly specific delivery of ^{131}I to human head and neck tumors has been demonstrated in xenografted nude mice (*3,4*). Recently we demonstrated a dose-dependent growth delay, regression and complete remissions of established tumors by injecting single doses of ^{131}I-labeled Mab E48 IgG in nude mice bearing HNSCC xenografts (*5*).

As a first approach to test the capability of Mab E48 for tumor targeting in patients with head and neck cancer, we decided to evaluate Mab E48-F(ab')$_2$ for its accuracy in detection of lymph node metastases. To this end we used a ^{99m}Tc-MAG3-labeled conjugate of Mab E48 F(ab')$_2$ which appeared highly capable of detecting metastatic and recurrent disease (*6*). Preliminary data indicate that Mab E48 IgG is also well suited for tumor targeting in patients: labeled with ^{99m}Tc, the percentage injected dose per gram (%ID · g^{-1}) of tissue was found to be ranging from 0.014% to 0.080% at 44 hr after injection. These data justify the

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further development of Mab E48 for therapeutic approaches.

So far, most clinical experience with radioimmunotherapy (RIT) has been obtained with ^{131}I-labeled immunoconjugates. Iodine-131 labeling of Mabs is easy since the isotope has an appropriate half-life (8 days) and beta particle energy (0.6 MeV) for utility in RIT. However, disadvantages are the reported instability of ^{131}I-labeled immunoconjugates and the gamma emission which represents 65% of the released energy. It has been hypothesized that ^{186}Re may be a better candidate isotope for radioimmunotherapy than ^{131}I (*7*). With its half-life of 3.7 days, its 9% gamma emission (which has an ideal energy (137 keV) for imaging), its 71% beta emission of 1.07 MeV and a 21% beta emission of 0.94 MeV (*8*), theoretically ^{186}Re seems to be better suited for RIT than ^{131}I.

The physical properties of ^{99m}Tc and ^{186}Re seem to be ideal for radioimmunoscintigraphy (RIS) and for RIT, respectively, and the chemical properties of ^{99m}Tc and ^{186}Re are considered to be similar. Therefore, at our institute effort has been put on developing analogous ^{99m}Tc and ^{186}Re labeling chemistry for Mabs directed to HNSCC, with the clinical option to use ^{99m}Tc imaging to identify ^{186}Re therapy candidates. In this concept it is clear that the Mab biodistribution should be the same regardless of whether it is labeled with ^{99m}Tc or ^{186}Re. Consequently, because the specific activity of ^{186}Re is several orders of magnitude lower than that of ^{99m}Tc, the applicability of the labeling procedure should not be restricted by the low specific activity of ^{186}Re.

The approach, which obviously gives the best chemical control over the labeling process leading to highly stable conjugates without aggregate formation, has been described by Fritzberg et al. (*9,10*). During this multistep procedure, an active ester of tetrafluorophenol and a N_2S_2-pentanoate or N_3S-butyrate carrying the radioisotope is prepared, which is subsequently conjugated to amino groups of the antibody. Either the ester is formed after prelabeling of the chelate in the presence of $S_2O_4^{2-}$ at pH 12, or the preformed active ester of the chelate is labeled via transchelation at pH 3 (the "pre-ester" method) (*9–12*). The obtained conjugates were found to have retained their immunoreactivity and to be stable when challenged by other chelating compounds (*9*).

Trying to apply this method using S-benzoyl-MAG3, the precursor of ^{99m}Tc-MAG3 used for renal function measurement, we found that both methods were not suitable for coupling ^{186}Re to the antibody. Rhenium-186-MAG3 was not formed with $S_2O_4^{2-}$ at pH 12 during the prelabeling method, while the pre-ester method failed due to severe hydrolysis of the esterbond, even in the presence of isopropanol.

In the present study we report on a solid phase synthesis of ^{186}Re-MAG3. A detailed technical protocol is provided for reproducible and aseptical labeling leading to stable and sterile Mab conjugates (either IgG or their fragments) with ^{186}Re to a high Re:Mab molar ratio. The chemical stability in connection with the radiolytic decomposition occurring when using strong beta emitters, is described. From comparative studies in HNSCC-bearing nude mice, biodistribution characteristics of E48 IgG and its $F(ab')_2$ labeled with ^{186}Re, ^{99m}Tc, and ^{131}I are provided.

MATERIALS AND METHODS

Materials

The 2,3,5,6-Tetrafluorophenol (TFP) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) used in the study were purchased from Janssen Chimica (Beerse, Belgium). S-benzoyl-MAG3 as a pure white solid, i.e., free from the presence of organic acids and not in "kit" form, was a gift from Mallinckrodt Medical (Petten, The Netherlands). S-benzoyl-MAG3 was dissolved in MeCN:H_2O (9:1) at a concentration of 1 $mg \cdot ml^{-1}$ and was found to be very stable especially with respect to loss of the sulphur-protecting group. Upon HPLC analysis no detectable amounts of benzoic acid or other compounds were observed even after 3 mo. (Details on HPLC analysis: 15 cm C18 Novapak column (Waters, Millipore, MA); eluent 25% (vol/vol) EtOH/6.25 m*M* (n-Bu)$_4NH_2PO_4$; flow rate 1 $ml \cdot min^{-1}$, R_t S-benzoyl-MAG3 = 14.5 min; R_t benzoic acid = 5.3 min; detection at 210 and 254 nm).

Rhenium-186 was obtained as a [^{186}Re]ReO_4^- solution from Mallinckrodt Medical. Quantitative measurement of the ^{186}Re activities in solutions was performed in glass vessels with an internal diameter of 13.5 mm in a dose calibrator. It was found that the amount of radioactivity measured in the ^{99m}Tc 140-keV channel needed to be multiplied with a factor of 2.5 to obtain the ^{186}Re activity.

The tube heater was made from a Cu/Zn block fitted to the glass vessel. Heating of the block was accomplished by a 240 V/150 W Watlow band element. Thermo-control was carried out by a West temperature controller (type PYZ4 TCY1), a solid-state relais and a PT 100 (platinum resistance thermometer). The tube heater avoids the use of a water or oil bath in the sterile flow hood. The pH measurements were carried out with a small volume glass/Pt electrode (Schott CG837, Φ 3 mm, Tannson B.V., Zoetermeer, The Netherlands).

For purification of the ester and the conjugate respectively, Sep-pak C_{18} cartridges (Waters) and PD-10 Sephadex columns (Pharmacia Biotech, Woerden, The Netherlands) were used. For final sterilization of the radioimmunoconjugate, 0.22-μm Acrodisk filters (Gelman Sciences Inc., Ann Arbor, MI) were used.

All solutions were sterile and pyrogen-free and were made from "water for injection."

Analysis

HPLC analysis of the ^{186}Re-labeled and ^{99m}Tc-labeled MAG3 and their corresponding MAG3 TFP esters was performed on a 25-cm Lichrosorb 10 RP 18 column (Chrompack) with a gradient elution. Solution A consisted of a 5:95 mixture of EtOH and a 0.01-*M* sodium phosphate buffer plus a 0.015-*M* sodium azide (pH 6) solution. Solution B was a 9:1 mixture of MeOH and H_2O. Gradient (flow rate 1 $ml \cdot min^{-1}$): 10 min 100% A, 10 min 100% A → 100% B, 10 min 100% B. Radioactivity was detected continuously by an Ortec 406A single-channel analyzer connected to a Drew 3040 Data collector (Betron Scientific, Rotterdam, The Netherlands) and fractions of 1 ml were collected on a LKB 2212 Helirac (Pharmacia Biotech, Woerden, The Netherlands). Comparison of the injection standard with the total effluent from the HPLC column showed a quantitative recovery of the activity (>98%) from the HPLC column in all cases. The HPLC retention

TABLE 1

Protocol for the Preparation of ^{186}Re-MAG3-Conjugated Mab IgG/F(ab')$_2$ Schematically Presented in Figure 1, with Reference to the Experimental Set-Up as Schematically Provided by Figure 2*

Step	Procedure
1.	Mix 150 μl 1 M Na_2CO_3 (pH = 11.7) with the appropriate amount of [^{186}Re]ReO_4^- solution (up to 8 ml).
2.†	Add 150 μl (120 μmole) of a freshly prepared Na_2SO_3 solution (100 $mg \cdot ml^{-1}$).
3.‡	Add 25 μl (68 nmole) S-benzoyl-MAG3 (1 mg in 1 ml $MeCN/H_2O$ (9:1)).
4.‡	Add 100 μl (442 nmole) freshly prepared $SnCl_2/2H_2O$ solution (1 $mg \cdot ml^{-1}$).
5.§	Heat mixture in tube heater (A) at 100°C. Evaporate solvent under a stream of N_2 until dry. Continue heating for another 15 min.
6.	Put mixture on ice for 3 min.
7.	Add 500 μl of water for injection, then vortex.
8.¶,**	Bring pH between 5.7–6.3 with 490 μl of 1 N H_2SO_4.
9.¶,††	Add 200 μl of 2,3,5,6-TFP, 100 mg in 1 ml $MeCN/H_2O$ (9:1) and 50 mg EDC as a solid. Vortex, check pH and when necessary readjust pH with 1 N H_2SO_4 to pH = 5.7–6.3. Incubate at room temperature for 30 min.
10.‡‡	Adjust the reaction mixture with water to a volume of about 8 ml.
11.	Suck the reaction mixture through two conditioned Sep-pak C18 cartridges in series via B,C,C' routing.
12.	Suck 20 ml of water for injection through the columns via B,C,C' routing for washing.
13.	Suck 30 ml 20% (vol/vol) EtOH/0.01 M sodium phosphate, pH 7.0, through the columns via B,C,C' routing for washing.
14.	Suck 10 ml of water through the columns via B,C,C' routing for removal of EtOH/phosphate.
15.	Suck 0.5 ml of ethyl ether through the columns via B,C,C' routing for removal of most of the remaining water.
16.¶,§§	Turn valve and elute active ester into the tube with 2.5 ml of MeCN via B,D,E routing.
17.	Evaporate solvent in the tube heater (A) at 30°C under a stream of N_2 via E,F routing.
18.¶¶***	Solve active ester in 0.5 ml of 0.9% NaCl and add antibody. Adjust pH with 0.05 M Na_2CO_3 to get pH 9.5; incubate at room temperature for 30 min.
19.†††	Purify antibody-conjugate by gel filtration on a PD-10 column equilibrated with 0.9% NaCl, and elute with 0.9% NaCl.
20.	Sterilize the antibody conjugate through a low protein binding 0.22-μm filter.

*Typical example starting from 27.9 nmole [^{186}Re]ReO_4^- solution (1.03 mCi; spec. act. 36.9 $\mu Ci \cdot nmole^{-1}$ Re).

†Sulphite is added to neutralize the effect of "aging" of the [^{186}Re]ReO_4^- solution.

‡When using higher nmole amounts of ^{186}Re, for optimal incorporation of ^{186}Re into the complex, the Re:MAG3 molar ratio should be taken at 1:2.3, the Re:Sn^{2+} molar ratio at 1:8.

§The tube heater avoids the use of a water or oil bath in the sterile flow hood.

¶For analysis of the different chemical reaction steps, HPLC analysis can be performed at the end of these steps.

**With 800 nmole of Re and consequently 6400 nmole of Sn^{2+}, at this point a certain amount of tin colloid formation occurs; however, this does not affect the esterification process, while the colloids are removed or lost during the Sep-pak procedure.

††The amounts used here are in high excess to speed up the esterification process and are present in such amounts that these do not need to be adjusted when using higher nmole amounts of Re.

‡‡This step dilutes the original CH_3CN/H_2O (1:5) mixture, necessary to avoid elution of a part of the ester into the waste flask.

§§In general, at this time 80% ± 5% of the activity is remaining in the form of pure ester.

¶¶Chemical half-life of the esterbond in 0.9% NaCl is 7 hr.

***The conjugation yield is 50% ± 5% when using 2 mg of Mab IgG/F(ab')$_2$ in a reaction volume of 2.5 ml; the conjugation yields are higher when using higher amounts of protein.

†††The first sample consists of the 2.5-ml reaction volume; the labeled protein is collected in the next 3.5-ml fraction; with high ^{186}Re activity, this collection vessel already contains 175 μl of 100 $mg \cdot ml^{-1}$ ascorbic acid solution (final concentration 5 $mg \cdot ml^{-1}$) for immediate protection against radiolytic decomposition. The next 2-ml fraction is discarded, whereafter the following 8-ml fraction contains the hydrolyzed ^{186}Re-MAG3 (and some $^{186}ReO_4^-$ formed by radiolytic decomposition during the conjugation process). This ^{186}Re-MAG3 can be reused immediately by adding 200 μl of TFP-solution and 50 mg of EDC in solid form; after 30 min of incubation, repeat steps 11–20, any $^{186}ReO_4^-$ present is removed during the Sep-pak procedure.

times were 3.0 min (most probably a 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).

Thin-layer chromatography (TLC) of the labeled Mabs was performed on silica gel-impregnated glass fiber sheets (Gelman Sciences Inc.), with a thickness of 0.3 mm, eluent 0.1 M citric acid pH = 7. After development (5 min), the chromatograms were cut into 1-cm segments and counted in a LKB Wallac Compugamma (Rf Mabs = 0; Rf [^{186}Re]ReO_4^- = 1).

Labeling Protocol

A detailed protocol for labeling of Mabs with ^{186}Re by using the MAG3 chelate is provided in Table 1. For the synthesis of ^{99m}Tc-MAG3, simply heating at 100°C for 10 min is sufficient (step 5, Table 1) while the presence of sulphite (step 2, Table 1) and step 7 (Table 1) are not necessary. Consequently, only 250 μl of 1 N H_2SO_4 is needed to bring the pH between 5.7–6.3 (step 8, Table 1). As such, steps 1–20 (Table 1) are the standard protocol for the ^{99m}Tc labeling of Mab E48 (*1*), 323A3 (*13*), K928 (*14*) and chimeric SF-25 (cSF-25) (*15*) IgG and F(ab')$_2$, which are currently being used in ongoing patient studies at our institute, part of which has been published recently (*6*). In general, 150–250 mCi of ^{99m}Tc are used for labeling, but higher amounts (tested up to 900 mCi) were labeled equally effective. Also, the volume in which the $^{99m}TcO_4^-$ is added allows a great deal of flexibility. Reaction volumes up to 8 ml did not appear to be a problem. Using 2 mg of Mab, ^{99m}Tc-Mab is thus obtained with a total yield of 40% ± 5% (decay corrected), and with a radiochemical purity >97%.

In Vitro Stability Measurements

For measurement of the in vitro stability in relation to the radiolytic decomposition ^{186}Re- and ^{99m}Tc-labeled aliquots of

MAG3-TFP and MAG3-Mab, were taken from a stock solution. For a meaningful comparison, all samples possessed the same geometry: 0.5-ml solutions in glass vessels with an internal diameter of 10 mm. As a result, the percentage of the energy escaping from the solution or deposited in the glass is the same for each individual isotope. Additives with the potential to retard the radiolytic decomposition gentisic acid (2,5-dihydroxybenzoic acid) and ascorbic acid (3-oxo-L-gulofuranolactone) were investigated. From these two additives, 25 $mg \cdot ml^{-1}$ stock solutions were made; to 100-μl samples (for the blank, this was 100 μl of water), an aliquot of radioactive solution was added, whereafter saline was used as the diluent to 0.5 ml (final concentration antioxidant 5 $mg \cdot ml^{-1}$).

Solutions of 5 $mg \cdot ml^{-1}$ ascorbic acid and gentisic acid are acidic solutions (pH 2.3). To assess the stability in 5 $mg \cdot ml^{-1}$ ascorbic acid or gentisic acid solutions at a higher pH, the solutions were brought to that pH with 1 *M* of Na_2CO_3 before adding the labeled protein or ester.

TLC analysis was performed after 0.5, 1, 2, 3 and 4 hr and subsequently every day until Day 21. HPLC analysis was performed once a day.

Monoclonal Antibodies

Production and selection of Mab E48 has been described previously. Mab E48 detects a 22 kDa surface antigen, which in normal tissue is present only in stratified squamous and transitional epithelium. So far, tested Mab E48 has reacted with 90% of the primary head and neck tumors (n = 110) and with the majority of cells within these tumors. A comparable reactivity pattern was observed in 26 tumor infiltrated lymph nodes from neck dissection specimens (*16*). Mab JSB-1 (*17*) which was used as an isotype-matched control antibody in biodistribution studies along with 323A3 (*13*), K928 (*14*) and cSF-25 (*15*) which were used to evaluate the general applicability of the ^{186}Re-labeling procedure, have been described in detail elsewhere. Purification of the antibodies and preparation of $F(ab')_2$ fragments by pepsin digestion of IgG has been described previously (*4*). Purity of whole IgG and $F(ab')_2$ preparations was evaluated by SDS polyacrylamide gel electrophoresis under nonreducing conditions and appeared to be >95%. The affinity constants were 1.5×10^{10} M^{-1} for E48 IgG and 1.2×10^{10} M^{-1} for E48 $F(ab')_2$ fragment as determined by Scatchard analysis.

Radioiodination

Radioiodination of $F(ab')_2$ fragments was performed as described by Haisma et al. (*18*); 500 μg of $F(ab')_2$ fragment was mixed with 1 mCi of ^{131}I or ^{125}I. After removal of unbound ^{131}I or ^{125}I (5%–10%), the radiochemical purity was 96% for Mab E48 $F(ab')_2$ and 97% for JSB-1 $F(ab')_2$.

Immunoreactivity

In vitro binding characteristics of Mab E48 $F(ab')_2$ fragment or IgG labeled with ^{131}I, ^{99m}Tc and ^{186}Re were determined in an immunoreactivity assay as described by Lindmo et al. (*19*). In short, UM-SCC-22B cells were fixed in 0.1% paraformaldehyde and six serial dilutions (ranging from 5×10^6 cells per tube to 3.1×10^5 cells per tube) were made with 1% bovine serum albumin (BSA) in PBS. Mab IgG or $F(ab')_2$ fragments labeled with 10,000 cpm of ^{131}I and ^{186}Re or 80,000 cpm of ^{99m}Tc, were added to the tubes. The samples were incubated overnight at room temperature. Excess unlabeled Mab IgG or $F(ab')_2$ fragments were added to the last sample to determine nonspecific binding. Cells were spun down and radioactivity in the pellet and supernatant was determined in a gamma counter and the percentage bound and free radioactivity was calculated (LKB-Wallac 1282 CompuGamma, Kabi Pharmacia, Woerden, The Netherlands). Data were graphically analyzed in a modified Lineweaver Burk plot and the immunoreactive fraction was determined by linear extrapolation to conditions representing infinite antigen excess. The UM-SCC-22B squamous cell carcinoma cell line was derived from a lymph node metastasis of a hypopharyngeal tumor and was provided by Dr. T. Carey, University of Michigan, Ann Arbor, MI (*20*).

Xenograft Line

Female nude mice (Hsd: Athymic nu/nu, 25–32 g, Harlan/CPB, Zeist, The Netherlands) were 8–10 wk old at the time of the experiments. The HNSCC xenograft line HNX-HN was established by subcutaneous implantation of tumor fragments (3 × 3 × 1 mm) in the lateral thoracic region on both sides of the nude mice. The HNSCC xenograft line had been established from a T4N2 squamous cell carcinoma of the base of the tongue from a 54-yr-old female patient. The expression of the E48 antigen in this xenograft line was demonstrated previously by immunohistochemistry using the biotin-avidin peroxidase technique (*4*).

Radioimmunoscintigraphy

Mice were killed by cervical dislocation and scanned with an Ohio gamma camera (Sigma 410S, General Electric, Utrecht, The Netherlands). For further analysis and production of images, 100,000 cpm were obtained and data were stored in a computer (PDP 1134 computer system, Digital Equipment Corp., Utrecht, The Netherlands).

Biodistribution

In vivo tissue distribution of radiolabeled Mab E48 IgG/$F(ab')_2$ was studied in nude mice bearing the HNSCC xenograft line HNX-HN (150–300 mm^3) or in tumor-free nude mice as described earlier (*4*). In short, mice were bled, killed and dissected 24 or 48 hr after intravenous injection of the radioimmunoconjugate. Organs were removed, placed in 5-ml plastic tubes and weighed. Samples were taken from blood, urine, tumor, liver, spleen, kidney, heart, stomach, ileum, colon, bladder, sternum, muscle, lung, skin and tongue. After weighing, radioactivity in organs and tumors was counted in a LKB-gamma counter. The antibody uptake in the tumor and other tissues was calculated as the percentage of the injected dose per gram of tissue ($\%ID \cdot g^{-1}$).

Three sets of biodistribution experiments were designed to investigate possible artifacts introduced by the described labeling procedures. In the first experiment, the biodistribution of ^{186}Re-E48 $F(ab')_2$ was compared with the biodistribution of equimolar amounts of ^{99m}Tc-E48 $F(ab')_2$ to determine the influence of the conjugation of the Re-MAG3 to the Mab on the biodistribution characteristics in tumor-bearing nude mice. The biodistribution of ^{131}I-E48 $F(ab')_2$ and the ^{125}I-labeled control Mab JSB-1 $F(ab')_2$ was described earlier and was taken in this study as a mutual control for the in vivo behavior of the ^{186}Re-labeled and ^{99m}Tc-labeled E48 $F(ab')_2$. In the second experiment, the influence of the number of Re-MAG3 molecules bound per Mab molecule on the biodistribution characteristics of E48 IgG labeled with different numbers of Re-MAG3 molecules was determined. For a comparison, the biodistribution of equimolar amounts of analogously prepared ^{99m}Tc-E48 IgG was determined. To avoid a differential influence of tumors on the biodistribution characteristics of these radioimmunoconjugates in normal tissues, this experiment was performed using tumor-free nude mice. Finally, in the third ex-

periment, the tumor targeting properties of ^{186}Re-E48 IgG with a high Re-MAG3:Mab molar ratio was determined.

In all experiments, the ^{99m}Tc-labeling was performed with the same amount of chemical ingredients as with the corresponding ^{186}Re labeling; both according to the protocols described above. For each experiment, the exact chemical conditions and ^{186}Re: Mab molar ratios are given in the legend of the corresponding figure.

RESULTS

Chemistry

In the solid-phase synthesis as outlined in Table 1 and Figures 1 and 2, [^{186}Re]ReO_4^- was reduced by $SnCl_2$ at a pH of 11.7 in the presence of Na_2SO_3 and S-benzoyl-MAG3 to give more than 90% of the ^{186}Re-MAG3. The ^{186}Re-MAG3 yield appeared highly dependent on the heating procedure applied. When the reaction mixture was heated for 10 min at 100°C, conditions suitable for high-yield ^{99m}Tc-MAG3 synthesis, the yield of ^{186}Re-MAG3 was found to be <1%. This yield did not increase upon prolonged heating. When the solvent of the reaction mixture was evaporated at 100°C under a stream of N_2 until dry and heating was continued, ^{186}Re-MAG3 formation occurred. In this solid-phase synthesis a yield of 90% or more ^{186}Re-MAG3 was obtained within 15 min. Once formed, ^{186}Re-MAG3 is a stable compound in aqueous solution. Under the reaction conditions described in the protocol (Table 1), the esterification is nearly quantitative. As a result, 80% ± 5% pure ester was obtained after the Sep-pak purification procedure, the experimental set-up of which is outlined in Figure 2. In a reaction volume of 2.5 ml, the conjugation yield was 50% ± 5% when using 2 mg Mab IgG or F(ab')$_2$, but was found to be higher when using increasing amounts of protein in the same reaction volume (e.g., 75% with 8–10 mg of IgG).

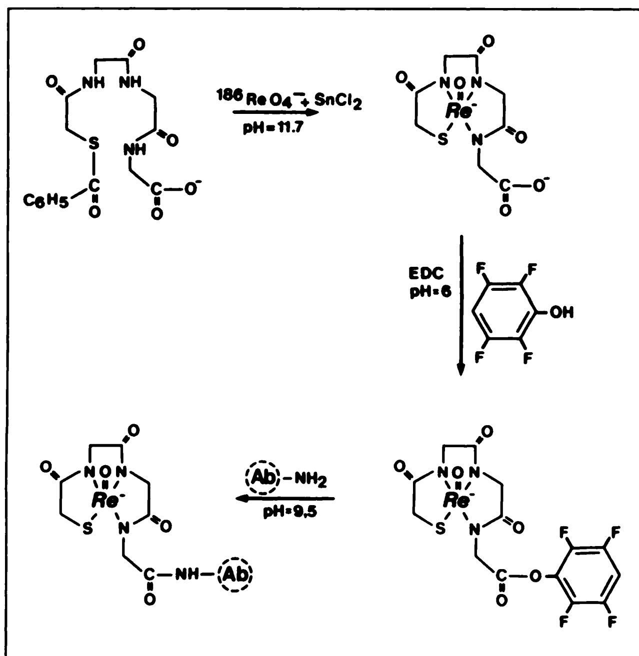


FIGURE 1. Schematic presentation of the solid phase synthesis of ^{186}Re-MAG3, its esterification and conjugation to Mabs. For description of the synthesis routings see Table 1 and Figure 2.

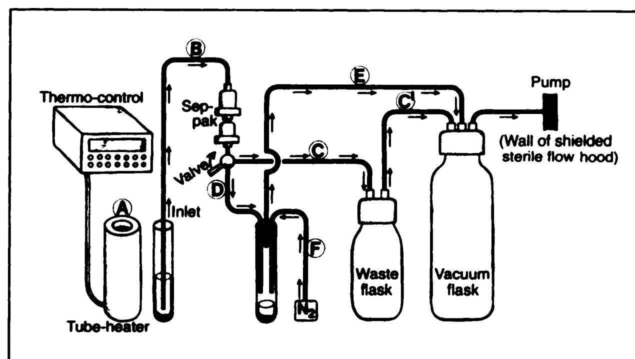


FIGURE 2. Schematic presentation of the experimental set-up for aseptical labeling of Mabs with ^{99m}Tc or ^{186}Re. For description of the synthesis routings see Table 1.

Depending on the amount of protein used, the overall yield was 40%–60%. The major proportion of isotope loss is caused by the hydrolysis of ^{186}Re-MAG3-TFP at a pH of 9.5 during the antibody conjugation step. However, ^{186}Re-MAG3 formed in this way can be recovered from the PD-10 column and be reused immediately by adding 200 μl of TFP solution and 50 mg of EDC in solid form. After a 30-min incubation, steps 11–20 of the protocol can then be repeated for another batch of ^{186}Re-labeled antibody.

The concentrations given in the protocol are the standard concentrations used in the ^{99m}Tc labeling of Mabs, and can be applied for Re up to 30 nmole. When using higher nanomole amounts of Re, optimal ^{186}Re-MAG3 formation was obtained when taking the molar ratios S-benzoyl-MAG3:Re as 2.3:1 and Sn^{2+}:Re as 8.0:1. For optimal esterification, no adjustments to the standard protocol, provided by Table 1, are necessary due to the high excess of EDC and TFP. As an example, for a ^{186}Re-MAG3-TFP preparation with 800 nmole of [^{186}Re]ReO_4^-, 1840 nmole of S-benzoyl-MAG3 and 6400 nmole of Sn^{2+}, the yield was routinely 80%. The addition of 8–10 mg of Mab E48, 323A3, K928 or cSF-25 to this ^{186}Re-MAG3-TFP preparation gave 7–8 Re-MAG3 molecules per Mab molecule without in vitro denaturation of precipitation of the protein. As expected (*9*), the addition of cysteine or desferal as "challenging" agent, did not affect the label.

In Vitro Analysis

Considering that ^{186}Re is a strong beta-emitter, the ^{186}Re-MAG3-Mab conjugate appeared to be fairly stable at a low radioactivity concentration; e.g., for a ^{186}Re-MAG3-E48 IgG preparation, 0.12 mCi·ml^{-1} (0.6 mg Mab·ml^{-1}, Re:IgG molar ratio 0.5:1), after 2, 24 and 120 hr at room temperature, the percentage of protein-bound ^{186}Re was 95.3%, 91.0% and 82.8%, respectively (TLC analysis). The effect of radiolytic decomposition was more prominent at higher radioactivity concentrations as demonstrated in Table 2 for a ^{186}Re-MAG3-E48 IgG preparation, 2.5 mCi·ml^{-1} (1.16 mg Mab·ml^{-1}, Re:Mab molar ratio 3.5:1). At 24 and 120 hr, the percentage of protein-bound ^{186}Re

TABLE 2
Stability of ^{186}Re-MAG3-E48 IgG in 0.9% NaCl With or Without Ascorbic Acid or Gentisic Acid

	% protein bound ^{186}Re						
	4 hr	24 hr	65 hr	96 hr	120 hr	144 hr	21 days
0.9% NaCl	90.2	65.3	29.0	17.0	10.7	7.5	—
+ gentisic acid (pH = 2.3)	95.6	94.1	93.5	92.6	92.4	92.4	90.5
+ ascorbic acid (pH = 2.3)	95.2	90.7	83.8	80.0	78.0	75.0	65.2
+ ascorbic acid (pH = 3.5)	95.7	94.7	93.8	92.7	92.5	92.0	86.6

The ^{186}Re-labeled protein was added to ascorbic acid and gentisic acid 45 min after end of synthesis. At that time, 96% of the ^{186}Re activity was protein-bound. The pH of the solution in the presence of Mab E48 has been indicated. Percentage protein-bound ^{186}Re was assessed by TLC analysis. Final radioactivity concentration: 2.5 $mCi \cdot ml^{-1}$ (1.16 mg $Mab \cdot ml^{-1}$, Re:Mab = 3.5:1, specific activity ^{186}Re 92.3 $\mu Ci \cdot nmole^{-1}$ Re). Final antioxidant concentration: 5 $mg \cdot ml^{-1}$

was 65.3% and 10.7%, respectively. The effects of the presence of the antioxidants gentisic acid or ascorbic acid (final concentration 5 $mg \cdot ml^{-1}$) under the same geometric conditions are also shown in Table 2. The antioxidative capacity of gentisic acid appeared to be strongly pH dependent. Although gentisic acid was found to be protective at a pH of 2.3, it became destructive at physiological pH and within 24 hr, all ^{186}Re was detached from the protein. In contrast, ascorbic acid still possessed protective capacity at physiological pH (half-life of the ^{186}Re-Mab bond was 4.5 days). The optimal antioxidative capacity of ascorbic acid was found to be at a pH of 3.5. Detachment of ^{186}Re from the protein was a consequence of reoxidation of the chelated ^{186}Re. HPLC analysis of the ^{186}Re-MAG3-TFP ester under aforementioned conditions revealed $^{186}ReO_4^-$ formation. For the ^{186}Re-MAG3-TFP ester stored in saline after the Sep-pak procedure (i.e., free from SO_3^{2-} and Sn^{2+}), a 75% $^{186}ReO_4^-$ formation had taken place within 92 hr. In the presence of gentisic acid or ascorbic acid, this percentage was 11.0% and 11.9%, respectively, while again gentisic acid at physiological pH appeared to be destructive. It is of note that for the ^{99m}Tc-MAG3-TFP ester in a radioactivity concentration of 40 $mCi \cdot ml^{-1}$, exactly the same phenomenon was observed. In saline, 19% $^{99m}TcO_4^-$ formation had taken place after 45 hr in the presence of gentisic acid or ascorbic acid (5% in both cases). Also for ^{99m}Tc, gentisic acid was found to be destructive at physiological pH.

Dose calculations show that for relatively short time periods (1 day), addition of ascorbic acid to a final concentration of 5 $mg \cdot ml^{-1}$ is sufficient for protection against radiolytic decomposition. For example, a solution consisting of 9.7 $mCi \cdot ml^{-1}$ (2 mg $Mab \cdot ml^{-1}$, Re:Mab molar ratio of 7.3:1), which had lost 6% of the label within 20 min (from 98% to 92%), only lost 0.9% of the label during the next 4 hr after addition of ascorbic acid, while after 24 hr, 89% of the label was still protein bound. After 3 days this percentage was 75%. A corresponding sample without ascorbic acid showed almost complete loss of protein label at that time. On the basis of these results, collection of the ^{186}Re-labeled Mab preparation in ascorbic acid was made part of the technical protocol.

The immunoreactive fractions of ^{186}Re-labeled, ^{99m}Tc-labeled or ^{131}I-labeled E48 IgG and $F(ab')_2$ fragments at infinite antigen excess were >95% in all experiments. This also holds true for ^{186}Re-immunoconjugates with the highest Re:Mab molar ratios prepared in these experiments, corresponding with seven to eight Re-MAG3 molecules per Mab molecule, as assessed for Mab E48, 323A3, K928 and cSF-25. These data indicate that upon coupling of ^{186}Re-MAG3, Mabs fully retain their binding potential.

Biodistribution Studies

Maintenance of biodistribution characteristics after coupling of ^{186}Re to Mab E48 was investigated in biodistribution studies with ^{186}Re-labeled Mab E48 $F(ab')_2$ in tumor-bearing nude mice. To this end the biodistribution of analogously prepared ^{186}Re-labeled and ^{99m}Tc-labeled E48 $F(ab')_2$, and of ^{131}I-labeled E48 $F(ab')_2$ prepared via the iodogen method, was compared in nude mice bearing the HNSCC xenograft line HNX-HN. Mice received either 10 μCi of ^{186}Re-E48 $F(ab')_2$ (Fig. 3A), 70 μCi of ^{99m}Tc-E48 $F(ab')_2$ (Fig. 3B), 10 μCi of ^{131}I-E48 $F(ab')_2$ (Fig. 3C) or 10 μCi of ^{125}I-JSB-1 $F(ab')_2$ control antibody (Fig. 3D). The biodistribution data at 24 hr after injection reveal a similar high and selective accumulation of Mab E48 $F(ab')_2$ in the tumor but not in any other organ, irrespective whether labeled with ^{186}Re, ^{99m}Tc or ^{131}I. The $\%ID \cdot g^{-1}$ in the tumor was 9.27 ± 0.58, 9.37 ± 0.60 and 7.22 ± 1.07 for E48 $F(ab')_2$ labeled with ^{186}Re, ^{99m}Tc and ^{131}I, respectively. Uptake in other organs did not exceed 1%. Control ^{125}I-labeled $F(ab')_2$ did not show any specific accumulation in the tumor (Fig. 3D). Mice injected with ^{186}Re-E48 $F(ab')_2$ were scanned with a gamma camera 24 hr following intravenous injection. Figure 4 shows a representative immunoscintigraphic image with isotopes localizing in the two xenografts only.

The biodistribution characteristics of ^{186}Re-E48 IgG in nude mice were studied in relation to the number of Re-MAG3 molecules attached to the antibody. Radioimmunoconjugates with either a low Re-MAG3:Mab molar ratio of

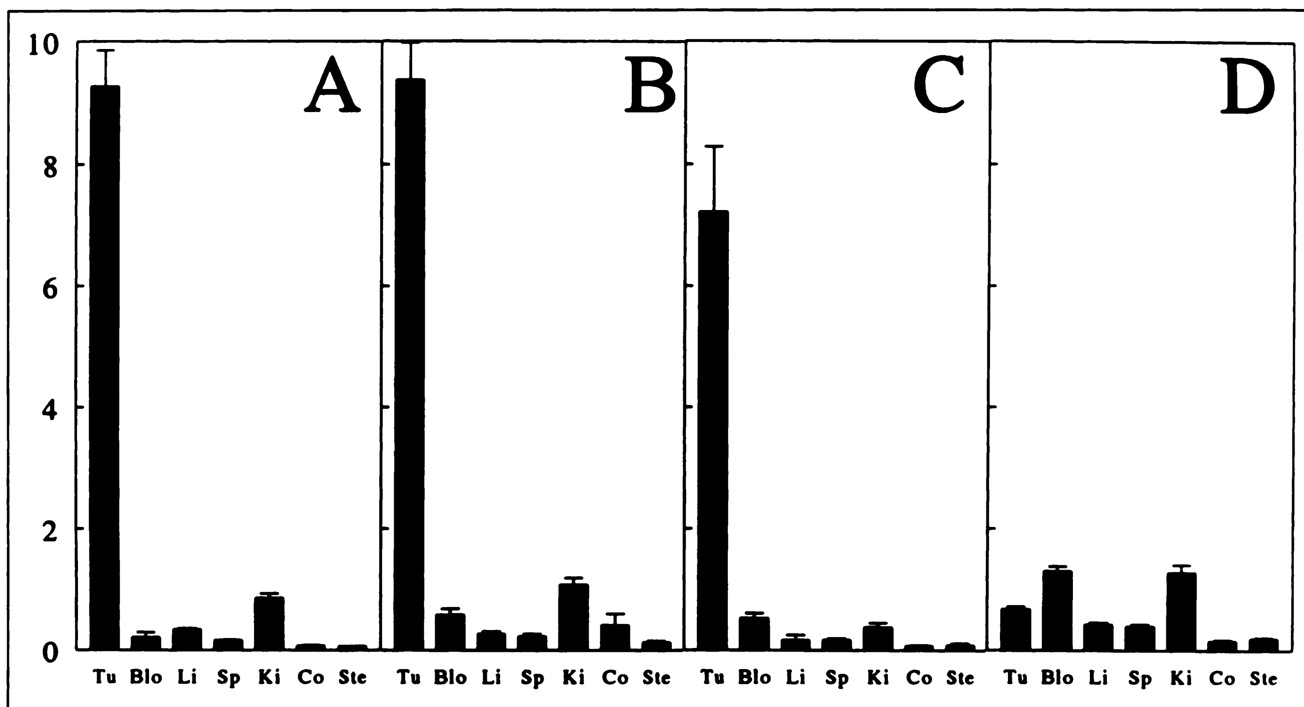
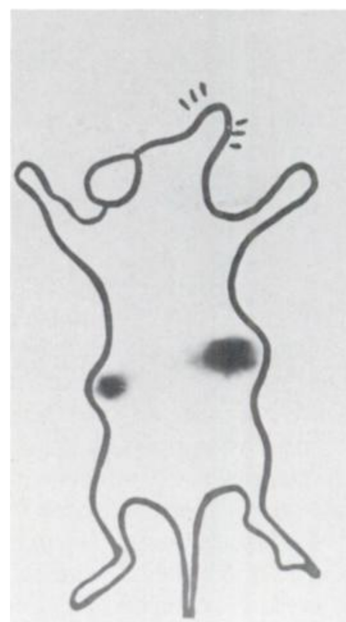


FIGURE 3. Biodistribution of radiolabeled E48 $F(ab')_2$ in HNSCC-bearing nude mice. (A) 10 μCi of ^{186}Re-, (B) 70 μCi of ^{99m}Tc-, (C) 10 μCi of ^{131}I-E48 $F(ab')_2$, (D) 10 μCi of ^{125}I-JSB-1 $F(ab')_2$ control antibody. At 24 hr following intravenous injection, mice (n = 4) were bled, killed, dissected and the $\%ID \cdot g^{-1}$ was calculated. Tu = tumor; Blo = blood; Li = liver; Sp = spleen; Ki = kidney; Co = colon; and Ste = sternum. Uptake in organs not shown did not exceed 1%. Starting chemical conditions: 27.9 nmole $[^{186}Re]ReO_4^-$ (spec. act. 36.9 $\mu Ci \cdot nmole^{-1}$ Re); 68 nmole S-benzoyl-MAG3; 442 nmole Sn^{2+}; 2 mg E48 $F(ab')_2$; final labeling result 203 $\mu Ci \cdot mg^{-1}$ Mab (molar ratio Re:Mab = 0.55:1). For ^{99m}Tc the same S-benzoyl-MAG3, Sn^{2+} and E48 $F(ab')_2$ amounts were used.

0.95:1 or a high molar ratio of 7.17:1 were injected intravenously at a dose of 27 and 230 μCi, respectively, into tumor-free nude mice. Mice receiving the ^{186}Re-conjugates were killed 24 hr after injection. Tumor-free nude mice were used to avoid variability in biodistribution introduced by the tumor. The amount of ^{186}Re activity delivered by the conjugates with low and high Re-MAG3:Mab molar ratio to the various organs, expressed as the average percentage of radioactivity of the $\%ID \cdot g^{-1}$, is shown in Figure 5A. No obvious differences were found between distribution of the conjugates with low and high Re-MAG3:Mab molar ratios and no selective accumulation of ^{186}Re in any particular organ was observed. The biodistribution of ^{186}Re-conjugates appeared to be similar to the biodistribution of ^{99m}Tc-conjugates prepared starting with the same S-benzoyl-MAG3, Sn^{2+} and Mab E48 IgG amounts and tested in a separate set of experiments (Fig. 5B).

The tumor-targeting properties of ^{186}Re-E48 IgG with a high Re-MAG3:Mab molar ratio were determined by injecting tumor-bearing nude mice with 200 μCi of ^{186}Re-E48 IgG. At 48 hr after injection, mice were killed and biodistribution of the radioimmunoconjugate was determined (Fig. 6). At this timepoint, 10.4 $\%ID \cdot g^{-1}$ was localized in tumor, while only 2.8 $\%ID \cdot g^{-1}$ localized in the blood. In all other tissues, the $\%ID \cdot g^{-1}$ did not exceed 1.2%.



186Re-E48 F(ab)2 ,HNX-HN

FIGURE 4. Whole-body scintigraphic image of a nude mouse bearing two subcutaneous HNX-HN xenografts in the flanks. Images were taken 24 hr following intravenous injection of 20 μCi of ^{186}Re-E48 $F(ab')_2$. Weight of the xenografts: left: 269 mg; right: 581 mg.

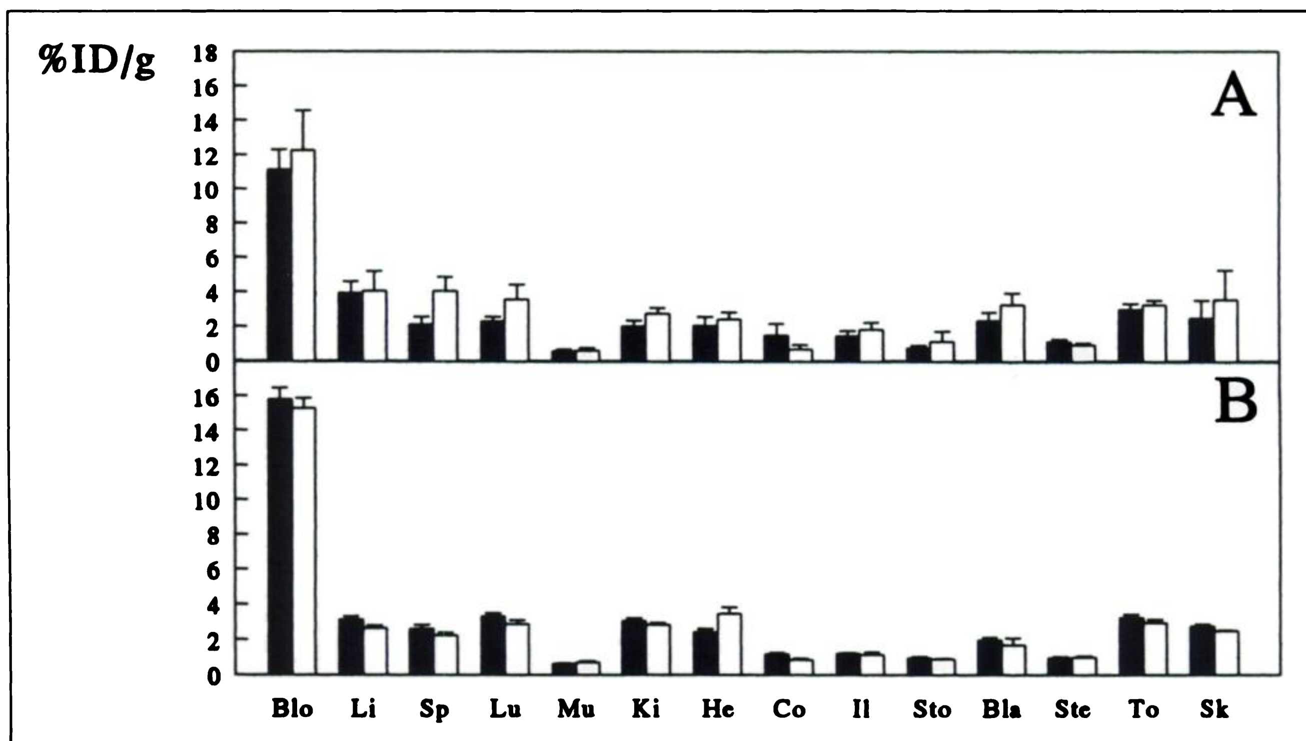


FIGURE 5. Biodistribution of ^{186}Re-E48 IgG labeled with different numbers of ^{186}Re-MAG3 molecules per Mab molecule and their corresponding ^{99m}Tc-conjugates in tumor-free nude mice. (A) 230 μCi of ^{186}Re-E48 at a Re:Mab molar ratio of 7.17:1 (black bars), compared to 27 μCi of ^{186}Re-E48 at a molar ratio of 0.95:1 (open bars), (B) 452 μCi of ^{99m}Tc-E48 (black bars) compared to 53 μCi of ^{99m}Tc-E48 (open bars) starting from the same amounts of S-benzoyl-MAG3, Sn^{2+} and E48 IgG. At 24 hr following intravenous injection, mice (n = 4) were bled, killed, dissected and the %ID · g^{-1} was calculated. Blo = blood; Li = liver; Sp = spleen; Lu = lung; Mu = muscle; Ki = kidney; He = heart; Co = colon; Il = ileum; Sto = stomach; Bla = bladder; Ste = sternum; To = tongue; and Sk = skin. Starting chemical conditions: 347 nmole [^{186}Re]ReO_4^- (spec. act. 54.3 μCi · $nmole^{-1}$ Re); 806 nmole S-benzoyl-MAG3; 2.54 μmole Sn^{2+}. At step 18, after dissolution in 0.9% NaCl, the activity was divided in two samples in a ratio of 8:1 and each sample was conjugated to 2 mg E48 IgG (steps 19, 20). Final labeling result Re-"high": 2.57 mCi · mg^{-1} Mab (Re:Mab = 7.17:1); Re-"low": 336 μCi · mg^{-1} Mab (Re:Mab = 0.95:1).

In all ^{186}Re-Mab biodistribution experiments described, TCA precipitation of serum samples revealed that 24 hr after injection, more than 95% of the ^{186}Re was still protein bound. This percentage did not change during subsequent storage for 10 days at 4°C.

DISCUSSION

Recent data from an ongoing Phase I/II trial with intravenously administered ^{99m}Tc-labeled Mab E48 $F(ab')_2$ and IgG in patients with HNSCC indicate that both are highly capable of detecting metastatic and recurrent disease (*6*). Moreover, Mab E48 was shown to be capable of eradicating HNSCC xenografts grown in nude mice when labeled with ^{131}I (*5*). As outlined in the introduction, the efficacy of radioimmunotherapy of head and neck cancer may improve when ^{186}Re-Mab conjugates become available.

In this paper, a reproducible labeling procedure leading to stable and sterile radioimmunoconjugates of Mab E48 with ^{186}Re using S-benzoyl-MAG3, the precursor of ^{99m}Tc-MAG3 used for renal function measurement, is provided in detail. High Re:Mab molar ratios were obtained without impairment of the immunoreactivity or biodistribution properties of the conjugate. For Mab 323A3, K928 and cSF-25 labeled to the same high Re:Mab molar ratio, a similar in vitro stability and retention of immunoreactivity was observed, indicating the general applicability of this labeling procedure (data not shown).

The labeling procedure described in this paper stems from a method developed by Fritzberg et al. (*9,10*) in which an active ester of TFP and N_2S_2-pentanoate carrying the radioisotope is prepared and then conjugated to amino groups of the antibody. When using the MAG3 chelator, the reported (*9,10*) reaction conditions to obtain ^{99m}Tc-MAG3 and its corresponding ester appear to give low and irreproducible yields. Optimal and reproducible yields were obtained when Sn^{2+} was used as reducing agent instead of $S_2O_4^{2-}$ while the preparation of ^{99m}Tc-MAG3-TFP was performed at pH 5.7–6.3 in the absence of phosphate buffer, and at room temperature instead of at 75°C. The presence of phosphate ions was found to retard the rate of esterification.

Technetium-99m-MAG3 conjugates of Mab E48, 323A3, K928, cSF-25 $F(ab')_2$ and IgG prepared in this way have been administered (750 MBq, 1 mg) to 40 head and neck cancer patients. The labeling procedure appeared to be highly reproducible and resulted in radioimmunoconjugates of constant quality. Of the conjugates prepared for these studies, TLC analysis of serum samples taken up to

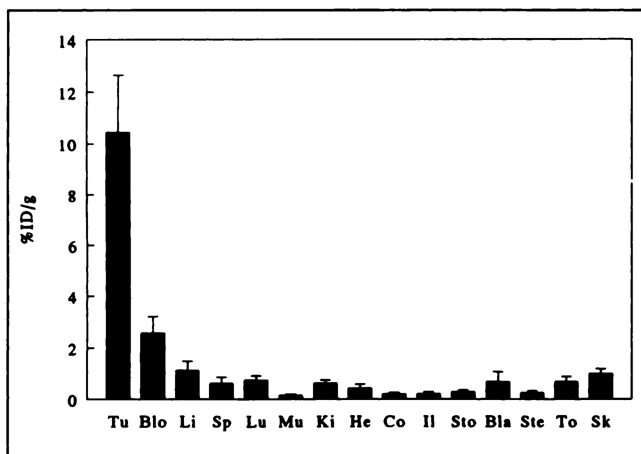


FIGURE 6. Biodistribution of 200 μCi of ^{186}Re-E48 IgG labeled at a Re:Mab molar ratio of 7.35:1 in nude mice bearing HNSCC xenografts. At 48 hr following intravenous injection, mice (n = 4) were bled, killed, dissected and the %ID $\cdot$ g^{-1} was calculated. Tu = tumor; Blo = blood; Li = liver; Sp = spleen; Lu = lung; Mu = muscle; Ki = kidney; He = heart; Co = colon; Il = ileum; Sto = stomach; Bla = bladder; Ste = sternum; To = tongue; and Sk = skin. Starting chemical conditions: 782 nmole [^{186}Re]ReO_4^- (spec. act. 77.3 μCi $\cdot$ $nmole^{-1}$ Re); 1792 nmole S-benzoyl-MAG3; 5.8 μmole Sn^{2+}; 8.9 mg E48 IgG. Final labeling result: 3.75 mCi $\cdot$ mg^{-1} IgG (Re:Mab = 7.35:1).

24 hr after injection revealed that >95% of the ^{99m}Tc was protein bound. HPLC analysis of these serum samples revealed that the protein-bound radioactivity was confined to the Mab. Reanalysis after storage at 4°C for 24 hr gave the same result, affirming that this conjugation method does not give transchelation (*9*). As determined by the modified Lineweaver-Burk plot, the immunoreactive fraction before injection was consistently >0.70, while the radioimmunoconjugates retained full binding capacity up to 24 hr after injection.

Although the chemistry of corresponding 4d and 5d metals is often similar, the reaction conditions to form the corresponding compounds are different in most cases (*21,22*). The MAG3 complexes of ^{99m}Tc and ^{186}Re form a good example of this difference. While ^{99m}Tc-MAG3 is formed within 10 min of heating at 100°C in water, ^{186}Re only forms its ^{186}Re-MAG3 complex after removal of all water molecules. With the Re concentrations used in this study (25–800 n*M*), it appeared that this could easily be accomplished by heating for 15 min after evaporation of the water.

For reproducible labeling results, the presence of SO_3^{2-} before the addition of S-benzoyl-MAG3 and Sn^{2+} is required. Aqueous [^{186}Re]ReO_4^- solutions suffer from the "aging" process. A variety of highly reactive radicals and H_2O_2 are formed, possibly up to micromole amounts, due to the intense radiation (e.g., a 1-ml solution of 200 mCi of [^{186}Re]ReO_4^- assuming that all beta particles are absorbed in the solution, has obtained a dose of 3400 krads after 1 day). The amount of reactive species present in the [^{186}Re]ReO_4^- solution to be used for labeling will vary with and depend on the amount/concentration of activity and the time delay between production and use for synthesis. Consequently, oxidation of sulphur atoms of the added MAG3 and consumption of Sn^{2+} ions necessary for the reduction of ^{186}Re will be variable and possibly complete. The large excess of SO_3^{2-} does not affect the ^{186}Re-MAG3 formation and is washed away during the Sep-pak procedure.

Once formed, the ^{186}Re-MAG3 complex is a stable compound in aqueous solution, i.e., at low specific activities and low radioactivity concentration. "In vitro stability" is often a vague description of a very complex situation. For ^{99m}Tc it can be calculated that about 2% of the total energy is deposited in the solution. At low radioactivity, this forms no problem with respect to radiolytic decomposition since ^{99m}Tc is nearly carrier-free. At high radioactivity, this amount cannot be neglected. However, often reducing additives like Sn^{2+} or beta mercaptoethanol are still present in the solution because a postpurification step was not carried out or the Mab contains internal reducing groups (SH-groups generated by the Schwartz or pre-tinning method) (*23,24*). For ^{186}Re the amount of energy deposited in the solution is several orders of magnitude higher than for ^{99m}Tc. As a result, the in vitro stability or speed of radiolytic decomposition of a ^{186}Re-protein bond is a function of the specific activity (with the same dose at low specific activity, the ^{186}Re compounds seem to be relatively more stable because more ^{185}Re atoms are reoxidized), the dose (the radioactivity concentration), time delay ("aging" of the solution), the pH (*25*), the presence of other oxidizable groups (the Mab concentration or added HSA) (*26,27*), and finally the presence of other constituents of the solution to produce secondary reactive species (e.g., when phosphate ions were present we observed a faster radiolytic decomposition). So although there are undoubtedly differences in rates and redox chemistry of ^{99m}Tc and ^{186}Re (*21,22,28*), these subtle differences are not the main reason why lower valent ^{186}Re compounds are apparently more easily reoxidized than their corresponding ^{99m}Tc compounds. The main reason lies in their differences in decay properties and their concomitant radiolytic decomposition. In this study, the use of antioxidants was shown to suppress the radiolytic decomposition. Addition of ascorbic acid or gentisic acid at a low pH ensures the stability of ^{186}Re-MAG3-Mab when stored in vitro until injection. In contrast to ascorbic acid, gentisic acid at a physiological pH caused rapid detachment of ^{186}Re from the protein. On the basis of these results we prefer to add ascorbic acid, which is a safe agent for parenteral administration, to ^{186}Re-MAG3-Mab prepared for in vivo application. The mixture of dissociated and undissociated ascorbic acid molecules at a pH of 3.5 apparently is the most effective antioxidative additive.

After injection of the ^{186}Re-MAG3-Mab into nude mice, no free ^{186}Re could be detected as became apparent after TCA precipitation of serum samples directly after bleeding. Even after prolonged storage of these samples no free

^{186}Re could be detected, indicating the stability of the conjugate in serum.

From the three sets of biodistribution experiments described here, it can be concluded that when using the aforementioned ^{186}Re labeling procedure, the intrinsic behavior of the antibody molecule is not obscured by label, chemistry or concentration related artifacts: (1) the tumor targeting capacity of ^{186}Re-E48 F(ab')$_2$, prepared with S-benzoyl-MAG3, Sn^{2+} and E48 F(ab')$_2$ amounts that are used routinely in the ^{99m}Tc protocol for patient studies was comparable to that of analogously labeled ^{99m}Tc-E48 F(ab')$_2$ and to that of ^{131}I-E48 F(ab')$_2$; (2) biodistribution characteristics and immunoreactivity did not alter for ^{186}Re-E48 IgG conjugates when the Re-MAG3:Mab molar ratio was increased to 7.3:1; and (3) these latter conjugates also appeared highly capable to selectively target HNSCC xenografts in a way similar to ^{131}I-E48 IgG as shown in previous studies (*3,5*).

One aspect that might need further investigation is the observation that ^{186}Re-conjugates appear to show a slightly faster blood clearance than their corresponding ^{99m}Tc-conjugates (Figs. 3 and 5). This phenomenon might originate from interexperimental variability (e.g., the use of a different batch of mice or different xenograft passages). However, it might be that this slightly faster blood clearance of the ^{186}Re-conjugates is a reflection of subtle differences in susceptibility to catabolic processes in the in vivo situation. Therefore, experiments in which ^{186}Re-labeled and ^{99m}Tc-labeled E48 are compared directly upon coinjection in nude mice are currently being initiated.

Several methods for coupling ^{99m}Tc to Mabs have been described (*29–33*). Besides methods for indirect coupling, methods for direct coupling are also used successfully in clinical immunoscintigraphy studies. Whether or not direct labeling methods will be suitable for preparation of ^{186}Re-Mab conjugates to be used for clinical RIT studies is questionable in view of the reported in vivo instability of directly labeled ^{186}Re as well as ^{188}Re-labeled Mabs (*26,34*). Also, it can be anticipated that for clinical application of directly ^{186}Re-labeled Mabs the Re:Mab ratio should be higher than the 1.34:1 that was recently reported by Griffiths et al. (*26*). Now that ^{99m}Tc-Mab imaging is a clinical option for identifying ^{186}Re therapy candidates and conducting dose calculations, we believe that the direct method for coupling is not the method of choice.

Methods for indirect labeling of ^{186}Re have also been reported by others. Najafi et al. described a method for ^{186}Re labeling using a N_2S_4 chelate precoupled to the protein (*27,35*). In contrast to the method used here in which the radiolabeled chelate is conjugated to amino groups of Mabs, in this procedure the chelate is coupled to the Mab via a disulfide bond. Although these bonds are potentially less stable in the presence of cysteine or SH-groups of proteins, Najafi et al. showed that Mabs labeled in this way were capable to eradicate tumors in nude mice (*27*). Whether this labeling procedure will have a value for general application of ^{186}Re is not clear. Also in this case it can be anticipated that for clinical application the N_2S_4:Mab molar ratio should be higher than the 1:1 molar ratio used. Higher molar ratios probably make the pre-coupled protein susceptible to aggregate formation.

Goldrosen et al. (*12*) described coupling of ^{186}Re to N_2S_2 or N_3S chelate esters via transchelation at a pH of 3 in the presence of Sn^{2+} and isopropanol at 95°C. As already mentioned in the introduction, for the MAG3 chelate this pre-ester method was found to be inconvenient due to rapid hydrolysis of the ester bond under these circumstances.

Rhenium-186-Mab conjugates prepared by this pre-ester method using the N_3S butyrate chelate have been evaluated in animal radioimmunotherapy studies in tumor-bearing nude mice (*36*), as well as in a clinical radioimmunotherapy trial by Breitz et al. (*37*). In an editorial accompanying this latter paper, Goldenberg and Griffiths pose some doubts about the clinical utility of the ^{186}Re chemistry developed by this group (*38*). For administration of 40–300 mCi of ^{186}Re, 36 to 47 mg of Mab were used. Assuming the use of ^{186}Re with a specific activity of 2.5 $Ci \cdot mg^{-1}$, Goldenberg and Griffiths calculated that two to three Re-MAG$_2$-GABA chelates had been coupled to each antibody molecule. They argued that when using commercially available ^{186}Re, which has a lower specific activity (900 $mCi \cdot mg^{-1}$, NEN Dupont, N. Billerica, MA), more antibody will be required than in the study of Breitz et al., thereby limiting the more general application of this labeling technique.

From a chemical point of view, the pre-ester method, implying a one-pot reduction, transchelation and conjugation, is less flexible than the prelabeled chelate approach with respect to the integrity of the ester bond and the antibody. The latter method allows a variety of chemical adjustments in chelation conditions before esterification, while the Sep-pak purification step removes all potentially disturbing chemical ingredients before conjugation. In view of the high Re-MAG3:Mab molar ratios of 7–8:1 that could be realized in this way without apparent impairment of the intrinsic behavior of the Mab, we regard the specific activity of ^{186}Re not necessarily a restrictive factor any longer. For administration of 300 mCi of ^{186}Re-E48 IgG, ^{186}Re with a specific activity of 900 $mCi \cdot mg^{-1}$ can be coupled to 36 mg of Mab, the same amount as administered in the study of Breitz et al. in which ^{186}Re of high specific activity was used. Obviously, the amount of antibody needed will be less when ^{186}Re of high specific activity becomes available more generally.

Applying the label strategies described in this paper, initial therapy experiments with ^{186}Re-labeled Mab E48 IgG in nude mice bearing established HNSCC have been started. Preliminary results indicate that ^{186}Re-conjugates of Mab E48 are better suited to eradicate HNSCC xenografts than ^{131}I-conjugates. Using a single bolus injection of 400–600 μCi of ^{186}Re-labeled Mab E48 IgG, all tumors regressed while 36% of the tumors showed complete remission without regrowth during follow-up (>4 mo). We feel that the synthesis as outlined in the technical protocol

is an important step in our aim to end up with an effective adjuvant radioimmunotherapy strategy for a group of HNSCC patients, who are at risk of developing distant metastases. Reaching the phase of routine clinical application, coupling of high doses of ^{186}Re to Mabs as schematically presented by Figure 2 can be easily automated in a way that recently has been realized at our institute for the synthesis of ^{18}FDG.

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REFERENCES

1. Quak JJ, Balm AJM, van Dongen GAMS, et al. A 22-kDa surface antigen detected by monoclonal antibody E48 is exclusively expressed in stratified and transitional epithelia. *Am J Pathol* 1990;136:191–197.
2. Schrijvers AHGJ, Gerretsen M, Quak JJ, et al. Evidence for a role of the monoclonal antibody E48 defined antigen in cell-cell adhesion in squamous epithelia and head and neck squamous cell carcinoma. *Exp Cell Res* 1991; 196:264–269.
3. Quak JJ, Balm AJM, Brakkee JGP, et al. Localization and imaging of radiolabeled monoclonal antibody against squamous cell carcinoma of the head and neck in tumor-bearing nude mice. *Int J Cancer* 1989;44:534–538.
4. Gerretsen M, Quak JJ, Suh JS, et al. Superior localization and imaging of radiolabeled monoclonal antibody E48 $F(ab')_2$ fragment in xenografts of human squamous cell carcinoma of the vulva and of the head and neck as compared to monoclonal antibody E48 IgG. *Br J Cancer* 1991;63:37–44.
5. Gerretsen M, Schrijvers AHGJ, van Walsum M, et al. Radioimmunotherapy of human head and neck squamous cell carcinoma xenografts with ^{131}I-labeled monoclonal antibody E48 IgG. *Br J Cancer* 1992;66:496–502.
6. Van Dongen GAMS, Leverstein H, Roos JC, et al. Radioimmunoscintigraphy of head and neck cancer using ^{99m}Tc-labeled monoclonal antibody E48 $F(ab')_2$. *Cancer Res* 1992;52:2569–2574.
7. Wessels BW, Rogus RD. Radionuclide selection and model absorbed dose calculations for radiolabeled tumor associated antibodies. *Med Phys* 1984; 11:638–645.
8. Coursey BM, Cessna J, Garcia-Torano E, et al. The standardization and decay scheme of rhenium-186. *Int J Radiat Appl Instrum Part A* 1991;42: 865–869.
9. Fritzberg AR. Advances in ^{99m}Tc-labeling of antibodies. *Nucl Med* 1987; 26:7–12.
10. Fritzberg AR, Abrams PG, Beaumier PL, et al. Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent. *Proc Natl Acad Sci* 1988;85:4025–4029.
11. Kasina S, Rao TN, Srinivasan A, et al. Development and biologic evaluation of a kit for preformed chelate technetium-99m radiolabeling of an antibody Fab fragment using a diamide dimercaptide chelating agent. *J Nucl Med* 1991;32:1445–1451.
12. Goldrosen MH, Biddle WC, Pancook J, et al. Biodistribution, pharmacokinetic and imaging studies with ^{186}Re-labeled NR-LU-10 whole antibody in LS174T colonic tumor-bearing mice. *Cancer Res* 1990;50:7973–7978.
13. Edwards DP, Grzyb KT, Dressler LG, et al. Monoclonal antibody identification and characterization of a Mr 34,000 membrane glycoprotein associated with human breast cancer. *Cancer Res* 1986;46:1306–1317.
14. Quak JJ, Schrijvers AHGJ, Brakkee JGP, et al. Expression and characterization of two differentiation antigens in stratified squamous epithelia and carcinomas. *Int J Cancer* 1992;50:507–513.
15. Takahashi H, Wilson B, Ozturk M, et al. In vivo localization of human colon adenocarcinoma by monoclonal antibody binding to a highly expressed cell surface antigen. *Cancer Res* 1988;48:6573–6579.
16. Quak JJ, Gerretsen M, Schrijvers AHGJ, Meijer CJLM, van Dongen GAMS, Snow GB. Detection of squamous cell carcinoma xenografts in nude mice with radiolabeled monoclonal antibodies. *Arch Otolaryngol/Head Neck Surg* 1991;117:1287–1291.
17. Scheper RJ, Bulte JWM, Brakkee JGP, et al. Monoclonal antibody JSB-1 detects a highly conserved epitope on the P-glycoprotein associated with multidrug resistance. *Int J Cancer* 1988;42:389–394.
18. Haisma HJ, Hilgers J, Zurawski VR. Iodination of monoclonal antibodies for diagnosis and therapy using a convenient one-vial method. *J Nucl Med* 1986;27:1890–1895.
19. Lindmo T, Boven E, Cuttitta F, et al. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J Immun Meth* 1984;72:77–78.
20. Grenman RG, Carey TE, McClatchey KD, et al. In vitro radiation resistance among cell lines established from patients with squamous cell carcinoma of the head and neck. *Cancer* 1991;67:2741–2747.
21. Davison A, Orvig C, Trop HS, Sohn M, DePamphilis BV, Jones AG. Preparation of oxobis(dithiolato) complexes of technetium (V) and rhenium (V). *Inorg Chem* 1980;19:1988–1993.
22. Cotton FA, Wilkinson G. Technetium and rhenium: group VII B. In: Cotton FA, Wilkinson G, eds. *Advanced inorganic chemistry, fifth edition*, New York: Wiley; 1988:847–867.
23. Schwartz A, Steinsträsser A. A novel approach to ^{99m}Tc-labeled monoclonal antibodies. *J Nucl Med* 1987;28:721–727.
24. Rhodes BA, Zamora PO, Newell KD, Valdez EF. Technetium-99m labeling of murine monoclonal antibody fragments. *J Nucl Med* 1986;27:685–693.
25. Portenlänger G, Heusinger H. Chemical reactions induced by ultrasound and gamma-rays in aqueous solutions of L-ascorbic acid. *Carbohyd Res* 1992;232:291–301.
26. Griffiths GL, Goldenberg DM, Knapp Jr FF, et al. Direct radiolabeling of monoclonal antibodies with generator-produced rhenium-188 for radioimmunotherapy: labeling and animal biodistribution studies. *Cancer Res* 1991; 51:4594–4602.
27. Najafi A, Alauddin MM, Sosa A, et al. The evaluation of ^{186}Re-labeled antibodies using N_2S_4 chelate in vitro and in vivo using tumor-bearing nude mice. *Nucl Med Biol* 1992;19:205–212.
28. Quadri SM, Wessels BW. Radiolabeled biomolecules with ^{186}Re: potential for radioimmunotherapy. *Int J Radiat Appl Instrum Part B* 1986;13:447–451.
29. Eckelman WC, Paik CH, Steigman J. Three approaches to radiolabeling antibodies with ^{99m}Tc. *Int J Radiat Appl Instrum Part B* 1989;16:171–176.
30. Verbruggen AM. Radiopharmaceuticals: state of the art. *Eur J Nucl Med* 1990;17:346–364.
31. Hnatowich DJ. Antibody radiolabeling, problems and promises. *Int J Radiat Appl Instrum Part B* 1990;17:49–55.
32. Srivastava SC, Mease RC. Progress in research on ligands, nuclides and techniques for labeling monoclonal antibodies. *Int J Radiat Appl Instrum Part B* 1991;18:589–603.
33. Rhodes BA. Direct labeling of proteins with ^{99m}Tc. *Int J Radiat Appl Instrum Part B* 1991;18:667–676.
34. Su F-M, Axworthy DB, Beaumier PL, Fritzberg AR. Pharmacokinetic comparison of Re-186 direct versus chelate labeled NR-LU-10 Mab in tumored nude mice [Abstract]. *J Nucl Med* 1992;33:910.
35. Najafi A, Alauddin MM, Siegel ME, Epstein AL. Synthesis and preliminary evaluation of a new chelate N_2S_4 for use in labeling proteins with metallic radionuclides. *Nucl Med Biol* 1991;18:179–185.
36. Beaumier PL, Venkatesan P, Vanderheyden J-L, et al. ^{186}Re radioimmunotherapy of small cell lung carcinoma xenografts in nude mice. *Cancer Res* 1991;51:676–681.
37. Breitz HB, Weiden PL, Vanderheyden J-L, et al. Clinical experience with rhenium 186-labeled monoclonal antibodies for radioimmunotherapy: results of phase I trials. *J Nucl Med* 1992;33:1099–1109.
38. Goldenberg DM, Griffiths GL. Radioimmunotherapy of cancer: arming the missiles. *J Nucl Med* 1992;33:1110–1112.