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International Journal of Pharmaceutics 248 (2002) 173–182

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Optimization of antibody labeling with rhenium-188 using a prelabeled MAG_3 chelate

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Received 6 June 2002; received in revised form 31 July 2002; accepted 1 August 2002

Abstract

We report the optimization of polyclonal IgG labeling by ^{188}Re using *S*-benzoyl–MAG_3 as a model for labeling monoclonal antibodies (MoAb). We examined the in vitro stability of the labeled protein and its localization and excretion in mice with induced focal inflammation. Stability in serum was greater than 85.5% after 24 h. Biodistribution and imaging studies following administration to mice showed mainly renal and hepatic excretion and high IT/NT ratios (4.5 and 4.6) at 24 and 48 h, respectively. This indirect method of labeling antibodies using a ^{188}Re-labeled active ester of MAG_3 produced ^{188}Re–MAG_3–IgG of high in vitro stability and favorable uptake at sites of focal inflammation.

Keywords: Rhenium-188; ^{188}Re–MAG_3–IgG; Radiolabeling; Antibodies; ^{188}Re–MAG_3; Radiotherapy

1. Introduction

The utility of radiolabeled monoclonal antibodies (MoAb) (Goldenberg, 2002) and small peptides (Paganelli et al., 2001) in various therapeutic applications has increased interest in radiolabeling procedures, in particular for the high energy β emitters ^{90}Y, ^{186}Re and ^{188}Re. ^{188}Re has several advantageous properties. It can be obtained carrier-free, relatively cheaply and on demand from an in-house ^{188}W/^{188}Re generator (^{188}W $T_{1/2}$ = 69.4 h; ^{188}Re $T_{1/2}$ = 17 h) (Ehrhardt et al., 1987), and it decays by emission of high-energy β particles (E_{max} = 2.11 MeV) suitable for radiotherapy, followed by emission of 155 keV gamma photons in 15.88% abundance. Average β particle penetration is 3.3 mm (maximum 10.8 mm), providing a tightly circumscribed region of high energy deposition with little or no damage to adjacent organs The gamma photon can be used to monitor biodistribution and estimate dosimetry with standard scintigraphic equipment.

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0378-5173/02/$ - see front matter
PII: S0378-5173(02)00434-9

As with ^{99m}Tc (Viaggi et al., 1999) there are two methods of labeling antibodies with rhenium: the direct method via endogenous or generated thiol groups (Griffiths et al., 1991) and the indirect method via chelators such as N_3S (van Gog et al., 1996), N_2S_2 (Glaser et al., 1999), PN_2S (Visentin et al., 1999), hydrazinonicotinyl (HYNIC) derivatives (Yokoyama et al., 1999) and *S*-benzoyl-mercaptoacetyltriglycine (*S*-benzoyl–MAG_3) (Visser et al., 1993). Using the indirect method both preconjugate and postconjugate approaches may be employed (Guhlke et al., 1998).

S-Benzoyl–MAG_3 has been used extensively in nuclear medicine (Taylor et al., 1986) because is easy to synthesize, has a long shelf-life, and its rhenium complex is stable, both in vitro and in vivo.

In the preconjugate approach, radiolabeled *S*-benzoyl–MAG_3 is chemically activated by esterification (producing ^{188}Re–MAG_3-activated ester) and this is coupled to the free amino groups of antibodies (polyclonal or monoclonal), peptides or biotin derivatives, maintaining their biological activity. In this multistep approach, the numerous variables influencing yield have not been evaluated systematically. The aim of the present study was to optimize procedures for labeling antibodies with ^{188}Re using an active ester of *S*-benzoyl–MAG_3. Quality assurance tests were performed on the products at each step and on the final radiolabeled antibodies. Stability studies in saline (0.9% NaCl) and human serum were also performed and the biodistribution of the labeled antibodies was evaluated in an animal model with inflammation.

2. Materials and methods

2.1. Labeling of S-benzoyl–MAG_3 with ^{188}Re

A saline solution of $^{188}ReO_4^-$ (ca. 370 MBq) was freshly eluted from a ^{188}W/^{188}Re generator (MAP Medical Technologies, Finland) and evaporated to dryness. The influence of stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) (range 6–5000 μg) and *S*-benzoyl–MAG_3 (kindly provided by CGM Nuclear, Chile) (range 100–2600 μg), on the efficiency with which *S*-benzoyl–MAG_3 was labeled by ^{188}Re was studied. For example in one experiment, 1500 μg of $SnCl_2$ $2H_2O$ (250 μl of a 6000 μg/ml solution in 0.1 M citrate buffer pH = 5.5) and 750 μg of *S*-benzoyl–MAG_3 (250 μl of a 3000 μg/ml solution in acetonitrile:water (6:4 v/v)) were added to the rhenium in a nitrogen atmosphere, the vial was sealed and heated for 30 min at 90 °C. The ^{188}Re–MAG_3 obtained was then analyzed.

HPLC was employed to check the labeling yield: a Waters 600 HPLC with radiometric and UV (diode array) detectors and a Deltapak C18 column. The gradient was: solvent A: acetonitrile, solvent B: water, TFA 0.1%, gradient: 0–3 min 100% B at 1 ml/min, linear increase of eluent A to 50% from 3 to 13 min at 1 ml/min, 13 to 18 min 50% A at 1 ml/min, 18 to 20 min linear increase of eluent A to 70% at 1 ml/min.

2.2. Synthesis of ^{188}Re–MAG_3–TFP ester

After optimization of the above reaction, the product (^{188}Re–MAG_3) was allowed to cool and molar ratios of 2,3,5,6-tetrafluorophenol (TFP) to MAG_3 in the range 7:1–70:1 were tried and the optimal yield obtained. For example, 74.5 μmol of TFP (125 μl of a 100 mg/ml solution in CH_3CN:H_2O, 9:1) and 265 μmol (50 mg) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were added and the mixture agitated at room temperature for 40 min. The ^{188}Re–MAG_3–TFP ester was subsequently purified with Sep-Pack C-18 Waters cartridge. The cartridge was washed successively with 5 ml ethanol and 5 ml water. The reaction mixture was loaded and the cartridge washed with 5 ml deionized water and two fractions of 1 ml acetonitrile. The majority of the eluted ester was collected in the first acetonitrile fraction and then analyzed by reverse phase (RP) HPLC. The solvent was evaporated under nitrogen at room temperature to obtain the dry active ester.

2.3. Preparation of ^{188}Re–MAG_3–IgG

The active ester and human polyclonal immunoglobulin (IgG) (Sandoz Pharma, Switzerland) were conjugated at molar ratios of 75:1, 136:1, 200:1 and 272:1 (Crudo et al., 2000a). Thus, 0.25–

6.3 mg of IgG (10 mg/ml in CO_3HNa 0.1 M pH 10) was added to either the active Sep-Pack C18 purified ester or non-purified ester, and the reaction mixture incubated 15 min at room temperature.

2.4. Quality control and purification of labeled IgG

The radiochemical purity of the labeled IgG was carried out by HPLC using a Protein Pack SW-300 (Waters) column and phosphate buffer 0.02 M pH 7.2 as eluent at 1 ml/min. The product was purified by HPLC using the same column and buffer as for radiochemical purity control. One ml fractions were collected and the fraction containing the labeled protein was used subsequently for the in vitro and in vivo studies.

2.5. Stability in saline and serum

Aliquots (100 μl) of ^{188}Re–MAG_3 and purified ^{188}Re–MAG_3–IgG were incubated in the saline solution and human serum (1:3 v/v, labeled product:human serum) for 19 and 24 h, respectively, at room temperature. The incubated mixture was analyzed by HPLC using the same system as for quality control and the results were also checked with instant thin layer chromatography (ITLC SG, Gelman Instruments Company) run in sodium citrate 0.1 M.

2.6. Experimental animals

Eight to 12-week-old inbred NIH mice from the National Atomic Energy Commission facility, average weight 25 g, were kept (five to ten animals per cage) with water and food ad libitum. Animal care was provided in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals (Guide, 1996).

2.7. In vivo tissue distribution of IgG–MAG_3–^{188}Re

Focal inflammation was induced by injecting 40 μl of turpentine in the right thigh. The animals were left for 48 h, following which normal and inflammed mice were then injected intravenously (lateral tail vein) with 11 MBq of IgG–MAG_3–^{188}Re. Whole body images were obtained 4, 24 and 48 h post injection, using a gamma camera equipped with a medium-energy collimator. Five lakh counts were obtained in a 128×128 matrix. The biodistribution in normal NIH mice, and those with induced focal inflammation, was determined at 4, 24, 48 and 120 h post iv injection of 0.4 MBq of labeled IgG. To do this, three animals per group were sacrificed by cervical dislocation at each time. Blood samples were taken and organs of interest (including inflamed thigh (IT) and normal thigh (NT)) were resected, rinsed, dried and placed into pre-weighed tubes. The total injected dose (ID) was calculated by measuring syringes before and after injecting each animal. The activity of all samples was counted together with the standard and results were expressed as percentage of the ID per tissue gram (%ID/g). A dilution (1:100) of the injected labeled product was used as a standard in order to correlate counts per min (cpm) with the activity units (MBq).

3. Results

3.1. Labeling of MAG_3 with ^{188}Re

The first synthesis step employed a solid phase reduction of perrhenate with stannous ion, with citrate as transfer ligand. Yields of more than 95% were obtained when the ratio of $SnCl_2\ 2H_2O$ to ^{188}Re activity was greater than 3.13 μg/MBq as shown in Fig. 1. Nearly quantitative yields were obtained when the ratio of *S*-benzoyl–MAG_3 to ^{188}Re activity exceeded 0.81 μg/MBq (Fig. 2). Specific activities up to 8713.5 MBq/mg MAG_3 (2960 MBq/μmol MAG_3) were obtained.

3.2. Esterification of ^{188}Re–MAG_3 with TFP

The best esterification yield was obtained when the molar ratio of TEP to ^{188}Re–MAG_3 was 35:1 in which case up to 75% of the activity was recovered after purification. The ester was stable in acetonitrile at room temperature for 24 h without loose of free ^{188}Re.

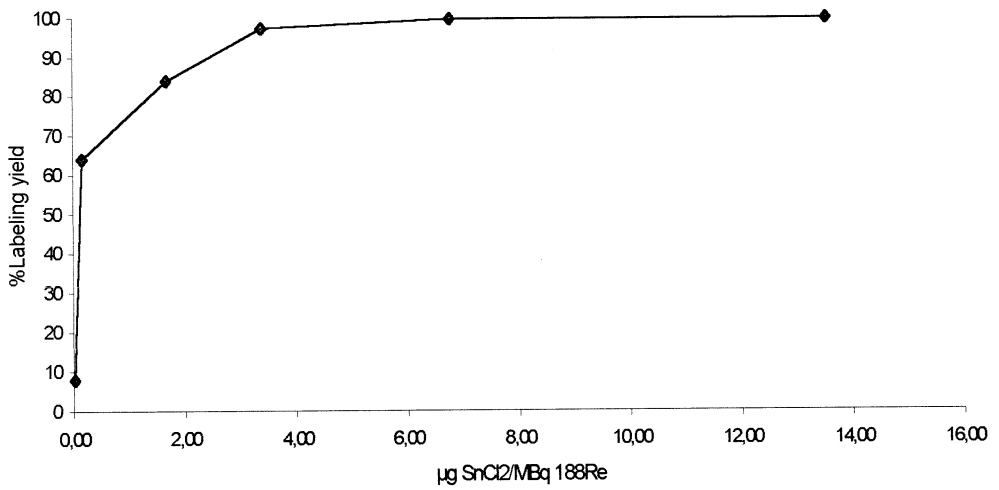


Fig. 1. Influence of $SnCl_2$ amounts per MBq ^{188}Re on labeling yield.

3.3. Conjugation of IgG with ^{188}Re–MAG_3–TFP

When non-purified ^{188}Re–MAG_3–TFP was reacted with IgG, only 73% of IgG was labeled (specific activity 1587.3 MBq/μmol, 10.14 MBq/mg IgG) and quality control (size exclusion HPLC) always showed the presence of protein aggregates.

Fig. 3 shows the various molar ratios of purified radiolabeled ester and IgG studied. The maximum efficiency (82.8%) was obtained when the molar ratio of ester to protein was 200:1, in which case the specific activity of labeled IgG was 9213 MBq/μmol (61.42 MBq/mg IgG). Protein aggregates were not detected on subsequent HPLC when purified ^{188}Re–MAG_3–TFP was used.

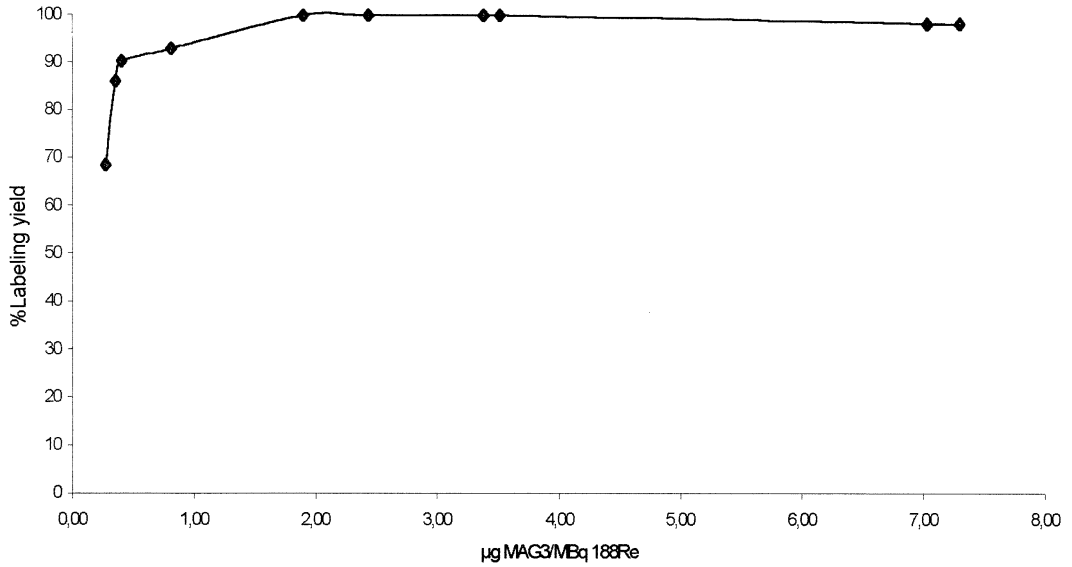


Fig. 2. Influence of MAG_3 amounts per MBq ^{188}Re on labeling yield.

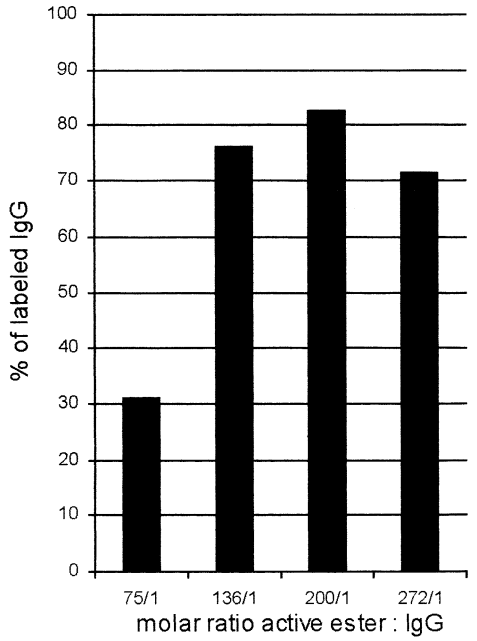


Fig. 3. Conjugation of IgG with ^{188}Re–MAG$_3$:TFP at different ratios.

3.4. HPLC studies

The HPLC retention times were 1.88, 11.55 and 15.59 min for $^{188}ReO_4^-$, ^{188}Re–MAG$_3$ and ^{188}Re–MAG$_3$–TFP, respectively, using a Deltapack C18 RP column.

The HPLC chromatograms of the labeled IgG using a Protein Pak column had a retention time of 7.73 min for the labeled protein, 12.43 min for the labeled ester, and 13.2 min for $^{188}ReO_4^-$.

3.5. Stability in saline and serum

The results of in vitro stability assays of ^{188}Re–MAG$_3$–IgG and ^{188}Re–MAG$_3$ are shown in Fig. 4. Over 96% of ^{188}Re–MAG$_3$ activity was recovered (RP-HPLC) after incubation for 24 h in saline and serum at room temperature.

Similarly 96.2 and 85.5% of the activity remained bound to IgG–MAG$_3$ (size exclusion HPLC) after incubation in saline and serum, respectively. ITLC data were consistent with these findings (data not shown). HPLC analysis after incubation of labeled IgG in saline also demonstrated that 3.8% of the radioactivity was associated with either ^{188}Re or ^{188}ReMAG$_3$. When ^{188}Re–MAG$_3$–IgG was incubated with human serum, 14.3% of the radioactivity was associated with serum proteins (Rt = 5.0–5.5 min).

3.6. Biodistribution

The biological distribution of ^{188}Re-labeled IgG was similar in control mice and in those with focal inflammation (Fig. 5a and b) with no significant differences in uptake for the majority of the organs. The uptake of the radiolabeled IgG to liver and kidney was similar and higher than in most other organs, indicating combined hepatic uptake and renal excretion. Clearance rates for liver and kidney were similar as for blood (Fig. 6). ITs had slower clearances than other organs. Uptake differences between IT and NTs were nearly constant over time so the IT/NT ratios were similar at all times (Table 1). Gamma camera images in mice with IT are shown in Fig. 7. The IT was clearly evident and the imaging data were consistent with the biodistribution results.

4. Discussion

Labeled MoAbs offer a powerful approach to cancer therapy in view of their specificity and targeting capability (Goldenberg, 2002).

Increased effort has been made to label MoAbs with ^{188}Re because of their potential role in the radiommunotherapy of cancer and the availability of ^{188}Re from a ^{188}W/^{188}Re generator (Rhodes et al., 1996).

Rhenium chemistry is dominated by redox reactions, and perrhenate, like pertechnetate, is a negatively charged anion that is rapidly excreted from living systems. When completed to MAG$_3$ a stable compound has been produced *S*-Benzoyl–MAG$_3$ was used because it is more stable to oxidation than MAG$_3$ with its free thiol group. As reported in Section 2, for efficient *S*-benzoyl–MAG$_3$ labeling, the $SnCl_2 \cdot 2H_2O$, *S*-benzoyl–MAG$_3$ and the $^{188}ReO_4^-$ mixture had to be heated for 30 min at 90 °C. At this temperature, *S*-benzoyl–MAG$_3$ loses the benzoyl protecting

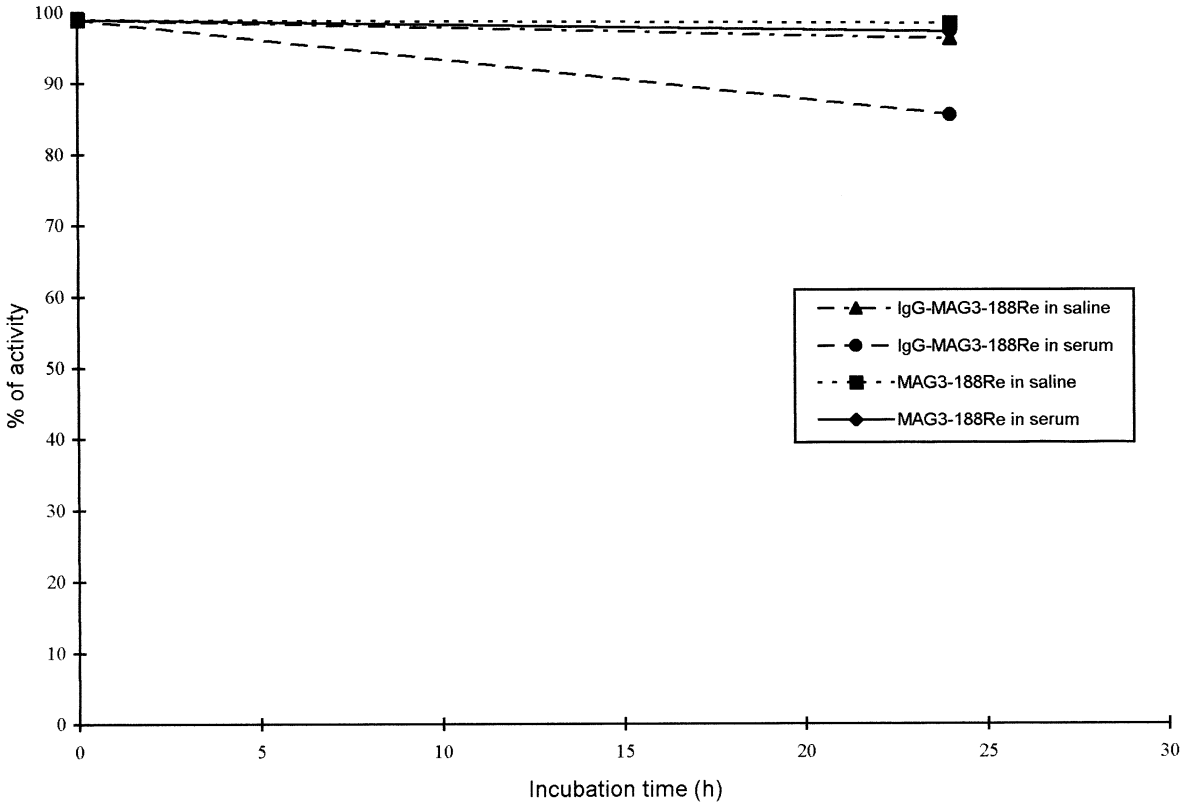


Fig. 4. In vitro stability of ^{188}Re–MAG_3–IgG and ^{188}Re–MAG_3 at 24 h.

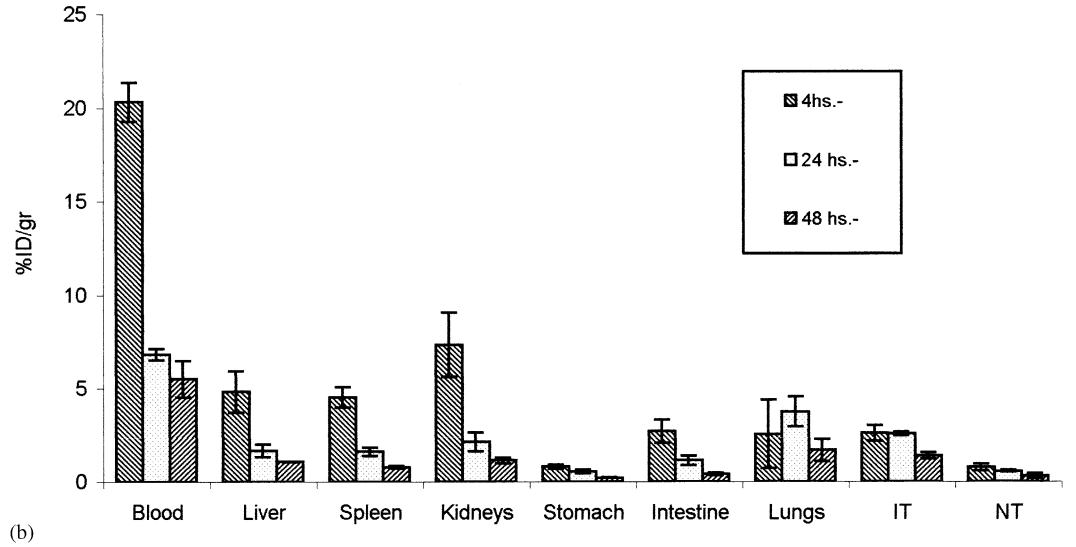
group and the reduced rhenium is complexed. However, at this temperature ^{188}Re labeling of MAG_3–IgG (or MAG_3–MoAb) is not feasible because of protein denaturation and consequent loss of immunoreactivity. Alternative protocols must, therefore, be used to label such labile biomolecules.

We, therefore, optimized the preconjugate approach in which radiolabeled MAG_3 was first chemically activated to obtain an active ester, following which the ester was coupled to free amino groups on the protein. Our studies showed that the complete reduction of $^{188}ReO_4^-$ requires 3.13 μg $SnCl_2 \cdot 2H_2O$ per MBq of ^{188}Re and 0.81 μg *S*-benzoyl–MAG_3 per MBq of ^{188}Re. This is approximately 620 times more $SnCl_2 \cdot 2H_2O$ than required for the reduction of the same activity of $^{99m}TcO4-$ (0.005 μg $SnCl_2 \cdot 2H_2O$ per MBq) (Viaggi et al., 1999). This fact is due to the difference between the redox potential of the two radionuclides.

Assuming a theoretical specific activity of ~ 4.97 GBq/μmol for ^{188}Re (Ehrhardt et al., 1992), our experimental data gave us a molar ratio of Sn^{2+}:^{188}Re of 115 471:1 and a molar ratio of *S*-benzoyl–MAG_3:^{188}Re of 41 017:1. These ratios differ markedly from those reported by van Gog et al. for labeling MoAb with ^{186}Re (van Gog et al., 1996) because its specific activity is lower than the one of ^{188}Re.

Variation of the molar ratios of active ester to IgG showed that optimal labeling (labeling efficiency 80%) occurred at a molar ratio of 200:1, when specific activities of 61.4 MBq/mg IgG were

(a)



(b)

Fig. 5. (a, b) Biodistributions of $^{188}Re–MAG_3–IgG$ in normal NIH mice and in NIH mice bearing an inflammation foci ($n = 3$).

obtained. From these data we assume that 4.2 $^{188}Re–MAG_3$ groups per IgG were present in the final product.

Our in vitro stability studies with labeled IgG showed that 96.2 and 85.5% of activity remained bound to the IgG when incubated in saline and serum, respectively, at room temperature for up to 24 h. The saline stability results are superior to the 77% obtained by Winnard et al. and quite similar to those for serum (86%) following direct labeling

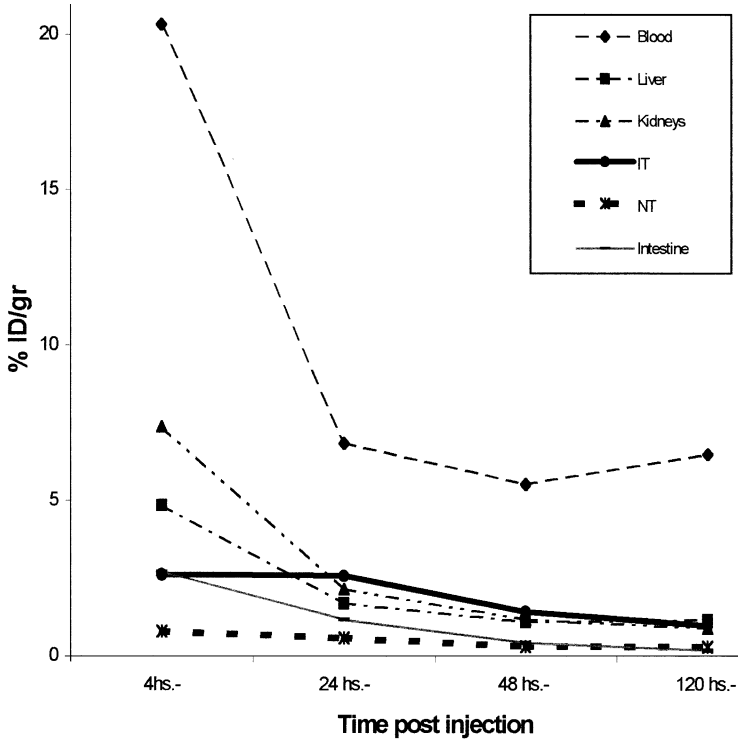


Fig. 6. Clearance curve in mice with thigh inflammation ($n = 3$).

Table 1
IT/NT ratios of IgG–MAG3–^{188}Re at different times post injection

Time post injection (h)	4	24	48
IT/NT ratio	3.4±0.3	4.5±0.2	4.6±1.5

of antibodies with rhenium (Winnard et al., 1996) suggesting that this indirect method produce a stable conjugate suitable for radioimmunotherapy.

When ^{188}Re–MAG_3 was incubated for 24 h in saline or serum over 98% of the radioactivity was recovered in the ^{188}Re–MAG_3 fraction. When labeled IgG was incubated with human serum, activity not associated with the IgG (14.3% of the total) was associated with serum proteins of higher molecular weight than IgG. Furthermore, IgG aggregates (an effect of β radiation) were not seen. We speculate that this reduced stability after conjugation may be due to lower co-ordination of the rhenium by chelator, since one bond is employed for protein conjugation.

We found high IT/NT ratios for IgG–MAG_3–^{188}Re at all the times following administration to inflamed and control mice. These ratios at 24 and 48 h (4.5 and 4.6, respectively) were higher than the IT/NT from those obtained for 99mTc polyclonal IgG (2.0), reported previously by our group following labeling using both direct and indirect methods (Crudo et al., 1998; Viaggi et al., 1999). This could be explained because of the transchelation of ^{99m}Tc to endogenous cysteine.

^{188}Re–MAG_3–IgG was excreted both from the liver into the gut and via the urine. We found a similar pattern of excretion for polyclonal ^{99m}Tc–MAG_3–IgG (Viaggi et al., 1999). Low levels of radioactivity were observed in the stomach, consistent with the absence of free perrhenate and with the stability of the labeled IgG as observed in the in vitro assays.

In preliminary experiments, the application of this optimized labeling procedure to an antimelanoma MoAb resulted in high specific activities 90.28 MBq/mg MoAb (Crudo et al., 2000b)

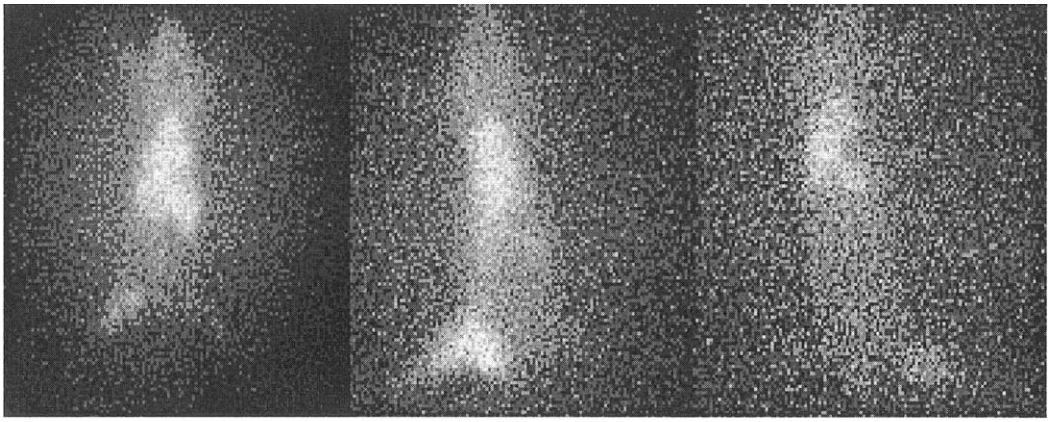


Fig. 7. Camera gamma images of mice carrying an inflammation foci in the right thigh using $^{188}Re–MAG_3–IgG$.

confirming that the conditions outlined in this paper can be extended to the ^{188}Re labeling of other molecules. This groundwork may be the starting point for radioimmunotherapy trials with β emitters.

Acknowledgements

The authors thank Jorge Arashiro for his assistance in animal images. This study was supported by the IAEA Co-ordinated Research Program 'Labeling Techniques of Biomolecules for Targeted Radiotherapy'.

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