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Macrocyclic chelates of radiometals for diagnosis and therapy

C.F. Meares[1], M.K. Moi[1,*], H. Diril[1], D.L. Kukis[1], M.J. McCall[1], S.V. Deshpande[2], S.J. DeNardo[2], D. Snook[3] & A.A. Epenetos[3]

[1]*Department of Chemistry, University of California, Davis, California 95616,* [2]*Department of Internal Medicine, University of California, Davis, Medical Center, Sacramento, California 95817, USA and* [3]*Imperial Cancer Research Fund, Department of Clinical Oncology, Hammersmith Hospital, Du Cane Road, London W12 0HS, UK.*

Summary Monoclonal antibody technology allows the specificity of an antibody for its antigen to be used in targeting cancer cells. Stable attachment of metal ions to antibodies by means of 'bifunctional' chelating agents can add the nuclear, physical and chemical properties of the metallic elements to these target-selective proteins. The conjugation of metals – particularly radionuclides – to monoclonal antibodies results in agents for radioimmunotherapy and other medical applications. Chelators that can hold radiometals with high stability under physiological conditions are essential to avoid excessive radiation damage to non-target cells. Derivatives of polyazamacrocycles (bearing a *C*-substituted functional group for antibody attachment) can exhibit remarkable kinetic inertness. We have developed a new synthetic route to these macrocycles via peptide synthesis and *intra*molecular tosylamide ring closure. Incubation of the yttrium complex of 2-*p*-nitrobenzyl-1,4,7,10-tetraazacyclododecane-*N*,*N'*,*N''*,*N'''*-tetraacetic acid (nitrobenzyl-DOTA) for 18 days in serum results in loss of so little yttrium from the complex (less than 0.5%) that the rate of loss cannot be measured under these conditions. In animal models, conjugates of this chelate with monoclonal antibodies show much lower levels of yttrium in the bone than are found with DTPA chelates prepared from the cyclic anhydride. The rates of loss of indium and cobalt from nitrobenzyl-DOTA in serum are slower than from previously studied chelates. Preliminary clinical imaging studies of ^{111}In-labelled monoclonal antibody conjugates of DOTA show highly encouraging results.

Radiolabelled monoclonal antibodies (MAbs) have shown considerable promise for the early detection and therapy of cancer (DeNardo *et al.*, 1983; Bloomer *et al.*, 1985; O'Grady *et al.*, 1986). Many radionuclides of interest for radioimmunoimaging and radioimmunotherapy are metals such as ^{111}In, ^{90}Y, ^{68}Ga, ^{99m}Tc, ^{67}Cu, ^{55}Co, etc. (Halpern *et al.*, 1983; Hnatowich *et al.*, 1983; DeNardo *et al.*, 1984; Wessels & Rogus, 1984; Order *et al.*, 1986; Pimm *et al.*, 1987; Deshpande *et al.*, 1988; Leichner *et al.*, 1988), and methods to stably attach these metal ions to MAbs have gained attention in the past few years (Yeh *et al.*, 1979*b*; Meares & Wensel, 1984; Meares *et al.*, 1984). Chelators that can hold radiometals with high stability under physiological conditions are essential to avoid excessive radiation damage to non-target cells (Klein *et al.*, 1988).

Bifunctional chelating agents are compounds with a strong metal chelating group at one end and a reactive functional group, capable of binding to proteins, at the other. When conjugated to MAbs, these agents act as carriers of radiometals for tumour targeting. We have recently developed new *macrocyclic* bifunctional chelating agents that hold yttrium and copper with extraordinary stability under physiological conditions in human serum. These macrocyclic compounds are exemplified by 2-(*p*-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-*N*,*N'*,*N''*,*N'''*-tetraacetic acid (nitrobenzyl-DOTA) and its protein conjugates (see Figure 1a).

The proteins of primary interest here are antibodies of the type immunoglobulin G (IgG). These are comprised of two pairs of polypeptide chains (light and heavy) with a total molecular weight of approximately 150 kD (Wasserman & Capra, 1977). For example, MOPC173 was the first mouse monoclonal IgG_{2a} to have its primary sequence determined (Fougereau *et al.*, 1974). The light chains of this antibody have 214 residues and the heavy chains have 447, giving a total (2L + 2H) of 1322 residues. The reactive groups we consider here are located at amino acid side-chains and at the polypeptide chain termini. Amino groups are particularly useful; in addition to four *N*-terminal NH_2 groups, the chains of MAb MOPC173 contain a total of 84 lysine ε-amino groups. As shown in Figure 1b, amino groups may be conveniently labelled with macrocyclic chelating agents by means of 2-iminothiolane. This provides a molecular spacer between protein and macrocycle, which has been found to facilitate addition of a metal ion to the conjugate.

Here we describe the properties of macrocyclic chelates of yttrium, indium and cobalt with 2-(*p*-nitrobenzyl)-1,4,7,10-tetraazacyclododecane-*N*,*N'*,*N''*,*N'''*-tetraacetic acid under physiological conditions. This work is correlated with biological studies of DOTA-antibody conjugates in the laboratories of Drs DeNardo and Epenetos, as noted below.

Materials and methods

Nitrobenzyl derivatives of EDTA, DTPA and DOTA were prepared in our laboratory by methods described previously

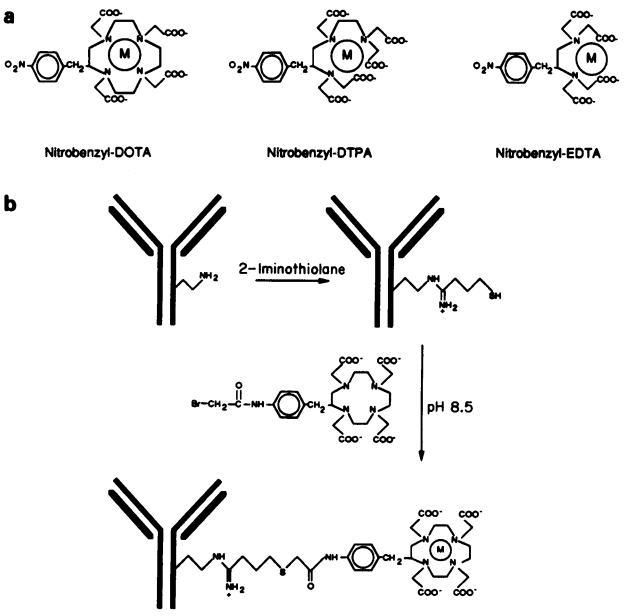

Figure 1 **a** Structures of three types of metal chelates whose properties are compared here. The metal ion is represented by a circled M. **b** Chemistry for attachment of bromoacetamidobenzyl-DOTA to an antibody via 2-iminothiolane.

*Present address: Hybritech Inc., San Diego, California, USA. Correspondence: C.F. Meares.

(Yeh *et al.*, 1979*b*; Moi *et al.*, 1988) and (where applicable) the nitro group was converted to a bromoacetamide as described (Meares *et al.*, 1984).

Preparation of indium and cobalt chelates

Carrier-free $^{57}CoCl_2$ and $^{114m}InCl_3$, each in 0.5 M HCl were purchased from ICN Radiochemicals and NEN Research Products, respectively. 3.6 μCi aliquots were dried under a heat lamp and to each were added 5 μl of 0.40 mM chelate solutions in 0.1 M ammonium acetate, pH 5.0. These solutions were incubated at room temperature for 4 h. Aliquots were drawn and diluted × 80 in 0.1 M ammonium acetate, pH 5.0; these diluted solutions were immediately examined by thin layer chromatography (TLC) to test for complete chelation. TLC was performed using plastic backed silica coated TLC plates (EM Science, DC-Plastikfolien Kieselgel 60F 254). The mobile phase was a mixture of equal volumes of 10% (w/v) aqueous ammonium acetate and methanol.

Stability of chelates in human serum

Once complete chelation was verified, aliquots of the undiluted chelate solutions were diluted × 10 in double deionized water which were in turn added to human serum. Typically, 5 μl of chelate solution was added to 1 ml or more of serum so that dilution was insignificant. Final concentrations of ligand in serum solutions of nitrobenzyl-EDTA-In, nitrobenzyl-DTPA-In, nitrobenzyl-DOTA-In, nitrobenzyl-EDTA-Co and nitrobenzyl-DOTA-Co were 200, 240, 240, 133, and 133 nM, respectively. Human serum was prepared by allowing blood collected from a healthy volunteer to clot for 1 h at room temperature in a closed tube. The sample was centrifuged and the supernatant serum was transferred to sterile plastic culture tubes. Fresh serum was used in the cobalt experiments; serum used in the indium experiments was frozen at −80°C. In both cases the serum was incubated overnight at 37°C in a humidified 5% CO_2, 95% air atmosphere and the pH measured 7.40 before the addition of chelate. The serum-chelate solutions were incubated at 37°C under a CO_2 atmosphere, with the serum pH ranging between 7.30 and 7.40 during the course of the experiments.

The stability of chelates in human blood serum was determined by measuring daily the extent of transcomplexation of radiometal from chelate to serum proteins. Size exclusion gel chromatography was used to separate indium chelates and proteins; polyacrylamide gel electrophoresis was used to resolve cobalt chelates and proteins.

Gel filtration chromatography

A Bio-Rad 0.7 cm × 10 cm column was packed with 9.0 cm BioGel P-60 in 0.1 M ammonium acetate, pH 7.0. Samples were eluted with 9 ml of 0.1 M ammonium acetate, pH 7.0, and four-drop (approximately 0.2 ml) fractions were collected. To calibrate the column, solutions of indium chelates of nitrobenzyl-EDTA, nitrobenzyl-DTPA and nitrobenzyl-DOTA in 0.1 M ammonium acetate, pH 5.0, were applied to columns and the eluent fractions counted on a Beckman 310 gamma counter; in no case were significant counts (>0.1% of total counts) found outside the range of 3.8–8.4 ml. Serum to which indium chloride had been added was similarly analysed, yielding significant counts only in the 1.0–3.8 ml range. The columns themselves were counted after elution of 9 ml, showing no significant residual radioactivity. Gel chromatography was performed on 50 μl aliquots of the

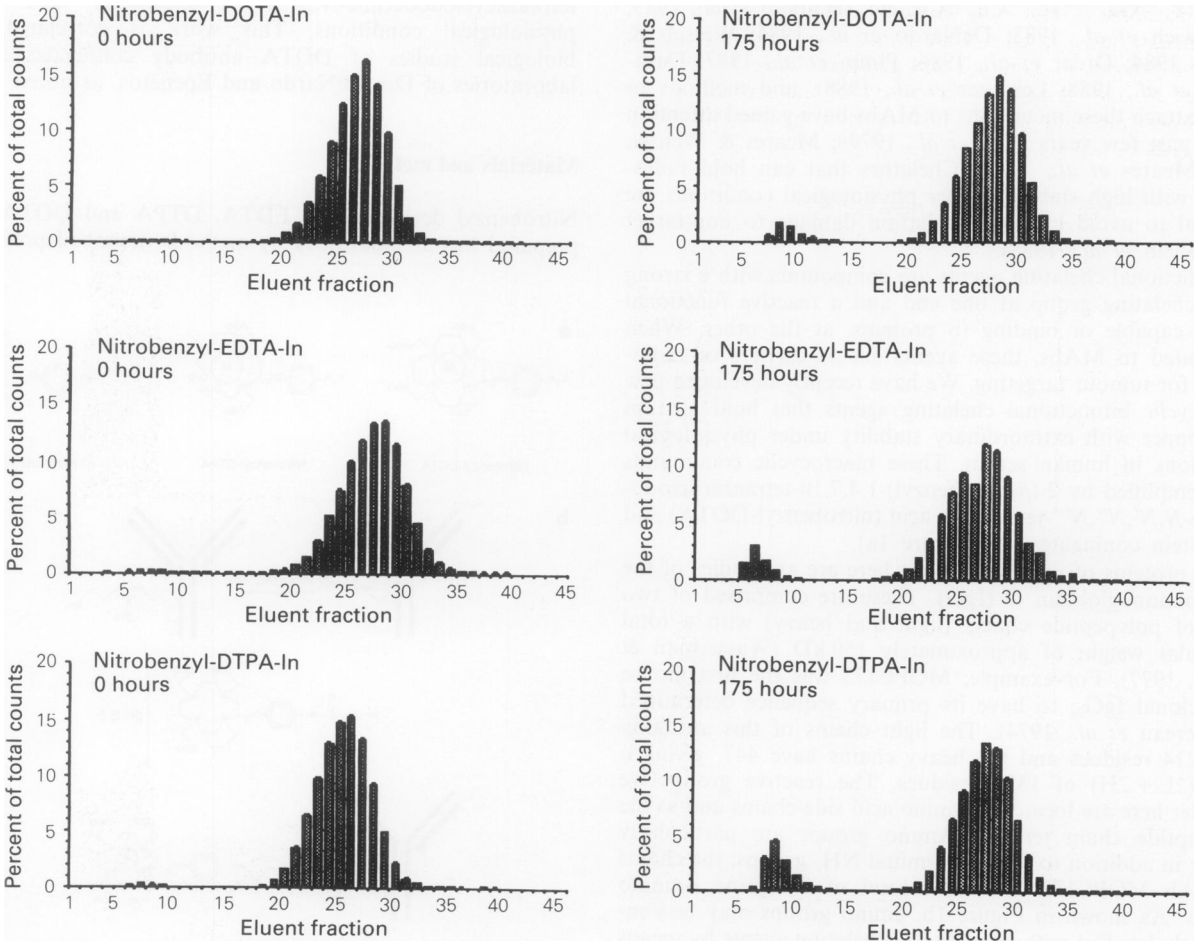

Figure 2 BioGel P-60 gel filtration column elution profiles of serum mixtures of three indium chelates, at the beginning of the experiments (left) and after 7 days of incubation under physiological conditions (right). Indium-transferrin elutes in early fractions, peaking near fraction 7; indium chelates elute in later fractions, peaking near fraction 26.

indium chelate in serum solutions immediately upon the addition of chelate and daily afterwards for 9 days.

Electrophoresis

Twenty-five μl aliquots of the serum were electrophoresed in 8% polyacrylamide slab gels under non-denaturing conditions. Samples were analysed immediately after addition of chelate to serum and at daily intervals for 16 days. The Tris-HCl buffered gels were of pH 8.48 and the Tris-glycine tank buffer of pH 8.89 (Yeh *et al.*, 1979*a*). Bromophenol blue solution in adjacent lanes marked the dye front (Rm = 1.0). The relative mobilities (Rm) of serum proteins were determined by electrophoresis and staining human serum albumin (purchased from Worthington Biochemical) and human serum transferrin (purchased from Sigma Chemical). The gels were dried and cut, and the pieces counted on a Beckman 310 gamma counter using a window of 0–0.17 MeV.

Results

The gel filtration column elution profiles of serum mixtures of three indium chelates at the beginning of the experiments and after 7 days of incubation under physiological conditions are shown in Figure 2. These clearly show differences in the loss of indium from nitrobenzyl-DOTA-In, nitrobenzyl-EDTA-In and nitrobenzyl-DTPA-In. The column fractions containing protein-bound indium (nos. 1–16) were compared with the fractions containing intact indium chelates (nos. 17–45) to determine the percentage of intact indium chelate as a function of time, plotted in Figure 3. Because the ^{111}In is present in trace quantities, the loss of indium from a chelate to transferrin is expected to follow pseudo-first-order kinetics. Therefore, the plots in Figure 3 should be fit by exponential functions as $\exp(-k_i t)$, where k_i is a pseudo-first-order rate constant and t is time. Rate constants corresponding to the curves shown in Figure 3 are given in Table I. Because the rates of loss of indium from any of these chelates are quite slow, the data are fit just as well by straight lines $[\exp(-k_i t) \cong 1 - k_i t + ...]$.

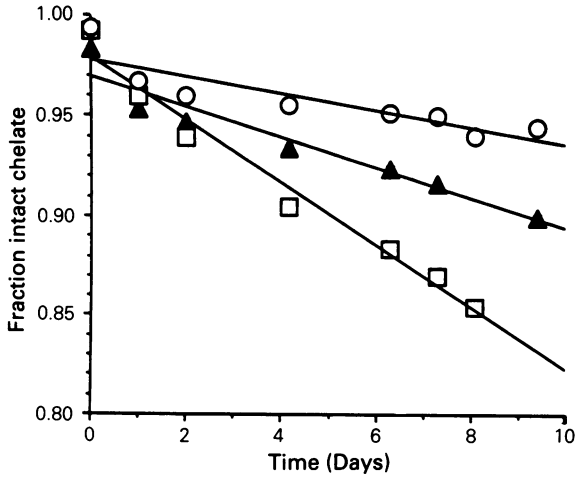

Figure 3 Percentage of intact indium chelates as a function of time of incubation in serum under physiological conditions, determined from gel filtration chromatography. Lines are least-squares exponential fits to the data; slopes and confidence limits are given in Table I: ○, nitrobenzyl-DOTA-In; ▲, nitrobenzyl-EDTA-In; □, nitrobenzyl-DTPA-In.

Table I Rates of chelate decomposition in human serum at pH 7.4, 37°C

Species	*Chelating agent concentration in serum (nM)*	*Rate constant for loss of radionuclide (day^{-1})*
Nitrobenzyl-DOTA-In	240	0.0045±0.0024
Nitrobenzyl-EDTA-In	200	0.0081±0.0026
Nitrobenzyl-DTPA-In	240	0.017±0.003
Nitrobenzyl-DOTA-Co	133	0.0016±0.0010
Nitrobenzyl-EDTA-Co	133	0.048±0.003

Errors are 95% confidence limits.

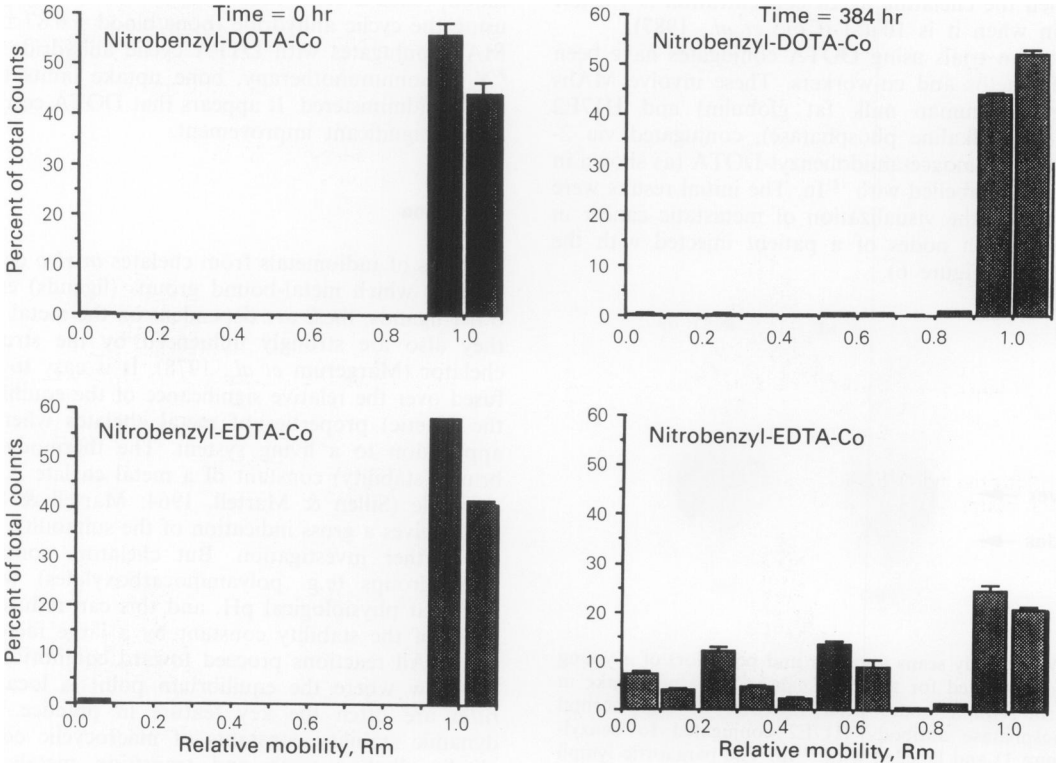

Figure 4 Non-denaturing polyacrylamide gel electrophoresis profiles of serum mixtures of two cobalt chelates at the beginning of the experiments (left) and after 16 days of incubation under physiological conditions (right). Note that cobalt lost from a chelate does not bind firmly to a single serum protein, but rather appears in many positions (lower right).

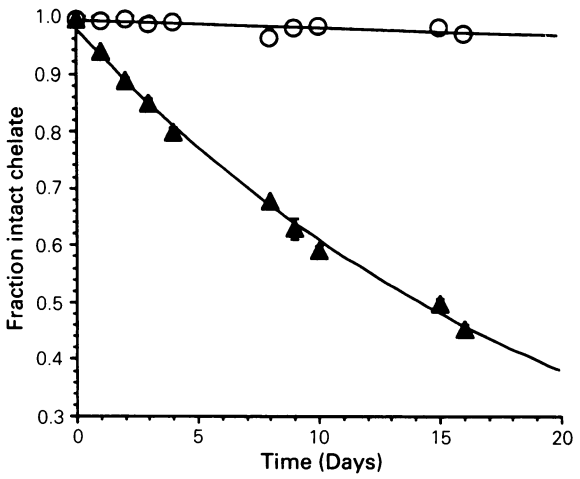

Figure 5 Percentage of intact cobalt chelates as a function of time of incubation in serum under physiological conditions, determined from non-denaturing polyacrylamide gel electrophoresis. Lines are least-squares exponential fits to the data; slopes and confidence limits are given in Table I: ○, nitrobenzyl-DOTA-Co; ▲, nitrobenzyl-EDTA-Co.

The gel electrophoresis profiles of serum mixtures of two cobalt chelates at the beginning of the experiments and after 16 days of incubation under physiological conditions are shown in Figure 4. These clearly show differences in the loss of cobalt from nitrobenzyl-DOTA-Co and nitrobenzyl-EDTA-Co. The gel slices containing protein-bound cobalt (Rm 0.0–0.9) were compared with the slices containing intact cobalt chelates (Rm 0.9–1.1) to determine the percentage of intact cobalt chelate as a function of time, plotted in Figure 5. Because the ^{57}Co is present in trace quantities, the loss of cobalt from a chelate to serum proteins is expected to follow pseudo-first-order kinetics, as for indium above. However, cobalt lost from a chelate evidently does not bind firmly to a single serum protein (see Figure 4), which makes it difficult to be sure of the value of the dissociation rate constant for cobalt chelates. Rate constants corresponding to the curves shown in Figure 5 are given in Table I. Note that the rate of loss of cobalt from a benzyl-EDTA chelate is much more significant when the chelating agent concentration is 133 nM (Table I) than when it is 10 μM (Cole *et al.*, 1987).

The first human trials using DOTA conjugates have been initiated by Epenetos and co-workers. These involve MAbs HMFG1 (against human milk fat globulin) and H17E2 (against placental alkaline phosphatase), conjugated via 2-iminothiolane to bromoacetamidobenzyl-DOTA (as shown in Figure 1) and then labelled with ^{111}In. The initial results were excellent, including the visualization of metastatic cancer in the paraaortic lymph nodes of a patient injected with the H17E2 conjugate (Figure 6).

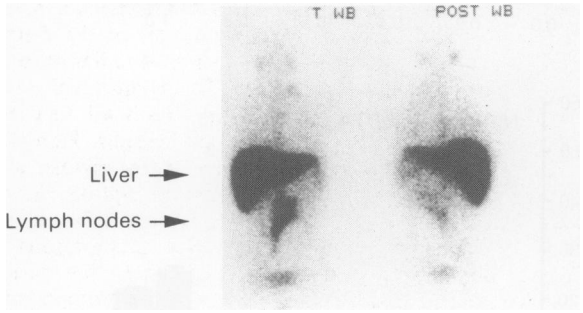

Figure 6 Whole-body scans (anterior and posterior) of a young man previously treated for testicular cancer, showing uptake in the paraaortic lymph nodes after injection of antiplacental alkaline phosphatase antibody H17E2 conjugated to benzyl-DOTA (Figure 1) and labelled with ^{111}In. The paraaortic lymph nodes lie below the liver, which shows very high uptake of the radiolabel. The lymph nodes were easily visualized 48 h after injection. The image improved over time; the scan shown was taken 7 days after injection.

Moi *et al.* (1988) compared Y(III) complexes of nitrobenzyl-DOTA with other ligands which have recently been reported to form complexes with Y(III) or In(III) appropriate for use in living systems (Hnatowich *et al.*, 1985; Vaughan *et al.*, 1985; Brechbiel *et al.*, 1986; Washburn *et al.*, 1986; Anderson-Berg *et al.*, 1987; Kozak *et al.*, 1989). At 10^{-7} M, the non-cyclic octadentate ligand 1-*p*-nitrobenzyl-DTPA loses Y(III) with a pseudo-first-order rate constant $k = 0.006$ day^{-1}. An analogue of the ligand formed by reaction of DTPA cyclic anhydride with a lysine residue, ^{88}Y-DTPA-mono-*n*-butylamide, loses Y(III) 45 times faster, having $k = 0.27$ day^{-1}. Incubation of the ^{88}Y(III) complex of nitrobenzyl-DOTA for 18 days results in loss of so little Y(III) from the complex (less than 0.5%) that the rate of loss cannot be measured under these conditions.

These results have also been confirmed using a conjugate of Y-benzyl-DOTA with MAb Lym-1. This mouse IgG_{2a} antibody binds to a membrane-bound glycoprotein which is strongly expressed on many human lymphoma and lymphocytic leukaemia cells (Epstein *et al.*, 1984). Prepared as shown in Figure 1 using 2-IminoThiolane and BromoAcetamidobenzyl-Dota, the conjugate Lym-1-2IT-BAD-^{88}Y has a rate constant for decomposition $k = 0.0003 \pm 0.0005$ day^{-1}, again showing no detectable loss of yttrium from the chelate. Five days after injection of Lym-1-2IT-BAD-^{88}Y into mice the radioactivity in bone is $2.1 \pm 0.5\%$ g^{-1}; this compares favourably with the value 2.53% g^{-1} reported by Kozak *et al.* (1989) for anti-TAC MAb conjugated to benzyl-DTPA-^{88}Y, and is far smaller than the $24.9 \pm 2.7\%$ g^{-1} that we observe in bone 5 days after injection of ^{88}Y-citrate.

Animal trials using DOTA conjugates and ^{90}Y have been carried out by Epenetos and Snook. These involve MAb HMFG1 (against human milk fat globulin), conjugated via 2-iminothiolane to bromoacetamidobenzyl-DOTA as shown in Figure 1, and then labelled with ^{90}Y. Because ^{90}Y is a pure β-emitter, it is more reliable to measure ratios of ^{90}Y in the organs to ^{90}Y in a standard organ (blood) and to compare tissue/blood concentration ratios, rather than to attempt to measure directly the fraction of ^{90}Y in tissues. Representative results are shown in Figure 7. Most important, these results show that 5 days after injection, bone uptake of ^{90}Y is ≈30 times less with the DOTA conjugate of HMFG1 (bone/blood $= 0.22 \pm 0.02$) than with a DTPA conjugate prepared using the cyclic anhydride (bone/blood $= 6.87 \pm 0.36$). When MAb conjugates with DTPA cyclic anhydride are used for ^{90}Y radioimmunotherapy, bone uptake limits the dose that can be administered. It appears that DOTA conjugates represent a significant improvement.

Discussion

The loss of radiometals from chelates *in vivo* depends on the rates at which metal-bound groups (ligands) exchange with other ligands; these are dependent on the metal involved, but they also are strongly influenced by the structure of the chelator (Margerum *et al.*, 1978). It is easy to become confused over the relative significance of the equilibrium (versus the kinetic) properties of metal chelates when the goal is application to a living system. The thermodynamic equilibrium (stability) constant of a metal chelate is often readily available (Sillen & Martell, 1964; Martell & Smith, 1974), and it gives a gross indication of the suitability of the chelate for further investigation. But chelators containing highly basic groups (e.g. polyaminocarboxylates) will bind H^+ avidly at physiological pH, and this can reduce the effective value of the stability constant by a large factor (Ringbom, 1963). All reactions proceed toward equilibrium, so it helps to know where the equilibrium point is located; however, *rates* are often the key feature in practice. The thermodynamic stability constants of macrocyclic complexes with alkali, alkaline earth and transition metals (Delgado & Frausto da Silva, 1982), and certain lanthanides (Loncin *et al.*, 1986), are remarkably high. More important, the macrocyclic structure of the ligand imposes rigidity; thus, the metal

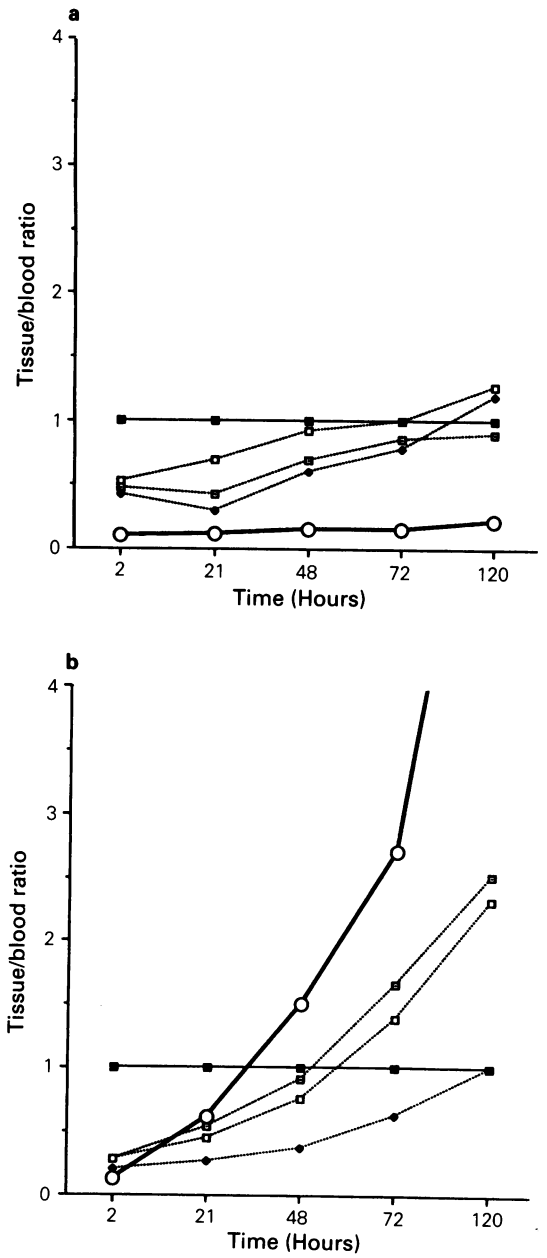

Figure 7 Tissue/blood concentration ratios of ^{90}Y in mice, after injection of monoclonal antibody HMFG1 conjugated either to: **a** DOTA, according to Figure 1, or **b** DTPA, via the cyclic anhydride method. Note that the bone/blood ratio remains very low over the course of 5 days after injection of HMFG1-DOTA-^{90}Y, but that the bone/blood ratio increases steadily after injection of HMFG1-DTPA-^{90}Y. At least three mice were used for each data point. Error bars have been omitted in the figure for clarity. At 2 h the bone/blood DOTA values were 0.10 ± 0.04 s.d. and the DTPA values were 0.13 ± 0.05 s.d.; at 120 h the bone/blood DOTA values were 0.22 ± 0.02 s.d. and the DTPA values were 6.87 ± 0.36 s.d.: ···⊡···, kidney; ···●···, spleen; ···□···, liver; ——○——, bone; ——■——, blood.

complexes of these macrocycles are expected to have high *kinetic* stability as well.

After radioactive metal chelates are injected into living systems, they are extremely dilute (e.g. injecting 10 mg of MAb into 3 l of plasma results in 2×10^{-8} M antibody). Blood contains substantial concentrations of molecules that bind metals very strongly (e.g. albumin [$\approx 10^{-3}$ M] and transferrin [$\approx 10^{-5}$ M]), and it also contains chelatable metals ($\approx 10^{-3}$ M Ca^{2+}, Mg^{2+}) that may compete for available chelating groups. Thus if a radioactive metal ion dissociates from its chelating group, it almost certainly will not return. The stability of a metal complex under physiological conditions may be assessed by dissolving the complex at high dilution in sterile human serum at 37°C, pH 7.4, and measuring the rate of transfer of the metal from the complex to serum proteins over the course of several days (Meares *et al.*, 1976).

Indium is a trivalent metal ion which is metabolized similarly to the lanthanides, yttrium(III) and iron(III). In blood the main carrier protein for these metals is transferrin. Studies of the loss of indium from metal chelates to transferrin in human serum have frequently been used to compare the suitability of chelates for use in medical applications (Yeh *et al.*, 1979). We have evaluated the loss *in vivo* of ^{111}In from Lym-1-benzyl-EDTA-^{111}In (an antibody conjugated with isothiocyanatobenzyl-EDTA) in normal mice. A monoclonal antibody (CHA 255) that binds to benzyl-EDTA-indium chelates, but not to other forms of indium, was used to measure the percentage of ^{111}In remaining in the chelate. Four days after injection, $97.4 \pm 2.2\%$ of the ^{111}In in the liver was still in the benzyl-EDTA chelate, as was $99.4 \pm 0.7\%$ of the ^{111}In in the urine, and $99.1 \pm 0.7\%$ of the ^{111}In in the blood (Deshpande *et al.*, 1990).

Also, preliminary evidence indicates that the use of benzyl-EDTA-^{111}In labelled monoclonal antibodies can lead to significantly faster clearance of indium from the human body than is observed when DTPA-^{111}In is the label (Goodwin *et al.*, 1986). In a small number of lymphoma patients imaged with the antibody Lym-1-benzyl-EDTA-^{111}In, the typical half-life observed for clearance of radioactivity from the body is a few days, much shorter than the times observed with ^{111}In antibodies labelled via the cyclic anhydride of DTPA (DeNardo *et al.*, 1986). Most of the radioactivity is excreted in the urine.

As shown in Table I, the nitrobenzyl-DOTA-^{111}In chelate decomposes slower than the nitrobenzyl-EDTA-^{111}In chelate in serum. Therefore, it is likely that DOTA-^{111}In chelates will remain intact *in vivo* as well. Also shown in Table I, our studies *in vitro* indicate that nitrobenzyl-EDTA-^{111}In is more stable in human serum than nitrobenzyl-DTPA-^{111}In. Other studies *in vitro* indicate that a benzyl-EDTA-^{111}In-antibody-chelate conjugate is more stable in human serum than a benzyl-DTPA-^{111}In conjugate in contrast to Brechbiel *et al.* (1986), and that both benzyl-chelate conjugates are much more stable than an unsubstituted DTPA conjugate (Deshpande *et al.*, 1990).

Moi *et al.* (1985) prepared a 14-membered macrocycle (nitrobenzyl-TETA) designed to stably complex ^{67}Cu for use in medical diagnosis and therapy. In the *in vitro* serum evaluation of copper chelates as potential radiopharmaceuticals, over an 8-day period Cu-nitrobenzyl-TETA decomposed with the very slow rate constant of 0.013 day^{-1}, while the rate of loss of copper from the 12-membered macrocyclic chelate, Cu-nitrobenzyl-DOTA was approximately 0.0043 day^{-1}, the lowest for any copper chelate we have studied in serum.

In fact, the macrocyclic DOTA structure binds an interesting variety of metals better than any other chelator studied so far. Because it binds both indium and yttrium with great stability, it should be possible to use DOTA chelates of the γ-emitting ^{111}In as tracers for DOTA chelates of the β-emitting ^{90}Y, permitting accurate measurements of radiation dosimetry. In addition, the stability of DOTA chelates with other metals such as cobalt and copper suggest that it will be useful in a variety of applications to biological systems. However, radiometal-labelled antibodies still have many problems to overcome: physiological barriers to tumour uptake, inconvenient clearance rates of radiometals from the body, and also the presence in antibody conjugate preparations of ineffective molecules that do not contribute to the desired results. We need to improve the delivery of the radiopharmaceutical to the tumour, as well as speed the removal of the radioactivity from normal tissues. The preparation of bifunctional chelating agents that hold metals with extreme stability under physiological conditions is an important step in that direction.

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