Preparation and preclinical evaluation of humanised A33 immunoconjugates for radioimmunotherapy

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Summary
A humanised IgG1/k version of A33 (hA33) has been constructed and expressed with yields up to 700 mg l⁻¹ in mouse myeloma N50 cells in suspension culture. The equilibrium dissociation constant of hA33 (Kₐ = 1.3 nM) was shown to be equivalent to that of the murine antibody in a cell-binding assay. hA33 labelled with yttrium-90 using the macrocyclic chelator 12N4 (DOTA) was shown to localise very effectively to human colon tumour xenografts in nude mice, with tumour levels increasing as blood concentration fell up to 144 h. A Fab' variant of hA33 with a single hinge thiol group to facilitate chemical cross-linking has also been constructed and expressed with yields of 500 mg l⁻¹. Triazolecide cross-linkers have been used to produce a trivalent Fab fragment (hA33 TFM) that binds antigen on tumour cells with greater avidity than hA33 IgG. Cross-linkers incorporating 12N4 or 9N3 macrocycles have been used to produce hA33 TFM labelled stably and site specifically with yttrium-90 or indium-111. These molecules have been used to demonstrate that hA33 TFM is cleared more rapidly than hA33 IgG from the circulation of animals but does not lead to accumulation of these metallic radionuclides in the kidney. ⁹⁹ᵐY-labelled hA33 TFM therefore appears to be the optimal form of the antibody for radioimmunotherapy of colorectal carcinoma.

Keywords: Radioimmunotherapy; antibody; yttrium; tri-Fab

Tumour localisation and therapy studies have now been performed using a considerable number of antibodies directed to tumour-associated antigens. These studies have demonstrated that uptake of radioimmunoconjugates by human solid tumours is generally very low, rarely exceeding 10% of injected dose per kilogram of tumour, and that therapeutic responses are observed only rarely. Our aim has been to develop a new generation of radioimmunoconjugates for successful treatment of solid tumours, and to this end we have attempted to optimise all aspects of the conjugate – the antibody targeting moiety, therapeutic effector and linkage between the two. We have addressed these issues in the construction and characterisation of novel radioimmunoconjugates for therapy of colorectal cancer metastases based on the antibody A33. This antibody recognises a poorly characterised antigen expressed by virtually all primary and secondary colon cancers. A33 labelled with ¹³¹I has shown impressive, selective tumour localisation in patients with hepatic metastases of colorectal carcinoma (Welt et al., 1990, 1994). A phase I/Ii study has been conducted (Welt et al., 1994) with this murine antibody, in which some tumour responses were observed at the maximum tolerated dose (75 mCi m⁻²). The major limiting toxicity was haematological, as observed in almost all therapy studies with radioimmunoconjugates. All patients treated developed a human anti-mouse antibody (HAMA) response after one administration, and this led to very rapid clearance of the conjugate upon retreatment, consistent with all previous results with rodent antibodies. These data suggest that A33 is a promising antibody for successful radioimmunotherapy of colon cancer, and the purpose of this study has been to design and develop a second generation reagent based upon it. The key to the development of successful radioimmunotherapy will be the identification of reagents capable of delivering a killing dose to tumour cells without unacceptable toxicity to normal tissues. To this end we are evaluating several alternative radioimmunotherapeutic strategies including the use of isotopes which require internalisation into the cell for cytotoxicity, such as ¹²³I (which are less toxic to normal tissues), and engineering the antibody for the optimal delivery of highly cytotoxic agents such as ⁹⁹ᵐY.

The radioisotope ⁹⁹ᵐY has been used in several radioimmunotherapy studies and is an attractive isotope for this purpose owing to its appropriate physical properties. As a pure high-energy β-emitter ⁹⁹ᵐY has advantages over the more commonly used ¹³¹I in terms of greater energy deposited and ease of patient handling. The half-life of ⁹⁹ᵐY (2.7 days) is sufficient for tumour localisation and short enough to minimise toxicity in organs involved in catabolism. Previous studies with ⁹⁹ᵐY have been limited by the use of poor acrylic chelators such as DTPA which allows leakage of ⁹⁹ᵐY from the chelator under physiological conditions with subsequently increased bone marrow toxicity (Hnatowich et al., 1988; Larson, 1991). Attempts to circumvent this problem by the co-administration of free EDTA to chelate free ⁹⁹ᵐY released have also been made but showed only a very limited improvement (Stewart et al., 1990). Stable macrocyclic chelators for ⁹⁹ᵐY have now been developed based on the macrocycle 12N4 (also known as DOTA), which essentially completely prevents loss of the isotope from the conjugate under physiological conditions (Deshpande et al., 1990; Harrison et al., 1991; DeNardo et al., 1994).

We have attempted to optimise the antibody part of the conjugate in two ways: by humanisation to overcome the HAMA response and by using a trivalent Fab fragment of the antibody, which has pharmacokinetic properties more suitable than those of whole antibody for delivering ⁹⁹ᵐY. It is now possible to replace most of the rodent-derived sequences of an antibody with sequences derived from human immunoglobulins without loss of antigen-binding activity. The first generation of humanised antibodies involved the fusion of the variable domains of the mouse antibody to human immunoglobulin constant regions to produce chimeric antibodies. Several such antibodies with specificity for tumour antigens have been administered to patients (LoBuglio et al., 1989; Baker et al., 1991; Saleh et al., 1992). In general an immune response still develops against these

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chimeric antibodies in a large proportion of patients, although the level of the response is usually lower than that seen with the parent mouse antibody and is directed against the variable regions. The reduced HAMA response to chimeric antibodies leads to the expectation that more extensive humanisation, i.e. humanisation of the variable region outside the antigen-binding site as well as the constant domain may further diminish the immune response, and preliminary data with the first few antibodies fully humanised in this manner are consistent with this view (Caron et al., 1992; Isaacs et al., 1992; Stephens et al., 1994). Full humanisation involves redesigning the variable domains so that the amino acids contributing to the antigen-binding site of the mouse antibody are integrated into the framework of a human antibody variable region. Several strategies have been employed for full antibody humanisation (reviewed in Mountain and Adair, 1992).

We have recently described the evaluation, in a nude mouse xenograft system, of chemically cross-linked antibody fragments for radioimmunotherapy when labelled with \(^{99}\text{Y}\) via the 12N4 macrocycle (King et al., 1994). In this study we demonstrated the potential targeting advantages of a tri-Fab fragment termed TFM. In this paper we describe the construction, expression and tumour cell properties of humanised variants of the antibody A33, together with studies on their biodistribution and pharmacokinetics in animals when labelled with \(^{99}\text{Y}\) or \(^{111}\text{In}\). Humanised A33 TFM labelled with \(^{99}\text{Y}\) is a promising reagent for therapy of colorectal carcinoma.

Materials and methods

Cloning and expression of genes for A33, humanised A33 IgG1 and humanised A33 Fab' 

A33 hybridoma cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and 1 mM glutamine. Total RNA was prepared from 10^6 hybridoma cells using guanidinium isothiocyanate and poly(A)\(^+\) mRNA isolated from this by oligo (dT) affinity chromatography. First strand cDNA was synthesised from 10 mg of mRNA using the Amersham International cDNA synthesis kit. DNA sequences encoding A33 variable domains (including signal sequences for secretion) were amplified from the cDNA using the PCR procedure described by Jones and Bendig (1991) but with primers designed to allow facile cloning of the PCR products for expression in mammalian cells. These vectors were derived initially from pEE6 (Stephens and Cockett, 1989). Figure 1 shows final NSO expression vectors. PCR-amplified fragments for the light variable domain were cloned between the BsrI and SpeI sites of PMR010, a pEE6 derivative constructed to allow expression of such sequences with kappa chimeric light chains. PCR-amplified fragments for the A33 heavy variable domain were cloned between the HindIII and Apal sites of PMRR011, a pEE6 derivative constructed to allow expression of such sequences as \(\gamma\)-1 chimeric heavy chains. The cloned variable region genes were sequenced by the double-strand dideoxy chain terminating method using T7 DNA polymerase (Sequenase version 2.0, United States Biochemical, Cleveland, OH, USA).

The humanised variable domains were assembled by the procedure of Daugherty et al. (1991), using primers which allowed facile cloning into pMMR010 and pMRR011 for the light and heavy chains respectively. These humanised variable region genes were sequenced by the same procedures used for the murine variable region genes. An expression vector for the hA33 Fab'\(^{\alpha}\)cs heavy chain with a single hinge thiol was constructed by replacing the \(\gamma\)-1 constant domains with the appropriate segments from the cB72.3 Fab'\(^{\alpha}\)cs gene described by King et al. (1994).

Transient co-expression of murine IgG heavy and light chains, or humanised IgG and Fab'\(^{\alpha}\) heavy and light chains were achieved by co-transfection of the separate expression vectors into CHO-L761h cells as described previously (Cockett et al., 1990). For stable cell line development the heavy and light chain expression units were combined in a single plasmid. This was accomplished by replacing the NorI–BamH1 stuffer fragment in the light chain expression plasmids with the NorI–BamH1 fragments carrying the hCMV promoter/enhancer and heavy chain genes from the heavy chain expression plasmids. The final expression plasmids were termed pAL71 and pAL72 for hA33 IgG1 and Fab'\(^{\alpha}\)cs respectively (Figure 1). Stable NSO cell lines for the production of hIgG1 and hFab'\(^{\alpha}\)cs were then established by transfecting these plasmids according to the procedure of Bebbington et al. (1992). After transfection the cells were plated at 2 \times 10^9 cells per 96-well plate in Dulbecco's modified Eagle medium containing 10% dialysed fetal calf serum and 2 mM glutamine. After 24 h cells were selected by the addition of methionine sulfoximine to the medium at a final concentration of 7 \(\mu\)M. After 21 days of culture, resistant colonies were picked and expanded for analysis of productivity. The highest producers were selected for production of recombinant antibody in roller bottle culture.

Preparation of humanised A33 IgG, Fab'\(^{\alpha}\), DFM and TFM

hIgG was purified from tissue culture supernatants of NS0 cells using protein A-Sepharose affinity chromatography and characterised by SDS–PAGE as previously described (King et al., 1992). hA33 Fab'\(^{\alpha}\)cs was purified from cell culture supernatant by chromatography on protein A-Sepharose using the low affinity protein A binding site on Fab'\(^{\alpha}\) as a basis for purification. A column of protein A-Sepharose was

![Figure 1 Vectors for the expression of hA33 IgG1 and hA33 Fab'\(^{\alpha}\)cs in NS0 cells.](image-url)
equilibrated with 100 mM borate buffer pH 8.0 containing 150 mM sodium chloride. The tissue culture supernatant from NSO cells expressing hA33 Fab'4cys was adjusted to pH 8.0 by the addition of 1 M Tris and applied to the column. Washing the equilibration buffer the Fab' was eluted with 0.1 M citric acid, collecting fractions directly into sufficient 1 M Tris to immediately adjust the pH of the fraction to between 6 and 7.

After dialysis and ultrafiltration hA33 Fab'4cys was cross-linked to DFM and TFM using the maleimide-based homobifunctional and homotrifunctional linkers CT25 and CT998 respectively, as described for CB72.3 Fab'4cys (King et al., 1994). These cross-linkers contain the 12N4 macrocycle for incorporation of 9Y. The DFM and TFM produced were purified by gel filtration using a Sephacryl S-200HR column (Pharmacia). Radiolabelling with 9Y was performed as described previously (King et al., 1994).

Antigen binding assays

These were performed using cells of two human colorectal tumour cell lines which express the A33 antigen, Colo205 and SW1222. These cells were cultured in DMEM containing 2 mM glutamine and 10% fetal calf serum (Gibco). Assembled antibody in culture supernatants and in purified preparations was quantitated in an ELISA (Whittle et al., 1987). Direct binding of murine and humanised antibodies to SW1222 cells was measured in a FACSscan assay. SW1222 cells were resuspended to remove them from culture flasks, washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 10% bovine serum and 0.1% sodium azide. Humanised A33 antibody was serially diluted in PBS containing 10% FCS and 0.1% sodium azide and added to 2 x 10^5 cells. Following a 1 h incubation on ice the cells were washed in PBS and then incubated with a rhodamine anti-(human Fc) conjugate (1:1000 in PBS, 10% FCS and 0.1% sodium azide) for a further 1 h on ice. After washing in PBS, the amount of rhodamine-labelled A33 antibody conjugate bound to the cells was measured in a FACSscan analyser (Becton-Dickinson). Direct binding of murine A33 was measured by FACSscan analysis after incubation of SW1222 cells with FITC-labelled antibody. Suitable non-specific antibody controls were carried out to demonstrate that A33 binds specifically via antibody–antigen interaction rather than non-specifically through Fc interactions.

Determination of affinities of murine and humanised antibody was based on the procedure described by Krause et al. (1990). Briefly, antibodies were labelled with fluorescein using fluorescein isothiocyanate (FITC) titrated from 1.3 mg ml^-1, then incubated with 2.8 x 10^5 SW1222 cells for 2 h on ice in 350 ml PBS containing 5% FCS and 0.1% sodium azide. The amount of fluorescence bound per cell was determined in a FACSscan and calibrated using standard beads (Flow Cytometry Standards Corporation). The number of molecules of antibody that had bound per cell at each antibody concentration was thus established and used to generate Scatchard plots.

Competition assays were performed by FACSscan quantitation of bound FITC labelled murine A33 after incubating Colo205 cells with a standard quantity of the murine antibody together with a dilution series of the humanised variants.

Radiolabelling and animal studies

Antibodies were labelled with 9Y via the macrocyclic ligand tetra-azacyclododecane tetra-acetic acid, (termed 12N4 or DOTA) coupled to the immunoglobulin via 12N4-maleimide linkers (Harrison et al., 1991) as previously described (King et al., 1994). Radiolabelling of hA33-12N4 conjugates with 9Y and 125I, and biodistribution studies in nude mice bearing subcutaneous SW1222 tumour xenographs were carried out also as described (King et al., 1994). Antibodies were labelled with 111In via a second macrocyclic ligand, 1,4,7-triazacyclononane triactic acid or 9N3 using 9N3-maleimide linkers as described previously (Turner et al., 1994).

Biodistribution studies in guinea pigs were carried out after i.v. administration to male outbred Dunkin-Hartley guinea pigs (Interfona, Huntingdon, UK) of approximately 250–300 g. Groups of four guinea pigs were injected with each 9Y-labelled component into the ear vein and sacrificed post administration at the time intervals indicated. Blood samples were taken and tissues processed as previously described for mice (King et al., 1994). Pharmacokinetic studies in cynomolgus monkeys of 5–7 kg (two per group) were carried out after i.v. injection of radiolabelled components. Blood samples were taken at 0.5, 1, 2, 4, 6, 8, 24, 48, 72, 96, 120, 144 and 168 h for counting.

Dose calculations

An exponential, non-linear least-squares fitting procedure was used to determine the blood clearance parameters for each monkey. Mean values of the blood clearance parameters for IgG, TFM and DFM were then used to set up appropriate integrals to calculate the per cent of absorbed dose as a function of time post administration. Estimates were then made as to the absorbed dose for red marrow that would be delivered by each form of the antibody (IgG, TFM and DFM) in humans when labelled with 9Y. The monkey data showed that at early times all of the administered activity was in the blood circulation and this was taken to be the case for humans. Calculations were made based on the assumptions that the pharmacokinetics of 111In- and 9Y-labelled antibodies are the same as each other and the same in monkeys and humans, also that there is no specific uptake of radiolabelled antibodies in the marrow so that the radioactivity in the blood and marrow are the same after a few (<5) h. To generate numbers representative of humans the following data for standard man were used: a total blood volume of 5000 ml, marrow spaces, absorbed fractions for 9Y β-particles and the thickness of the endosteal layer were taken from Whitwell and Spiers (1976). Owing to the high energy of the 9Y β-particles, the radiation absorbed doses in the marrow and the endosteal layer are for all practical purposes the same.

Results

Cloning of A33 variable region genes

DNA sequences encoding the light and heavy chain variable domains were amplified by PCR from cDNA prepared from mRNA isolated from the A33 hybridoma and cloned into vectors allowing expression as a chimeric IgG1. Direct binding assays were performed on culture supernatants following transient co-expression of the heavy and light chains. The antigen recognised by A33 is poorly characterised and has not been isolated, so all binding assays used a human colorectal tumour cell line. The results of these binding assays showed that the chimeric antibody bound to cells expressing the antigen as well as murine A33 (data not shown), confirming that the cloned genes correspond to those of A33. Figure 2 shows the amino acid sequences of the heavy and light chains deduced from the DNA sequence of the cloned variable domain genes. N-terminal protein sequencing of the first 11 amino acids gave the expected sequence, in accordance with these deduced amino acid sequences for both heavy and light chains, confirming the appropriate genes had been cloned.

Humanisation of A33

The γ-1 isotype was chosen for the humanised heavy chain because this isotype best matched the murine γ-2a of the parent antibody. Antibodies with either human γ-1 or murine γ-2a heavy chains are able to fix complement (CDC) and mediate cellular cytotoxicity (ADCC) via interaction with FcR1 on phagocytic mononuclear cells (Burton and Woof, 1992).
The murine variable regions of A33 were humanised according to the strategy described by Adair et al. (1991). This strategy involves using as frameworks heavy and light chains with the greatest overall homology to the murine antibody, and transferring into these frameworks all the residues from the murine antibody predicted to be involved in antigen binding. The $V_\text{H}$ of A33 shows closest homology (70%) to the consensus sequence of human subgroup $V_\text{H}$III, while the $V_\text{L}$ shows greatest homology to the consensus sequence of human subgroups $V_\text{L}$I and $V_\text{L}$IV (62%). From these subgroups LAY, which has a $V_\text{L}$III heavy chain and $V_\text{L}$II light chain, was chosen as the human framework. Figure 2 shows the amino acid sequences of the humanised light and heavy chains. For the light chain residues 1–23, 35–45, 47–49, 57–86, 88 and 98–108 inclusive were derived from the LAY sequence, (numbering as in Kabat et al., 1987) and the residues 24–34, 46, 50–56, 87 and 89–97 inclusive were derived from the murine sequence. Residues 24–34, 50–56 and 89–97 correspond to the complementarity determining regions (CDRs, Kabat et al., 1987). Residues 46 and 87 are predicted to be at the interface of the light and heavy variable regions. Residue 46 is usually a leucine in human antibody sequences and residue 87 is usually either a phenylalanine or tyrosine.

For the heavy chain, residues 2–26, 36–49, 66–71, 74–82a, 82c–85, 87–93 and 103–113 inclusive were derived from the LAY sequence while residues 1, 27–35, 50–65, 72, 73, 82b, 86 and 94–102 inclusive were derived from the murine sequence. Residues 31–35, 50–65 and 95–102 in the heavy chain correspond to the CDRs. The murine-derived amino acids in the framework regions were included for the following reasons. Residue 1 is usually solvent accessible and in the vicinity of the CDR region (residues 27–30) LAY has a residue, alanine, not normally found at this position in human or murine $V_\text{H}$ sequences and therefore the murine residue was used. At positions 72 and 73 the murine residue was used because of the predicted proximity to CDR2 and also, in the case of residue 72, to remove the possibility of introducing an N-linked glycosylation site into the variable domain by the use of the LAY framework (see also Co et al., 1991). The murine sequence was also used at the interdomain residue 94, where A33 has a proline, not normally found at this position. Murine residues were used at positions 82 and 86 because of the chance of the human amino acids at these positions in a humanised antibody with LAY frameworks has previously been found to be deleterious for the expression of the heavy chain (Adair et al., 1992).

Preparation and in vitro characterisation of hA33 IgG1 and TFM

Small amounts of the humanised antibody were produced in a transient expression system in CHO cells to establish that it bound SW1222 cells expressing the antigen. Stable NSO cell lines were then isolated to produce larger quantities of purified material, for both hA33 IgG and Fab'Acys. The best cell lines produced approximately 700 mg 1^{-1} hA33IgG1 and 500 mg 1^{-1} Fab'Acys in suspension culture.

Figure 3 shows SDS–PAGE analysis of the purified antibodies under non-reducing conditions. It demonstrates that the purified hIgG was homogeneous and fully assembled. High-performance liquid chromatography (HPLC) analysis also demonstrated it was free of aggregates (data not shown). As expected the hFab'Acys was recovered largely in the form of monovalent Fab' with little in the form of F(ab')2, consistent with results for other recombinant Fab' fragments (King et al., 1992, 1994). Cross-linking of Fab'Acys to TFM was achieved with a yield of 60–65%, as shown by HPLC analysis in Figure 4. SDS–PAGE analysis of the purified TFM (Figure 3) showed a single species of approximately 150 kDa under non-reducing conditions.

Figure 5 shows Scatchard analysis for the murine antibody and hlgG binding to SW1222 cells. These data suggest both antibody forms have equilibrium dissociation constants ($K_d$s) of 1.3 nM and have approximately 300 000 sites per cell. The antigen-binding activity of hTFM was compared with those of monovalent hFab'Acys and hlgG in competition binding assays in which these species were asked to compete with murine IgG for binding to Colo205 cells expressing the antigen. The results (Figure 6) demonstrate that the monovalent Fab' fragment binds less well than the bivalent IgG as expected. The trivalent hTFM, on the other hand, showed approximately 2-fold better binding than hlgG, presumably as a result of increased avidity due to the extra antigen binding site. This finding is consistent with results for chimeric B72.3, for which TFM also showed 2- to 3-fold better binding to antigen than IgG (King et al., 1994).
Biodistribution and pharmacokinetics of hA33 IgG1, DFM and TFM

Immunonojugates of hIgG were prepared for \(^{99m}Tc\) labelling by derivatisation with the 12N4-maleimide linker CT77. An average macrocycle loading of 1.2 per molecule was achieved, and the immunonojugate was shown to be fully immunoreactive using the competition-based FACS assay both before and after radiolabelling (data not shown). For biodistribution experiments, radiolabelling was achieved to a sp. act. of 2\(\mu\)Ci \(\mu\)g\(^{-1}\) with >95% incorporation of \(^{99m}Tc\).

Figure 7 shows the biodistribution of \(^{99m}Tc\)-labelled hA33 IgG at 3, 24, 48 and 144 h in mice bearing subcutaneous SW1222 xenografts, with tissue uptake plotted as per cent injected dose per gram of tissue. In general, a favourable biodistribution was achieved, with high levels of activity localised to the tumour and little or no accumulation in any normal tissue. The biodistribution of the humanised A33 immunoconjugate was not significantly different from that of the murine antibody in the same xenograft system at these time points (Antoniw et al., manuscript in preparation). For both the murine and humanised antibodies the level of activity localised to the tumour increased with time, even though levels in all other tissues were falling, which led to increasing tumour to normal tissue ratios over time (Figure 8). To assess whether this was a feature of the antibody itself or the radioisotope used, a biodistribution experiment was also carried out with humanised A33 labelled with \(^{125}I\). In this experiment the absolute levels of isotope retained by the tumour were slightly lower but the tumour to blood ratios were very similar, suggesting that the increasing localisation is a property of the A33 antibody rather than the nature of the isotope/chelator system. The lower absolute levels of \(^{125}I\) labelled hA33 localised to the tumour are probably the result.
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were killed at each time point and the amount of activity was determined in tumour and normal tissues. Data are expressed as the mean value with error bars denoting standard deviation of the mean (n = 4). The value for muscle with 9Y-labelled hA33 at 144 h is 127 ± 38. , 3 h; , 24 h; , 48 h; , 168 h.

of dehalogenation of the radiiodinated antibody (Brown et al., 1987). A series of experiments was performed to compare the biodistribution and pharmacokinetics of hA33 IgG, DFM and TFM. It was consistently observed that humanised TFM and all other humanised fragments examined cleared aberrantly quickly from the circulation of mice, far more quickly than the equivalent murine fragments. This phenomenon was shown to be specific to mice and did not occur in rats, guinea pigs or monkeys (data not shown). Figure 9 shows a comparison of biodistribution for hA33 IgG, DFM and TFM in guinea pigs. It demonstrates the more rapid blood clearance of the DFM and TFM, with blood activities falling to 0.01 and 0.02% i.d. g⁻¹ respectively at the 144 h time point. Blood activity for hIgG was much higher, at 0.4% i.d. g⁻¹, at this time point. Figure 9 demonstrates very clearly that for DFM much higher levels of radioactivity are taken up by the kidney than for IgG and TFM. This high activity for the DFM clears much more slowly from the kidney than from the blood. At early time points kidney levels were a little higher for TFM than IgG but much lower than for DFM, and the activity cleared much faster from the kidney for TFM than for DFM. These results for A33 are consistent with the view that the kidney is the major organ of clearance for TFM.

The pharmacokinetics of hA33 IgG, TFM and DFM were compared in cynomolgus monkeys. Owing to safety considerations, these components were labelled with 111In rather than 9Y, previous work having suggested that 9Y-labelled IgG and fragments show pharmacokinetics and biodistribution very similar to those labelled with 111In (data not shown). The plasma clearance profiles are shown in Figure 10 with the alpha and beta phase half-life values in Table I. As expected from pharmacokinetic data in mice and guinea pigs both TFM and DFM cleared faster from the circulation than IgG. In addition, DFM cleared more quickly than TFM. When plasma clearance was examined without error correction for the isotope (Table II) the data was most consistent with monophasic kinetics for IgG and TFM, with biphasic kinetics for DFM. Dosimetric calculations based on these data suggest that when labelled with 9Y at equivalent amounts of radioactivity injected, the DFM would give approximately a 5-fold lower absorbed dose to the bone marrow than IgG, while TFM would give a 2-fold lower absorbed dose (Table II).

Discussion
In general, the antibodies directed to tumour-associated antigens that are currently available have been produced from rodent hybridomas and are immunogenic in man. This has been a major obstacle to radioimmunotherapy, although the first doses of some immuneconjugates have given promising biodistribution and partial therapeutic responses, subsequent doses have been rendered ineffective by rapid clearance due to the HAMA response elicited (Welt et al., 1990; Mountain and Adair, 1992).

We constructed the humanised variant of A33 by substituting into the frameworks of the human antibody LAY all the residues of the murine antibody, which we predict may contribute to antigen binding. These residues comprise the CDRs together with two residues on the light chain and five on the heavy chain which may contribute to the precise positioning of the CDRs for antigen binding (Adair et al., 1991). Scatchard analysis suggested the murine and humanised variants of the antibody have equivalent affinity for the antigen on the surface of colorectal tumour cells.

As yet there are still few data concerning the effectiveness of full humanisation in overcoming the patient immune response to rodent antibodies. In three reports on the immunogenicity of humanised antibodies in monkeys (Hakimi et al., 1991; Singer et al., 1993; Stephens et al., 1994) the immune response was much reduced compared with the parent murine antibodies. In each case, an anti-idiotype response (i.e. directed to the CDRs) developed on repeat dosing. In the case studied in greatest detail (Stephens et al., 1994) the CDRs appear less immunogenic when presented in the human framework since the anti-idiotype response to the humanised antibody was greatly reduced compared with the anti-idiotype component of the immune response to the murine antibody. As yet there are only three reports on the administration of fully humanised antibodies to patients. Two of these studies concerned the humanised antibody CAMPATH-1H. Two non-Hodgkin lymphoma patients treated with 1–20 mg doses of this antibody for up to 10 days showed no anti-CAMPATH-1H response during the course of treatment (Hale et al., 1988). These results should not be over-interpreted, however, because such patients are somewhat immunocompromised before treatment and because the treatment itself is likely to be immunosuppressive. More recently, however, results have become available for eight rheumatoid arthritis patients repeatedly administered with 8–15 mg doses of this antibody for 3 to 43 days (Bis et al., 1992). Significant clinical benefit was seen in seven of the patients, and anti-CAMPATH-1H antibodies were not detectable in any of the patients after this one course of treatment. Of four patients given a second course of treatment, three showed a detectable anti-CAMPATH-1H response. No data are yet available concerning the nature of this response, whether it interferes with efficacy, and if so, whether such interference can be overcome using larger doses. The immune response to the humanised anti-tumour
necrosis factor antibody CDP571 has been examined after administration of single doses of 0.1–10 mg kg⁻¹ to human volunteers (Stephens et al., 1994). Administration of the lower doses led to the development of a weak anti-idiotypic response, predominantly of the IgM isotype. Anti-CDP571 antibodies were very low or undetectable after administration of the higher doses. The limited clinical data available for fully humanised antibodies therefore suggest they will show substantially longer half-lives and greatly reduced immunogenicity compared with murine antibodies.

As described previously (Harrison et al., 1991) the 12N4 macrocycle gives extremely stable chelation of ⁹⁰Y, with no significant escape of the isotope in vivo. The potential immunogenicity of the macrocycle, however, as well as that of the humanised antibody carrier is a significant issue. It is far from clear whether macrocyclic chelators are likely to be immunogenic in patients. Kosmas et al. (1992) reported the rapid development of an anti-macrocycle immune response in ovarian cancer patients administered with antibody conjugates carrying ⁹⁰Y or ¹³¹I in the macrocycle p-nitrobenzyl-DOTA. Curiously, administration conditions favouring immunogenicity (i.p. injection of relatively large doses with a high proportion of aggregates) led to a stronger immune response, leading to substantially lower antibody titres.

**Table I** Mean plasma clearance half-life values for ¹¹¹In-labelled hA33 IgG, TFM and DFM in groups of two cynomolgus monkeys (decay corrected)

| Antibody form | t½(h) | t½(h) |
|---------------|-------|-------|
| IgG           | 15.8  | 129.0 |
| TFM           | 12.8  | 53.7  |
| DFM           | 10.8  | 42.0  |

Values were obtained using a two-compartment model (SIPHAR).

**Table II** Pharmacokinetics of ¹¹¹In-labelled hA33 IgG, TFM and DFM in cynomolgus monkeys

| Antibody form | Effective half-life (h) | Absorbed dose to red marrow and endostem (rad mCi⁻¹) |
|---------------|-------------------------|--------------------------------------------------|
| IgG           | 40.5                    | 12.0                                             |
| TFM           | 21.9                    | 6.3                                              |
| DFM           | 4.7±23.8β               | 2.6                                              |

Values for effective half-life and estimates of absorbed dose in red marrow and endostem in man for ⁹⁰Y-labelled antibodies were determined as described in Materials and methods.
response to the macrocycle than to the antibody carrier, and some of these patients manifested symptoms of serum sickness.

More recently Watanabe et al. (1994) have examined the immunogenicity of a similar chelator in rabbits, and concluded that an anti-macrocycle response develops in animals injected with the macrocycle conjugated to immunogenic carrier proteins such as murine antibodies, but not in those injected with rabbit antibody conjugates. Studies by Kosmas et al. (1992) to characterise the anti-macrocycle response in patients concluded that it is predominantly directed to the DOTA ring structure itself rather than to the linker, which contains an aromatic ring. We have conducted a study to examine the immunogenicity of the 12N4 macrocycle in mice. The only major difference between the chelator used in these studies and that used by Kosmas et al. (1992) is the presence of the aromatic ring on the latter. The results (TS Baker et al., manuscript in preparation) were similar to those of Watanabe et al. (1994), in that no anti-macrocycle response could be detected in mice injected with the macrocycle conjugated to a mouse antibody even using conditions very favourable to effective immunisation. Mice injected with the macrocycle conjugated to an immunogenic protein such as a chimeric antibody developed an immune response with components directed to both the carrier protein and the macrocycle. We conclude it is unlikely that immunogenicity of the 12N4 macrocycle will prove to limit the effectiveness of humanised antibodies carrying 9Y administered at reasonable doses intravenously.

Uptake of the 9Y-labelled humanised antibody by human colorectal xenografts in nude mice was similar to that reported for murine A33 labelled with 111In via the same methodology (P Antoni et al., manuscript in preparation). This high tumour uptake augurs well for the prospects of achieving therapeutically effective radiation doses in colorectal cancer patients. 9Y continued to accumulate in the tumour for the humanised antibody throughout the 144 h after injection over which measurements were made. This continued accumulation was also observed for hA33 with both of the other colorectal tumour cell lines studied (P Antoni et al., unpublished). hA33 labelled with 125I shows less accumulation, and the increased retention of 9Y by the tumour cells may result from the inability of the macrocyclic chelator to egress from the cells after internalisation.

Clinical studies with chimeric and humanised antibodies have also suggested that these antibodies have a substantially longer circulating half-life than murine antibodies. Humanised antibodies for radioimmunotherapy may therefore deliver an increased dose to the bone marrow from the circulating conjugate, which would give decreased therapeutic ratios unless tumour uptake is correspondingly increased. The production of TFM is an attempt to circumvent this problem. The binding avidity of TFM is increased compared with IgG and pharmacokinetic studies show faster blood clearance in both guinea pigs and cynomolgus monkeys. The mean beta-phase half-life of the IgG1 in cynomolgus monkeys (129 h) is in the range previously observed for humanised antibodies in this species (Hakimi et al., 1991; Singer et al., 1993; Stephens et al., 1994). The mean beta-phase half-life for TFM in cynomolgus monkeys (53.7 h) is less than half that of IgG, and dosimetric calculations indicate that for an equivalent injected dose of 9Y this shorter half-life would give an approximately 2-fold benefit for TFM in terms of the radiation dose to the bone marrow. Since the latter is usually the dose-limiting toxicity in clinical studies with therapeutic radioimmunoconjugates, it is likely TFM would allow a higher maximum tolerated dose.

Unlike all other antibody fragments carrying metallic isotopes which have been examined (see for example Sharkey et al., 1990) the TFM does not lead to high-level accumulation of the isotope in the kidney or other non-specific tissues. In the present studies DFM showed faster blood clearance and projected bone marrow doses lower than those for both IgG and TFM, but is clearly not an acceptable vehicle for delivering 9Y because it leads to unacceptably high accumulation of this isotope in the kidney. Such kidney accumulation does not occur for DFM labelled with radiodiode (data not shown), and it is likely that the DFM is the most appropriate delivery vehicle for therapy with 111In.

Clinical success with radioimmunotherapy has so far largely been restricted to haematological malignancies such as lymphomas and leukaemias. hA33 TFM carrying 9Y in the 12N4 macrocycle represents one of the first attempts to optimise all components of a radioimmunoconjugate for treatment of solid tumours. Evaluation of this second generation immunonoconjugate in colorectal cancer patients may reveal new potential for radioimmunotherapy of solid tumours. As the first stage in clinical evaluation of this technology, a quantitative biodistribution study in colorectal cancer patients using hA33 TFM carrying 111In is in progress.

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