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13. ABSTRACT (Maximum 200 Words)

In Antibody-Directed Enzyme-Prodrug Therapy (ADEPT), antibody - enzyme constructs localize to tumor tissue the toxification of non-toxic prodrugs. Recombinant fusion proteins are expected to overcome the limitations of chemical conjugates.

We have constructed fusion proteins of antibodies against the tumor antigens A33, Lewis-Y and fibroblast associated protein (FAP) with cytosine deaminase (CD), which converts 5-fluorocytosine (5-FC) into cytotoxic 5-fluorouracil (5-FU). After initial inefficient expression in an E. coli system, the intended use of a Pichia pastoris system made necessary the replacement of E.coli CD with the yeast isoenzyme. In addition, a second line of fusion constructs with green fluorescent protein instead of cytosine deaminase was designed for histological and intracellular distribution studies. With the A33 antibody construct as a pilot project, the desired fusion proteins were cloned, expressed in E. coli and purified. In vitro function assays confirmed bispecific activity of the fusion proteins.

Based on these results, experimental mice tumor models were established based on antigen-positive cell lines, and tumor targeting and biodistribution experiments were completed again with A33 as the pilot system. These experiments showed good tumor localization and favorable biodistribution, but little anti-tumor effect, which may be due to particular conditions of this pilot system. Encouraged by these results, additional animal experiments are currently underway.

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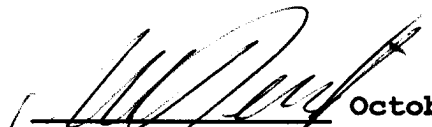

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October 27, 2003
Date

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Prodrug Therapy for Breast Cancer Targeted by Single-Chain Antibodies F19 and 3S193

Introduction

Antibody-directed enzyme-prodrug therapy (ADEPT) employs antibody-enzyme constructs to achieve tumor-targeted activation of nontoxic prodrugs. In this feasibility study, recombinant fusion proteins of single-chain antibody fragments (scFv) and prodrug-activating enzymes such as cytosine deaminase (CD; prodrug – drug, 5-fluorocytosine – 5-fluorouracil), thymidine kinase (TK; prodrug, ganciclovir), and carboxypeptidase A (CPA; prodrug, methotrexate-phenylalanine) are being constructed and evaluated based on the antibodies A33 as a pilot project and F19 and 3S193 as potential breast cancer targeting molecules.

In investigating the general feasibility of the expression and application of scFv-based recombinant fusion constructs, this project also sought to address some of the general limitations of ADEPT, i.e. poor diffusion of macromolecules into tumor tissue, leading to low tumor-to-blood ratios, and immungenicity of the administered protein constructs, limiting the number of possible treatment cycles.

Therefore, the experimental program included the whole span from designing and developing suitable scFv-enzyme constructs and their expression and purification to the reduction of immunogenicity and the in-vivo testing of the ADEPT system.

Considerable deviations from the original time line were incurred for two reasons. First, bacterial expression systems as employed for the A33 scFv alone proved inefficient for the production of the described fusion proteins. Thus, the whole expression system was moved to the eukaryotic Pichia pastoris system. This, in turn, required a change of the cytosine deminase (CD) component of the fusion protein from the bacterial to the yeast isoenzyme.

In addition, the principal investigator moved from New York to Berlin in 2000. While this provided an excellent career opportunity in accordance with the educational aims of this project, it lead to a temporary interruption in funding. Due to the generous cooperation of the USAMRAA, however, the unused funds could be transferred in 2001, and it has been possible to advance this project to meet most of the specific aims with a revised time line.

Report Body

Task 1: Expression of recombinant fusion proteins. A recombinant single chain antibody of humanized A33 was available from Ch. Rader and C. Barbas III, Scripps Research Institute, La Jolla, Ca. By PCR-cloning, suitable restriction sites were introduced for the insertion into the multilinker-site of the pET bacterial expression vector, and later into the pPIC9-K Pichia pastoris expression vector. Due to this procedural advantage, A33 was used throughout the project as a pilot sample for testing the feasibility of the expression and purification systems. In parallel, scFv-constructs were designed from the cDNAs of the humanized 3S193 and F19 antibodies and PCR-cloned in frame into the pET expression vector in collaboration with Ch. Renner, University Medical Center, Homburg, Germany. These constructs were equipped with the same flanking restriction sites as the A33scFv, so that they could be exchanged for each other in subsequent fusion constructs (see figure 1).

Three effector fusion partners were cloned into these vectors downstream of the scFv: green fluorescent protein (GFP) as a marker for histological and intracellular distribution studies, and cytosine deaminase (CD) and thymidine kinase (TK) as prodrug-activating enzymes.

After testing several expression systems, functional scFv and fusion proteins were produced with the pET expression system based on a T7 promotor-restricted bacterial vector (Novagen, Madison, WI). The fusion proteins, however, were almost exclusively retrieved as inclusion bodies, requiring a separate refolding procedure. Several refolding strategies had been tested, which lead to sufficient retrieval of refolded fusion protein for functional in-vitro-assays. Still, the low overall yield of these systems rendered them inefficient for the production of material for animal experiments.

As eucaryotic expression systems would facilitate correct folding of the protein and allow the sequestered expression and secretion by specialized cellular compartments, the Pichia pastoris system was selected for larger-scale production. Pilot experiments with A33-GFP

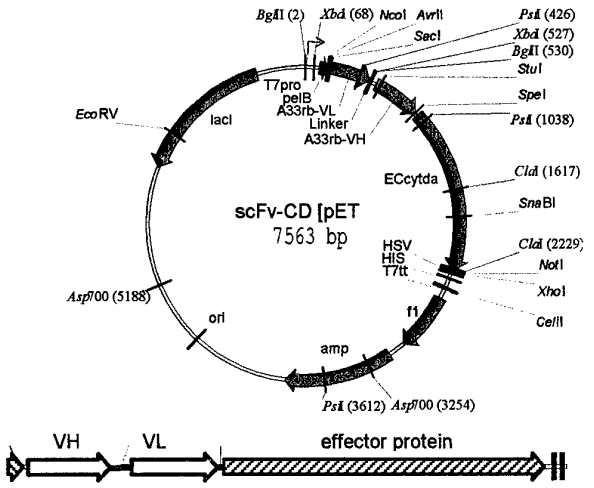

Fig. 1: General vector design and protocypic fusion construct

yielded stable expression results with functional protein secreted into the culture medium. In addition, the recombinant protein proved highly stable even under adverse conditions.

This production result was not transferable to constructs with bacterial CD instead of GFP. Here, only intracellular expression of non-functional material could be achieved, which appeared to be due to the bacterial origin of the CD isoenzyme. Hence, we started the isolation of cytosine deaminase from Sacharomyces cerevisiae by PCR-cloning, as this isoenzyme was expected to be produced easily in P. pastoris since this yeast strain and S. cerevisiae share most of their transcription and translation characteristics. In addition, this enzyme has previously been reported to show superior kinetics for prodrug activation over the bacterial isoenzyme in gene therapy approaches. Yeast CD has then been inserted into the fusion plasmid vectors described above and has been used for expression.

In parallel, thymidine kinase from Herpes simplex virus (TK) and bovine carboxypeptidase A (CPA) have been tested for their suitability as fusion partners. These experiments were carried out analogously to those described above, albeit only in the bacterial cloning and expression systems. With CPA, a principal problem lies in its natural expression as a pre-proenzyme, which has been demonstrated by others to be essential for correct conformation and function, requiring post-translational cleavage for activation. As the cleavable activation region is at the N-terminus of the protein, this hindered the downstream cloning position used for the other effector proteins. Preliminary experiments on the reverse protein order, i.e. N-enzyme-scFv-C, have been conducted but did not reveal any active protein to date.

Task 2: Validation of the proposed ADEPT concept *in vitro*. In vitro assays for testing the functionality of the fusion proteins and the complete ADEPT system included Biosensor binding assays, hemadsorption assays, fluorescence microscopy and cytometry, and cytotoxicity and colony forming assays. Naturally, the latter two forms of assays were not applicable for the GFP constructs.

Antibody binding assays (Biosensor, hemadsorption, fluorescence cytometry) were positive for all tested constructs. The scFv-GFP proteins showed consistent bifunctional activity with specific binding as demonstrated by fluorescence microscopy and flow cytometry with antigen-positve and antigen-negative cell lines.

Cytotoxicity assays with thymidine kinase showed no effective cytotoxicity to date. This preliminary result may be due to technical problems or to general features of this enzyme-prodrug system, e.g. lack of intracellular accessibility of the prodrug activator, which is currently under investigation.

The antigen binding and enzymatic activity of scFv–CD fusion proteins was assessed in cytotoxicity assays using the complete ADEPT system. The cytotoxicities of 5-FC and 5-FU showed no significant differences between antigen-positive and antigen-negative cell lines.

The complete ADEPT system was tested by first incubating antigen-positive and antigen-negative cell lines with serial dilutions of the scFv–CD, and then, after washing, with the 5-FC prodrug at a fixed concentration. In these assays, the fusion proteins had dose-dependant cytotoxic effects on antigen-positive cells, but not on A33-negative ones. No cytotoxicity was observed with scFv–GFP as a control (fig. 2).

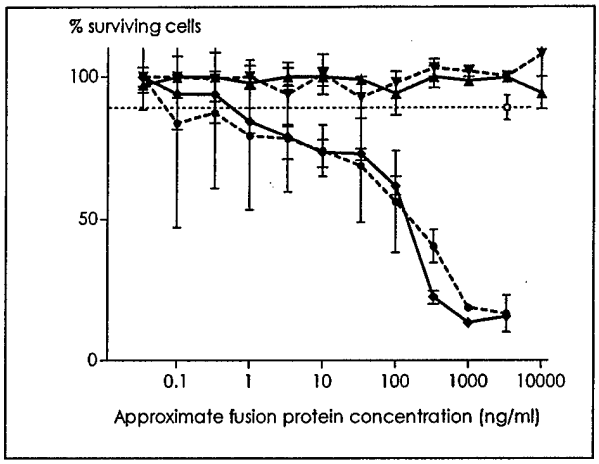

Fig. 2: A33-CD-mediated cytotoxicity on A33 antigen-positive and negative cells: A33-positive LIM1215 cells and A33-negative HT29 cells were incubated with a dilution series of A33-CD fusion protein and, after washing, with the 5-FC prodrug. Survival was measured by the MTT assay. A33-CD fusion protein from two different preparations was used on HT29 cells (▲ and ▼) and on LIM1215 cells (● and ♦). As a control, a single, high concentration of A33-GFP (°) was used instead of A33-CD. Mean and standard deviations of triplicate samples.

Without subsequent prodrug incubation, even the highest tested concentration of fusion protein had no cytotoxic effect on antigen-positive cell lines (fig. 3). When binding of scFv–CD was blocked by preincubation with the corresponding GFP-construct, subsequent 5-FC incubation showed reduced cytotoxicity compared to controls containing an irrelevant control antibody. In summary, these results demonstrate the feasibility of the proposed ADEPT concept in vitro.

Task 3: Localization, biodistribution, and elimination studies *in vivo*. Xenograft tumor models were established by subcutaneous inoculation of nude mice with established antigen-positive cell lines, and the radiolabelled fusion proteins or – to validate the CPA-concept in the absence of a recombinant enzyme – conjugates were injected when the tumors had reached a diameter of 5 mm.

After defined intervals, mice were sacrificed and radioactive doses in blood, tumor and relevant organs were determined. In summary, these experiments showed moderate tumor-to-blood ratios with chemical conjugates and recombinant fusion proteins between 3.5 and 14. These data were concordant with similar studies in the literature (see fig. 3).

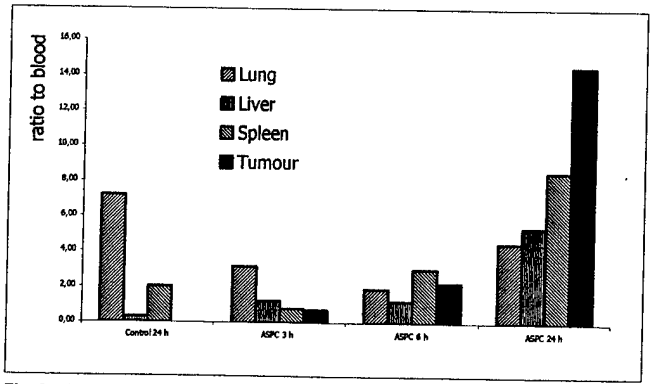

Fig. 3: Tumor-to-blood and tissue-to-blood ratios of radiolabelled A33-CD fusion protein in mice treated inoculated with A33-positive tumor cell lines. Negative control and results 3 h, 6 h and 24 h after injection of antibody construct.

Task 4: Validation of the proposed ADEPT concept *in vivo*. Nude mice in groups of three were inoculated with tumor xenografts and treated with fusion proteins as described above, albeit unlabelled. After allowing 48 hours for the clearance of unbound fusion protein from the circulation, the animals were treated with various

prodrug doses. All mice tolerated the treatment well, so after 24 and 48 hours, the same doses were applied again. Tumour growth appeared to be retarded in the mice with the highest prodrug doses as compared to controls, but this effect was not significant according to both the Mann-Whitney test and the non-paired t-test with Welsh's correction, and no remissions or stabilisations were observed before the mice had to be euthanised due to large tumour burden.

Task 5: Polyethylene-glykol modification of proteins. Polyethylene-glykol conjugation (PEGylation) has been used to reduce immunogenicity and to enhance the circulating half-life of therapeutically administered foreign proteins. Especially in the light of the in-vivo results outlined above, these issues will be important in the future development of protein constructs for ADEPT.

To establish the PEGylation method, humanized A33 (huA33) was again used as a model system. It was conjugated with methoxy-PEG of different molecular masses (M_r 5000 to M_r 20,000) and in different conjugation ratios. Antibody binding was then determined by mixed hemadsorption assay, and at optimized PEGylation ratios > 50 % binding to colon cancer cells was preserved when compared to native huA33.

In mice, initial serum clearance of both PEGylated forms was moderatly decreased compared to native huA33. After repeated immunization with PEG-huA33, anti-antibody titers in immunocompetent mice were < 5 % of those in huA33 treated controls. Both PEG-huA33 forms reached ~ 75 % of the maximum tumor dose of huA33 in SW1222-xenografted mice, but their tumor:blood ratios were considerably reduced. To demonstrate immunologic specificity of PEG-huA33 targeting in SW1222 tumor-bearing mice, antigenic sites were pre-saturated by injecting excess native huA33. This reduced subsequent uptake of PEG-huA33 by up to 80 %, whereas pre-saturation with hu3S193 control antibody had no significant effect. Finally, to assess the microdistribution of antibody uptake in the same xenograft model, tumor tissue was resected at different time points after antibody administration and examined for human IgG by immunohistochemistry. Compared to native huA33, both PEG preparations achieved the same peak staining intensity and homogeneity with a delay of 3 - 24 h. These results demonstrate that the tumor targeting potential of huA33 *in vivo* is preserved at PEGylation levels sufficient to suppress immunogenicity. Corresponding studies on the produced recombinant fusion proteins are currently underway.

Educational aims and Summary

Naturally, this report can only give a rough sketch of the actual methodological problems and their solutions as well as the results obtained from this study. In addition, results reported here for F19 and 3S193 constructs are still in part preliminary and not yet mature for publication. In summary, however, it has been possible to establish a stable and robust expression system for recombinant scFv-

effector fusion proteins and to investigate the proposed ADEPT systems in vitro and in vivo. While the results were not uniform across the different constructs (and could not be reported here in more detail for lack of space), they point in the same direction:

The proposed ADEPT systems based on antibody-CPA conjugates and scFv-CD fusion proteins were functional in vitro with immunological and catalytic specificity, demonstrating the feasibility of the overall concept in vitro. In vivo, the antibody-enzyme constructs localized to tumor tissue with immunological specificity in mice bearing antigen-positive tumor xenografts. Tumor-to-blood ratios, however, were considerably lower than those achieved with the unconjugated respective antibody, and in some cases organs such as lung, liver, and spleen accumulated doses similar to those in tumour. This latter finding may be due to non-specific uptake by diffusion, to redistribution of tumor-bound antibody during the elimination phase, or to cross-reactivity with yet unknown pulmonary or hepatic antigens, or to a combination of all three. While the very rationale of immunological tumor targeting is specificity, i.e. high tumor-to-blood and tumor-to-organ ratios, the observed organ uptake did not translate into toxicity: None of the mice showed adverse effects even with the highest prodrug doses. However, the findings of low tumor-to-blood ratios and high organ doses go along with a lack of efficacy in vivo. If at all, only a small difference in tumor progress between treated animals and controls could be observed.

These results are not a final summary of the proposed ADEPT systems at large, however, as a number of questions remain to be addressed. Thus, so far we were only able to test one fusion protein design, a linear, monovalent scFv-enzyme construct. Both the special conformation of the enzyme components and the antigen binding avidity may require different construct designs. For these reasons, we are currently developing divalent recombinant fusion constructs, which may be of more favorable kinetic characteristics. In addition, we did not have enough capacity yet to test a number of possible dosage schedules in vivo with regard to biodistribution and efficacy.

As a final note, the lack of anti-tumor effect in this study may be due to limitations of the mouse xenograft model as such: The xenografts grew much faster than cancers in humans do, reaching the equivalent of a 1.6 kg burden in an 80 kg person within four to six days. Thus, even if perfectly efficient targeting were achieved on day 1, administration of the prodrug on day 1+x will find the fusion construct diluted by the growth of new tumor in the meantime. Thus, our mouse xenograft model may be overly pessimistic.

As these remarks imply, the work on this project is going to continue beyond the current funding period. As to the wider educational aims, this project and its funding have allowed the principal investigator to establish a functional work group on tumor targeting at his new institution in Berlin, Germany. In this, it has been crucial to his development as a scientist.

Key Research Accomplishments

- Cloning and expression of scFv-enzyme fusion proteins based on antibodies huA33 (control and pilot project) and F19 and 3S193 (therapy project).
- Cloning and expression of additional fusion constructs with green fluorescent protein as control reagents and probes for future histological and intracellular distribution studies.
- Establishing of a highly efficient expression system for the production of soluble, functional fusion proteins in Pichia pastoris
- PEG-conjugation of proteins
- Validation of the general ADEPT concept introduced here by in vitro data
- Completion of preliminary in vivo studies, highlighting limitations of the current constructs and directions for future research

Reportable Outcomes

Publications:

Deckert, P.M., Jungbluth, A., Clark, M., Montalto, N., Williams, C.Jr., Carswell-Richards, E., Bertino, J.R., Old, L.J., and Welt, S.
Pharmacokinetics and Microdistribution of Polyethylene-Glycol-Modified Humanized A33 Antibody Targeting to Colon Cancer Xenografts (2000)
Int.J.Cancer 87(3), 382-390. 2000

Deckert PM, Renner C, Cohen LS, Jungbluth A, Ritter G, Bertino JR, Old LJ, Welt S.
A33scFv-cytosine deaminase: a recombinant protein construct for antibody-directed enzyme-prodrug therapy.
Br J Cancer. 2003 Mar 24;88(6):937-9.

Manuscript submitted:

Deckert PM, Bornmann WG, Williams Jr C, Franke J, Keilholz U, Thiel E, Old LJ, Bertino JR, Welt S
Specific Tumour Localisation of a huA33 Antibody – Carboxypeptidase-A Conjugate and Activation of Methotrexate-Phenylalanine

Manuscript in preparation:

Petrausch U, Dernedde J, Fuchs H, Coelho V, Heufelder K, Thiel E, Keilholz U, Deckert PM
Recombinant A33scFv fusion constructs for specific tumor targeting and detection of microlocalization

Tangibles:

Pichia pastoris strain A-GFP, secreting the huA33scFv-GFP fusion protein
Pichia pastoris strain A-CD, secreting the huA33scFv-CD fusion protein

Publications and tangibles based on the other scFv-constructs will be prepared or made available, respectively, once sufficiently stable and reproducible data are reconfirmed.

Academic degrees:

Ulf Petrausch, a doctoral student in the laboratory of the principal investigator, has received his doctorate with the mark "excellent" (magna cum laude).

Employment:

The principal investigator received a position as *wissenschaftlicher Mitarbeiter* (Research Associate) at the Charité Campus Benjamin Franklin, Berlin, Germany.

Funding applied for:

Based on the work of the funded project, the principal investigator has applied for a research contract grant of the Deutsche Forschungsgemeinschaft research foundation.

Int. J. Cancer: **87,** 382–390 (2000)

Publication of the International Union Against Cancer

PHARMACOKINETICS AND MICRODISTRIBUTION OF POLYETHYLENE GLYCOL-MODIFIED HUMANIZED A33 ANTIBODY TARGETING COLON CANCER XENOGRAFTS

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Therapeutic proteins have been conjugated with polyethylene glycol (PEGylation) to reduce immunogenicity and enhance circulating dose. Here we have investigated the effect of PEGylation on immunogenicity, pharmacokinetics, and histologic microdistribution of tumor-targeting antibodies with humanized A33 antibody (huA33) as a model system. Conjugation of huA33 with methoxy-PEG of M_r 5,000 (32%–34% of primary amines modified) or M_r 20,000 (16%–18% modification) preserved >50% of native huA33 binding to SW1222 colon cancer cells. In mice, both PEGylated forms cleared from serum moderately slower than native huA33. After repeated immunization with PEG-huA33, anti-antibody titers in immunocompetent mice were <5% of those in huA33-treated controls. Both PEG-huA33 forms reached approx. 75% of the maximum tumor dose of huA33 in SW1222-xenografted mice, but their tumor:blood ratios were considerably reduced. To demonstrate immunologic specificity of PEG-huA33 targeting in SW1222 tumor-bearing mice, antigenic sites were presaturated by injecting excess native huA33. This reduced subsequent uptake of PEG-huA33 by up to 80%, whereas presaturation with hu3S193 control antibody had no significant effect. To assess the microdistribution of antibody uptake in the same xenograft model, tumor tissue resected at different time points after antibody administration was examined for human IgG by immunohistochemistry. Both PEG preparations achieved the same peak staining intensity and homogeneity as native huA33 with a delay of several hours. Given the measured reduction in immunoreactivity *in vitro*, these results demonstrate that the tumor targeting potential of huA33 in vivo is preserved at PEGylation levels sufficient to suppress immunogenicity. *Int. J. Cancer* 87:382–390, 2000.
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Induction of immune reactions is a major obstacle to repeated clinical administration of antibodies. Chimeric or humanized mouse monoclonal antibodies (MAbs) (King et al., 1995) and antibodies derived from human DNA libraries by phage display (Hoogenboom et al., 1992) have been developed to overcome this limitation. However, even fully humanized antibodies have been found to elicit human-anti-human immune responses (Welt et al., 1997). Furthermore, humanization may not be feasible with fusion proteins of antibodies and heterologous effector proteins. First, many of these effector proteins are of non-human origin and have no human counterpart, such as bacterial toxins (Reiter and Pastan, 1998) and prodrug-activating enzymes (Melton and Sherwood, 1996). Second, even if the components of these constructs were human-derived or fully humanized, their junction region may still represent immunogenic epitopes.

Conjugation of therapeutic drugs with poly[ethylene glycol] (PEG) has been successfully employed to increase their circulating half-life and solubility as well as to reduce immunogenicity and toxicity. This approach has been applied clinically, allowing bacterial enzymes such as L-asparaginase to be administered repeatedly even in patients who had previously displayed hypersensitivity to the foreign protein (Ettinger et al., 1995).

While increased serum half-life and reduced immunogenicity are widely accepted as general effects of PEGylation on drug molecules, its role in tumor targeting is less clear. Several groups have reported increased passive tumor uptake of liposomes, cytokines (reviewed in Francis et al., 1996), and non-peptide drugs (Senter et al., 1995; Westerman et al., 1998) and suggested a potential role for PEG in increasing passive targeting. This effect has been proposed to be due to the leakiness of tumor neovasculature (Jain, 1990), facilitating extravasation into tumor but not into normal tissue with intact vasculature. However, tumor:blood ratios were markedly reduced compared with the non-PEGylated product, and in one study that has investigated localization histologically, the drug was predominantly found in or around tumor vasculature (Westerman et al., 1998).

In active targeting, i.e., tumor uptake due to specific binding, preclinical studies of PEG-conjugation have mainly focused on Fab′ and $F(ab)_2$ antibody fragments. The effect of PEGylation on tumor localization appeared to depend on the protein size: While tumor localization of PEGylated complete IgG was reduced compared with the native antibody (Kitamura et al., 1991), several groups have reported increased absolute tumor uptake of Fab′ or $F(ab)_2$ fragments (Delgado et al., 1996; Eno-Amooquaye et al., 1996; Kitamura et al., 1991). As in passive targeting, however, reduced tumor:blood ratios were observed after PEGylation of Fab′ or $F(ab)_2$ fragments (Delgado et al., 1996; Eno-Amooquaye et al., 1996). Two factors are thought to be responsible for this effect: reduced clearance rate and reduced diffusion of macromolecules. Diffusion characteristics have been investigated in detail for non-modified antibodies and $F(ab)_2$ fragments, and several authors have concluded that tumor-directed macromolecules in general will not be able to achieve homogeneous distribution in tumor tissue due to elevated convective intratumoral pressure and low diffusion capacity of macromolecules (Francis et al., 1996; Jain, 1990; van Osdol et al., 1991). Together, these observations raise the question whether active targeting is feasible with PEG-conjugated antibodies, or if the previously described increase in

Abbreviations: **Ab**, antibody; **Fab′**, **$F(ab)_2$**, antibody fragments comprising one or two antigen-binding domains, respectively; **CDR**, complementarity-determining region; **huA33**, **hu3S193**, humanized A33 and 3S193 antibodies, repectively; **PBS**, phosphate buffered sodium chloride solution; **PEG 5**, **PEG 12**, and **PEG 20**, methoxy-polyethylene glycol succinimidyl-succinate of M_r 5000, M_r 12,000, and M_r 20,000, respectively.

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tumor localization is a result of nonspecific accumulation in the interstitial space surrounding tumor vasculature.

MAb A33 recognizes a newly characterized cell-surface differentiation antigen of approximately 43 kDa molecular weight that belongs to the immunoglobulin superfamily. It is expressed on normal human gastrointestinal epithelium and on approx. 95% of primary or metastatic colon cancers but is absent in most other normal tissues (Heath et al., 1997). Some colon cancer cell lines, such as SW1222, express large amounts of the A33 antigen, binding up to 800,000 antibody molecules per cell. Upon binding to the A33 antigen, mAb A33 is internalized into a yet incompletely characterized vesicular compartment, and a significant fraction of the internalized antibody is recycled back to the cell surface (Daghighian et al., 1996). The A33 antigenic system has been the focus of several clinical studies in patients with colon cancer. Phase I/II clinical trials have shown that murine mAb A33 (i) localizes with high specificity to colon cancer tissue; (ii) is retained for prolonged periods of up to 6 weeks in the cancer but clears within 5 to 6 days from normal colon; and (iii) has anti-tumor activity as a carrier of ^{125}I or ^{131}I (Welt et al., 1994, 1996). A humanized version of the A33 antibody (huA33) has been constructed (King et al., 1995) and is currently being evaluated in clinical trials (Welt et al., 1997).

In this study we investigate the effect of PEGylation on tumor targeting and immunogenicity of huA33 in an established mouse xenograft model (Barendswaard et al., 1998).

MATERIAL AND METHODS

Animals and cell lines

Eight-week-old female outbred CD-1 mice (Charles River Laboratories, Wilmington, MA) and 8-week-old female athymic NCr-*nu*BR mice (nude mice; Taconic, Germantown, NY) were maintained at the Memorial Sloan-Kettering Cancer Center (MSKCC) Research Animal Resource Center. All animal experiments were performed under protocol 90-07-016, approved by the MSKCC Institutional Animal Care and Use Committee.

The human colon carcinoma cell lines SW1222 and HCT15 were from the cell bank of the Ludwig Institute for Cancer Research at MSKCC. Cells were maintained at 37°C and 5% CO_2 in Eagle's minimum essential medium supplemented with 1% (v/v) non-essential amino acids and 10% (v/v) FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and harvested using 0.1% (v/v) trypsin and 0.02% (v/v) EDTA (all reagents: GIBCO, Grand Island, NY).

SW1222 xenografts in nude mice

Nude mice were injected with 10^7 washed SW1222 cells in 150 μl sterile buffer (0.15 M NaCl and 0.1 M sodium phosphate, pH 7.4) into the left thigh muscle. Subsequent experiments were conducted when the tumor mass had reached a diameter of 0.4 to 0.6 cm, corresponding to a weight of 350 to 400 mg.

PEG modification of humanized antibodies

Methoxy-PEG-succinimidyl-succinate of M_r 5,000, 12,000, or 20,000 (Shearwater Polymers, Huntsville, AL) was weighed directly into the reaction tube, and 10 mg humanized A33 (King et al., 1995), or, for control experiments, 10 mg hu3S193 (Kitamura et al., 1994) were added in 10 ml of 100 mM sodium phosphate buffer, pH 7.4. The mixture was immediately vortexed vigorously for 30 sec and allowed to react for 60 min at room temperature under moderate shaking. Unreacted PEG was removed by ultrafiltration with a cutoff of 50 kDa (Ultrafree cartridges, Millipore, Marlborough, MA). The protein concentration was adjusted to 1.0 mg/ml, and purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 6% or 4%–12% precast gels and Coomassie staining (Novex, San Diego, CA).

Determination of modified primary amines. Unreacted primary amines were detected by mixing 150 μl of the purified conjugate with 50 μl of 1 mg/ml fluorescamine (Sigma, St. Louis, MO) in acetone and measuring fluorescence at an excitation wavelength of 360 nm ± 20 and an emittance wavelength of 460 nm ± 20 (modified after Stocks et al., 1986). The proportion of modified primary amines was calculated based on native (unconjugated) antibody as a standard.

Antibody binding activity (mixed hemadsorption assay). Binding of immunoglobulin to SW1222 tumor cells was detected by erythrocyte-bound protein A as previously described (Welt et al., 1994). The antibody binding titer was defined as the highest dilution that produced unequivocal rosetting of erythrocytes on tumor cells.

Radiolabelling of huA33 and hu3S193 antibodies

In the context of clinical imaging and therapeutic studies of radioiodinated A33, iodine 131 was selected for radiolabelling. Native and PEG-conjugated antibodies were labelled and assayed as described previously (Welt et al., 1994), using the chloramine T method. One milligram of the antibody was mixed with sodium iodide 131 (^{131}I) (1 mg/74 MBq) in 400 μl chloramine T solution (2 mg/ml). The reaction was terminated with 400 μl sodium metabisulfite (10 mg/ml), and the product was purified using a sterile Sephadex G25 column (Pharmacia) that had been preconditioned with sterile saline solution containing 5% human serum albumin. The MAb fractions were pooled and passed through a 0.2-μm filter. The specific activity was 7.4 to 9.3 MBq/mg. Each freshly prepared sample was tested for radiochemical purity by radio-thin-layer chromatography, for binding of ^{131}I to the antibody by trichloroacetic acid precipitation (>95% counts bound) and for immunoreactivity by absorption of 0.1 μg/ml ^{131}I-antibody with sequential tubes containing 2×10^7 antigen-positive cells (Welt et al., 1994). Background activity was determined by pretreating cell pellets with a >100-fold excess of unlabelled antibody prior to adding radiolabelled antibody. Immunoreactivity was calculated by subtracting background radioactivity (counts per minute, CPM) from cell-bound ^{131}I radioactivity after washing twice in PBS and dividing the remaining fraction by the total CPM added. Immunoreactivity measured by this method was 50%-70% for huA33 and 34% for hu3S193.

Determination of antibody immunogenicity in mice

Groups of 5 CD-1 mice received 4 weekly I.V. injections of 25 μg native or PEG-modified huA33 antibody equalized for pure protein concentration (days 1, 8, 15, and 22). Blood samples were obtained before the first injection and after 5, 9, and 13 weeks.

Immunoreactivity against A33 was assessed by ELISA. Microtiter plates coated with 10 μg/well of huA33 and blocked with 1% w/v bovine serum albumin were incubated with mouse blood samples in duplicate dilution series. Bound murine antiserum was detected photometrically after incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG,A,M serum (Sigma, St. Louis, MO) and subsequent chromogenic reaction as the highest dilution that produced an absorbance greater than twice the background.

Tissue dosimetry of [^{131}I]huA33 in mice

Tumor xenograft-bearing nude mice and naive controls were injected I.V. with a single protein-equalized dose of 5 μg (approx. 10 μCi) radiolabelled native or PEGylated antibody in 100 μl sterile buffer (0.15 M NaCl, 0.1 M sodium phosphate, pH 7.2). For circulation clearance studies, blood samples of 10 to 30 μl were taken repeatedly at the indicated time points (see Results) from the retro-orbital plexus opposite of the injection site. To study organ distribution and tumor uptake, mice were sacrificed and blood, lung, liver, spleen, kidney, and tumor samples were obtained and weighed. The radioactive dose was measured by an automated gamma counter (model 1282 CompuGamma CS, LKB Wallac, Gaithersburg, MD) and compared with an aliquot of the injected preparation as a standard. Relative in vivo doses, expressed as percent of the injected dose per gram of tissue, were calculated as

% injected dose/g
= 100 × (sample CPM ÷ sample mass [g]) ÷ injected CPM.

Morphological studies

SW1222 tumor-bearing nude mice were treated as described above with native or PEG-conjugated huA33 or 3S193 antibody as indicated (see Results) and sacrificed 1, 4, 24, and 72 hr after injection. Tissues were harvested immediately after death, embedded in cryomolds filled with OCT compound (Tissue-Tek, Torrance, CA), and snap-frozen in dry ice-precooled isopentane. The frozen blocks were stored at −75°C. Cryostat sections (5 μm) were adhered to slides (Superfrost, Fisher Scientific, Pittsburgh, PA) and dried at room temperature for 30 min. Fixation was performed with acetone (4°C) for 10 min immediately before immunostaining. Immunohistochemistry was performed with an avidin-biotin system (ABC, Vector Laboratories, Burlington, CA). As primary reagent, a goat anti-human antibody (1:100; Jackson Laboratories, West Grove, PA) was applied at 4°C overnight followed by a biotinylated horse anti-goat secondary antibody (1:200, Jackson Laboratories). A control slide without the goat anti-human antibody incubation was included for all assays. For visualization, the chromogen 3,3'diaminobenzidine (DAB, BioGenex, San Ramon, CA) was used. Endogenous peroxidase was suppressed with 1% H_2O_2 for 30 min prior to application of the avidin-biotin complex. The slides were counterstained with Meyer's hematoxylin (Sigma) and dehydrated. As a staining control, one slide derived from a control animal treated only with buffer solution was stained directly with huA33, followed by the detection system described.

The slides were evaluated by a histopathologist, and the extent of staining was assessed semiquantitatively by visually estimating the proportion of positive tumor tissue in 25% increments as follows: −, no staining of tumor cells; +, <25% of tumor cells stained; ++, 25%–50%; +++, 50%–75%; and ++++, >75% of tumor cells stained.

Statistical analysis

Due to the small sample sizes, a permutation test was used to compare groups over time whereby random reordering of observations determined the significance level of a test. The test statistic used to perform this comparison was the difference in means summed over time. If the observed difference was extreme relative to the null permutation distribution, we could conclude that a statistically significant difference exists between the groups in the experiment.

RESULTS

Chemical characterization and immunoreactivity of PEG-huA33

In optimizing the conjugation process, we sought the highest PEG:Ab ratio for each PEG size that would not diminish antibody binding by more than 50% (one titration step). PEG 5, PEG 12, and PEG 20 were examined in PEG:Ab reactant ratios from 5 to 100. The reaction products were heterogeneous in size, reflecting different conjugation ratios achieved in the reaction. As PEG migrates slower than proteins of the same mass in polyacrylamide gel (Francis et al., 1996), electrophoresis could not reveal the actual m.w., but was used only to estimate the amount of unreacted antibody and to compare different PEG preparations. This showed that the result was dependent on the PEG size (Fig. 1A) as well as the molar reactant ratio (Fig. 1B). The actual percentage of modified primary amines was quantified using the fluorescamine assay (Stocks et al., 1986), which demonstrated almost complete reaction of PEG in PEG:Ab ratios of up to 70:1, resulting in an effective conjugation ratio close to the PEG:Ab ratio in the reaction (data not shown).

The immunoreactivity of PEG-Ab conjugates was determined by mixed hemadsorption assay on A33-antigen-positive SW1222 colon cancer cells. With PEG:Ab reactand ratios of up to 30:1 for PEG 5 and up to 15:1 for PEG 12 and PEG 20, no inhibition of antibody binding was observed that exceeded the limit of one titration step (Fig. 2), and these reactand ratios were selected for subsequent experiments. The corresponding conjugation results according to the fluorescamine assay were 32%–34% modified primary amines (average molar ratio, 28–30 PEG/antibody) for PEG 5 and 16%–18% for PEG 20 (molar ratio, 14–16 PEG/antibody; the apparent excess over the reactand ratio is within the error of the assay). At 4°C the conjugates were stable for at least 8 weeks according to gel electrophoresis and activity tests (data not shown).

PEG modification reduces immunogenicity of huA33 in mice

Immunocompetent CD-1 mice were immunized 4 times with 25 μg (1.25 mg/kg) of native, PEG 5-conjugated, or PEG 20-conjugated huA33, and anti-huA33 antibody titers were determined by ELISA. Mice that had received native huA33 produced increased

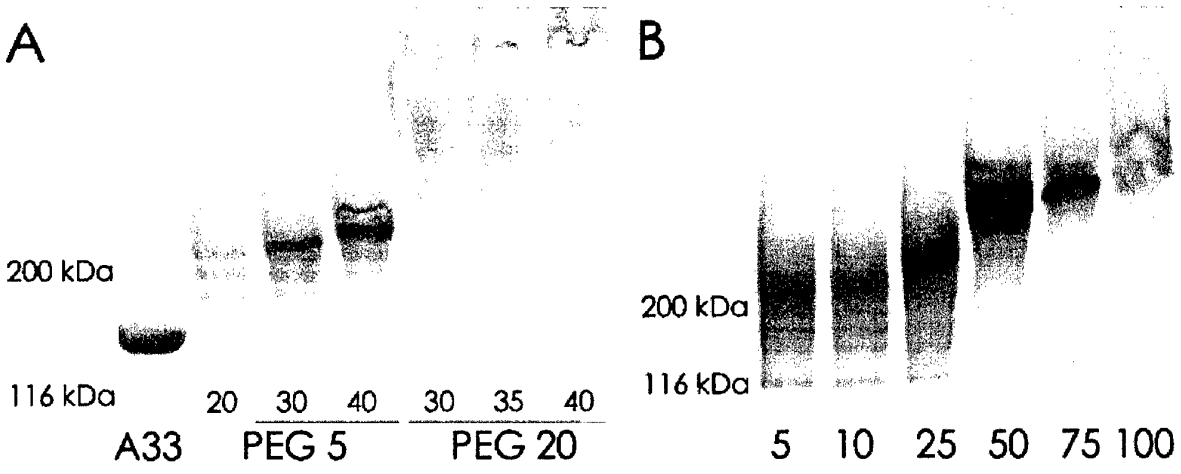

FIGURE 1 – PEG-huA33 conjugation results with different PEG sizes and molar ratios: huA33 conjugated with PEG as described in the text was run on 6% (A) or 4%–12% gradient (B) tris-glycine gels under nonreducing conditions. (A) huA33 conjugates with PEG 5 or PEG 20 in molar PEG:Ab reactant ratios as indicated. First lane (A33), native huA33 control. (B) Conjugates with PEG 12 in molar PEG:Ab ratios from 5 to 100.

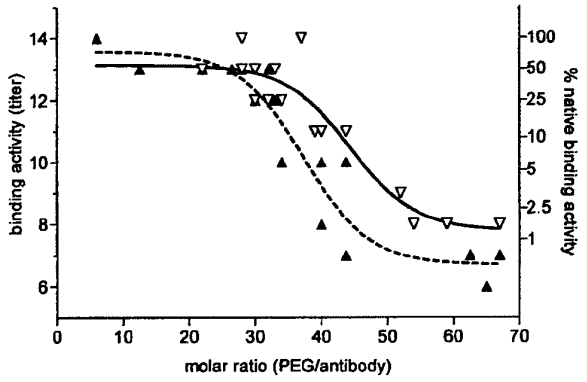

FIGURE 2 – Molar PEG:Ab ratio and binding activity: huA33 modified with either PEG 5 (▽—) or PEG 20 (▲- - -) at the indicated molar ratios as measured by fluorescamine assay was tested for binding to A33 antigen-positive SW1222 colon cancer cells. Average PEG ratio of the conjugate was measured by fluorescamine assay and antibody binding titer determined by mixed hemadsorption assay as described in the text. From these results, molar ratios of 30:1 (PEG 5) and 15:1 (PEG 20) were selected for subsequent experiments. Left-hand scale, dilution titer; right-hand scale, percent of activity (unmodified huA33 100%).

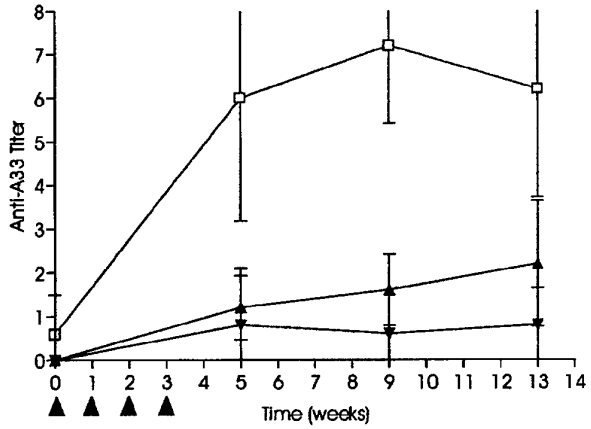

FIGURE 3 – Immunogenicity of huA33 and PEG-huA33. Groups of 5 mice each were injected with a protein-equalized dose of 5 μg of either native (□), PEG 5-conjugated (▼), or PEG 20-conjugated (▲) huA33 preparations at the indicated time points (arrows). Anti-huA33 binding activity was determined in an ELISA using unmodified huA33 as the target antigen (see text). Error bars indicate standard deviation. One-tailed paired *t*-test: native vs. PEG 5-huA33, P = 0.0217; native vs. PEG 20-huA33, P = 0.0211; PEG 5- vs. PEG 20-huA33, > 0.05.

antibody levels on day 35, and 4 of 5 mice reached a maximum titer of 256 on day 63 (Fig. 3). With both PEG preparations, however, a titer of 4 was not exceeded, and the highest titer was only observed on day 91 (significance levels over time, native huA33 vs. PEG 5-huA33, $P < 0.001$; vs. PEG 20-huA33, $P < 0.02$; no significant difference between PEG preparations).

To exclude immune reactions against epitopes formed by the PEG itself, we attempted to establish an ELISA with PEG-conjugated instead of native huA33. However, it was not possible to detect PEG-huA33 either directly or indirectly using the above murine sera or anti-human control sera.

PEG-conjugation increases the circulating dose of humanized antibodies but reduces huA33 dose in tumor

The radioactive dose in blood was measured from 20 min to 7 days after injection of trace-labelled native or PEG-conjugated antibody preparations in non-tumor-bearing CD-1 mice (Fig. 4A). At the selected PEGylation ratios of 15:1 for PEG 20 and 30:1 for PEG 5, an increase in serum dose was observed compared with non-PEGylated A33 antibody, resulting in dose levels of PEGylated antibody that were 125% of the corresponding dose of native huA33 after 6 hr and 165% after 48 hr, converging thereafter (native huA33 vs. either PEG preparation, $P < 0.001$). Only when a considerably higher conjugation ratio, 40:1 with PEG 20, was used on the isotype control antibody 3S193 was a more marked increase in serum dose observed (Fig. 4B).

To determine tumor and organ uptake, groups of 5 SW1222 xenograft-bearing nude mice each were injected with 0.5 μg of trace-labelled native or PEGylated huA33. Animals from each group were sacrificed at 24, 48, 72, 96, and 168 hr post injection to measure blood, tumor, and organ doses, which were expressed as percent injected dose per milliliter of blood or gram of tissue, respectively. With all antibody preparations, the maximum tumor dose was reached at 24 hr, declining thereafter (Fig. 4C). Tumor uptake of PEGylated huA33 reached 73% to 82% of the uptake of native huA33 at corresponding time points (native huA33 vs. PEG 5, $P = 0.013$; vs. PEG 20, $P < 0.001$). Tumor:blood ratios also were significantly higher with native huA33 compared with either PEG-conjugate (PEG 5, $P = 0.001$, PEG 20, $P = 0.014$). At the last time point, the PEG 20-conjugate appears to reach a higher ratio than PEG 5-huA33, but over the time course no significant difference between PEG-conjugates was found (Fig. 4D).

In normal tissues, only small differences in uptake were observed between native and PEGylated huA33 (Fig. 5). While isolated significant differences could be calculated for kidney, spleen, and lung at the 96-hr time point, over time only in lung PEG 5-huA33 was retained at significantly higher levels than native huA33 ($p = 0.0316$).

In vivo tumor localization of ^{131}I-PEG-A33 is immunologically specific

In order to assess the immunologic specificity of antibody localization to tumor in vivo, the previous xenograft experiment was modified by pretreating mice with excess native antibody (unlabelled, non-PEGylated) to presaturate antigenic sites. Tumor-bearing nude mice were injected with 250 μg of either huA33 or hu3S193 control antibody. Six hours later, 5 μg of ^{131}I-labelled PEG 20-huA33 was injected, and animals were sacrificed after 21, 45, and 68 hr. No significant difference between the 2 pretreatment groups was observed for radioactive dose in blood, kidney, spleen, liver, or lung (data not shown). In tumor tissue, however, blocking with huA33 significantly reduced ^{131}I-PEG-A33 binding down to the levels of nonspecific binding in organ tissues ($P < 0.001$ for difference to unblocked control), whereas pretreatment with hu3S193 control antibody had no effect on PEG-huA33 binding (hu3S193-block vs. unblocked control, $P = 0.678$; vs. huA33-block, $P < 0.001$; Fig. 6).

PEG-A33 targets tumor cells with the same microdistribution pattern as unconjugated huA33

The results of the immunohistochemical staining are shown in Table 1 and Fig. 7. All tissues showed variable degrees of intravascular and stromal staining due to the presence of humanized antibody in blood vessels and connective tissue. No staining was present in the neoplastic xenograft tumor cells of the animals treated with hu3S193. Mice treated with native huA33 showed intense tumor cell staining at all time points. The largest difference in the extent of immunohistochemical staining was seen at 1 hr after injection. At this time point, only restricted distribution was visible with all 3 PEG conjugates of huA33, which showed homogeneous or almost homogeneous tumor localization from the 4-hr time point on.

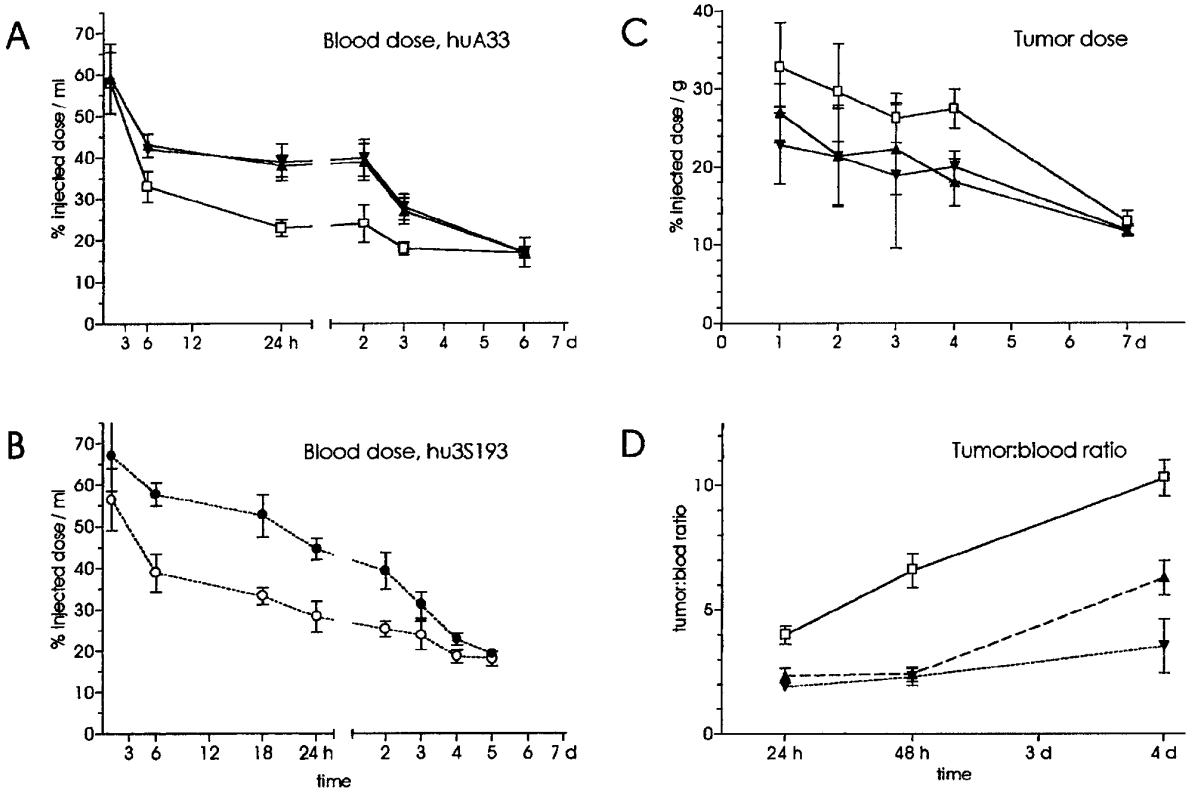

FIGURE 4 – Blood and tumor doses of native and PEG-modified antibodies in mice. Groups of 5 mice were injected with protein-equalized doses of 5 μg of one of the following ^{131}I-labelled antibody preparations: native (□), PEG 5-conjugated (▼), or PEG 20-conjugated (▲) huA33, or native (○) or PEG 20-conjugated (●) hu3S193. Blood and/or tumor tissue was obtained at the time points indicated, and radioactive dose per gram was measured and normalized for injected dose. (A, B) Elimination of native and PEGylated antibody preparations from the blood of non-tumor-bearing Swiss mice. (A) huA33; (B) hu3S193 control antibody. The PEG:Ab ratios were 15:1 for PEG 20-huA33, 30:1 for PEG 5-huA33, and 40:1 for PEG 20-3S193. Summarized data from 3 experiments. (C) Injected huA33-dose in tumors of nude mice bearing SW1222 xenografts of defined size. Mice were sacrificed at the time points indicated and the tumors resected. Summarized data from 2 experiments. (D) Tumor:blood ratios at indicated time points after injection of radiolabelled antibody into SW1222-tumor-bearing mice.

DISCUSSION

The A33 antigenic system has shown promising tumor-targeting in clinical trials (Welt et al., 1994, 1996). To reduce its immunogenicity, the A33 antibody has been fully humanized by CDR-grafting (King et al., 1995). However, in an ongoing clinical study, even this humanized version has induced immune reactions against the antibody in 4 of 11 patients (Welt et al., 1997). We have therefore explored PEG-conjugation as a means to overcome or reduce this limitation. This study demonstrates that PEG-conjugated huA33 antibody localizes to tumor tissue in vivo with immunological specificity. At conjugation ratios sufficient to suppress immunogenicity, PEG-huA33 showed homogeneous targeting to tumor tissue comparable to the native antibody. However, the proportional tumor dose of PEG-huA33 was reduced to approximately 75% of the dose achieved with the native antibody. Although elimination rates from tumor were similar for native and PEGylated antibody, tumor:blood ratios of the PEG conjugates were about one-third to one-half those of unmodified huA33, increasing over time for all 3 preparations as circulating antibody was eliminated from the vascular compartment.

In this study, we observed 3 phases in the micro-localization of non-PEGylated huA33: (i) initial targeting: as early as 1 hr post-injection, huA33 localized with high intensity to peripheral tumor cells; (ii) distribution in tumor tissue: staining throughout the tumor nodule was observed after approx. 4 hr; and (iii) clearance of nonspecific staining: stroma and vasculature were almost completely unstained after 24 hr, while tumor tissue remained homogeneously stained. With PEGylated huA33, the targeting process followed the same consecutive pattern with a delay of several hours.

Theoretical models have predicted that antibodies may not be able to achieve tumor targeting beyond the periphery of a tumor, as the inward directed concentration gradient would be insufficient to overcome the outward directed convective pressure gradient (Jain, 1990; van Osdol et al., 1991). However, this is not the case with the A33 antigenic system, since homogeneous distribution of A33 antibody throughout colon cancer tissue has been demonstrated in mice and humans (Barendswaard et al., 1998; Welt et al., 1994, 1996). The present study confirms these findings for PEG-conjugated huA33. A possible explanation for the fast and homogeneous distribution may be the high internalization rate of antigen-antibody complexes documented for A33 (Daghighian et al., 1996). The binding-site barrier model postulates that a high-affinity antibody to an abundantly expressed antigen will form a gradient from periphery to center, with most antibody binding at the entry site in the periphery, preventing further diffusion into tumor tissue (van Osdol et al., 1991). Therefore, one might

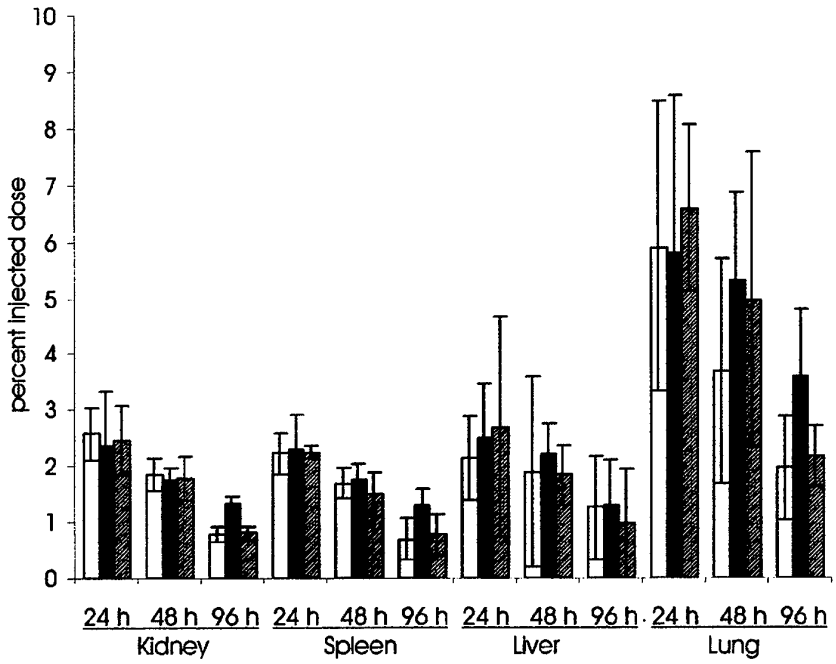

FIGURE 5 – Organ distribution of different huA33 preparations. Tumor-bearing nude mice were injected with a protein-equalized dose of 5 μg of ^{131}I-labelled native huA33 (white), PEG 5-conjugated huA33 (black), or PEG 20-conjugated huA33 (hatched). Percent injected dose per gram of tissue.

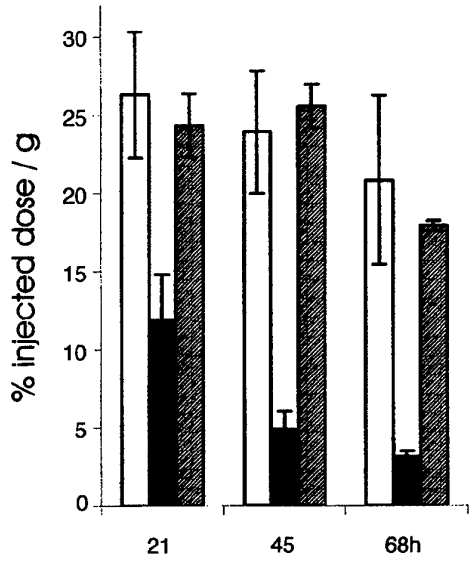

FIGURE 6 – Blocking of radiolabelled PEG 5-huA33 binding to tumor tissue by presaturation with unlabelled antibodies. Tumor-bearing nude mice were treated with unmodified huA33 (black) or control antibody hu3S193 (hatched) before being injected with ^{131}I-labelled PEG-huA33. Tumor doses were measured at the time points indicated and expressed as percent injected dose per gram of tissue. White: control without presaturation.

hypothesize that internalization of antigen-antibody complexes and the consequent depletion of antigenic binding sites would permit deeper penetration of antibody into the tumor. As long as a sufficient amount of antibody is present over time, antibody localization would thus progressively advance towards the core of a tumor nodule.

TABLE I – SCORE OF IN VIVO STAINING OF SW1222 TUMOR XENOGRAFTS BY DIFFERENT ANTIBODY FORMULATIONS

	1 hr	4 hr	24 hr	72 hr
Buffer	–	–	–	–
Native hu3S193	–	–	–	–
Native huA33	++++	++++	++++	++++
PEG 5-huA33	+	++++	++++	+++
PEG 12-huA33	++	++++	++++	++++
PEG 20-huA33	++/+++	+++	++++	+++

For details on experiment and staining, see text. Slides from Fig. 7 were evaluated for distribution of tumor staining and assigned one of the following scores: completely negative (–), <25% (+), 25% to 50% (++), 50% to 75% (+++), and >75% (++++) of tumor cells stained.

Several authors have described increased passive, i.e., not antigen-specific, tumor targeting as an effect of PEGylation of various proteins and non-protein drugs (Francis et al., 1996; Senter et al., 1995; Westerman et al., 1998). To exclude that the tumor localization we observed represented mere passive uptake, we demonstrated antigen-specific binding of PEGylated huA33 in tumor-xenografted mice by presaturation of antigenic sites with unconjugated, unlabelled antibodies. Native huA33 reduced subsequent detection of radiolabelled PEG-huA33 to the level of nonspecific uptake in organ tissues, while pretreatment with a control antibody had no significant effect. In addition, tumor:blood and tumor:organ ratios were highest during the elimination phase of the antibody, which is consistent with a binding force that retained PEG-huA33 in tumor against a concentration gradient. These results allow the conclusion that PEG-huA33 targeting is immunologically specific and not due to nonspecific pharmacokinetic characteristics of a PEGylated protein.

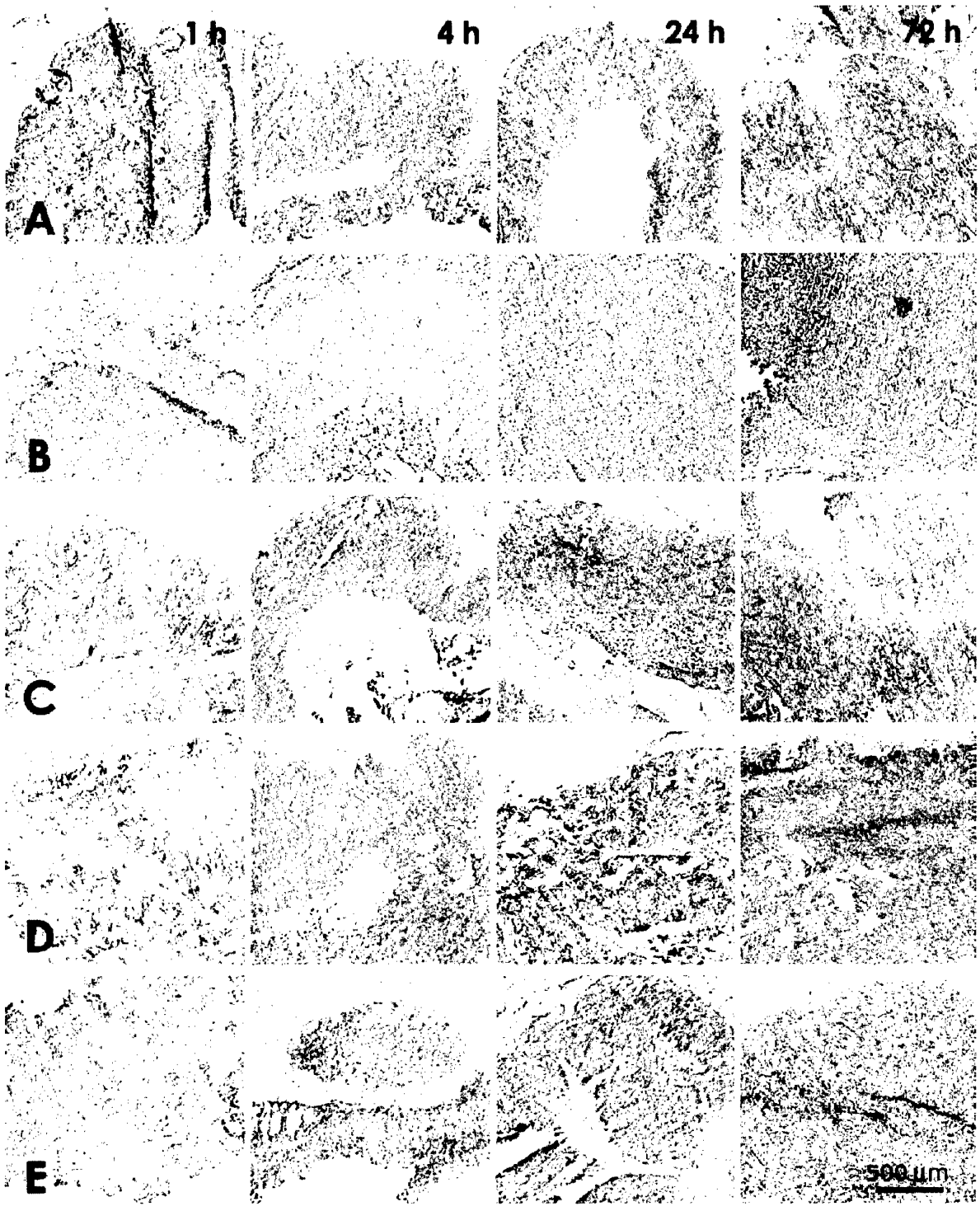

FIGURE 7 – Morphological localization of different antibody preparations in SW1222 colon xenografts in mice. Mice bearing tumors of defined size were injected with a protein-equalized dose of 5 µg of the antibody solutions listed below. At the time points indicated, tumors were resected, and thin sections were subsequently stained with IgG-specific goat anti-human primary and biotinylated horse anti-goat secondary antibodies and a streptavidin-alkaline phosphatase conjugate, which was detected by reaction with a chromogenic substrate. Representative fields. Antibodies: (A) native huA33; (B) hu3S193 control; (C) PEG 5-huA33; (D) PEG 12-huA33; (E) PEG 20-huA33.

The objective of PEG-conjugation in this study was to reduce the immunogenicity of a therapeutic antibody. Induction of antibodies against huA33 as a xenogenic protein in mice was reduced by more than 95% after modification of 32%–34% of primary amines with PEG 5 or of 16%–18% of primary amines with PEG 20.

Formally, our results do not exclude the possibility of antibodies against new epitopes formed by the introduction of PEG, as we only tested for murine antibodies against huA33, not PEG-huA33. However, it was not possible to establish an ELISA using PEG-huA33 as the target antigen. Probably this failure to detect PEG-huA33 immunologically reflects the same mechanisms that have been reported to reduce antigenicity of PEGylated proteins in vivo (Chaffee et al., 1992).

Using the sulfhydryl-methoxy-PEG method, we have optimized the conjugation conditions so as to achieve the highest possible PEGylation degree while leaving little or no antibody unconjugated and not incurring more than 50% (one titer step) loss in antibody binding activity. However, as we have shown that tumor uptake of PEG-huA33 is dependent on its antigen specificity, lower binding activity was likely to contribute to the observed reduction of tumor:blood ratios compared with native antibody. Reduced protein function is not necessarily an effect of the presence of PEG per se but may also be due to linker moieties and to harsh conditions during the conjugation reaction. These adverse effects may both be possible to eliminate by techniques such as the linker-less Tresyl-mPEG method (Francis et al., 1996).

Still, considering the distribution of primary amines as potential conjugation sites, impairment of immunoreactivity may be inevitable with all random linking methods. Of the 88 primary amines provided by lysine residues in each huA33 molecule, 20 are found in the variable regions. While only 4 of these are located in CDRs, close proximity of primary amines in the variable region framework will be likely to account for various degrees of steric hindrance in a sizeable proportion of PEGylated antibody molecules. New site-specific rather than random linking techniques (Lee et al., 1999), however, may lead to an improved balance of immunogenicity and function.

This being an exploratory study, it was not designed to determine conclusively the optimal combination of PEGylation degree and PEG type. However, conjugation with PEG 20 displayed a trend towards higher immunogenicity than PEG 5, but it also showed a trend towards higher tumor:blood ratios of the PEG 20 conjugate during the elimination phase. While these observations were not statistically significant, it seems plausible that both the reduction of immunogenicity and of antibody binding should depend more on the number of attached PEG molecules than on their size. Moreover, should this finding be reproducible, it would imply that the putatively better binding function of the PEG 20 conjugate may be sufficient to overcome the substantial diffusion obstacle constituted by the 2-fold higher total increase in molecular mass compared with the PEG 5 conjugate. Resolving these questions in a detailed comparative approach will be the objective of future studies using the improved conjugation methods mentioned above. Beyond comparing various PEGylation methods and PEG sizes, these studies also will have to question our preliminary assumption that the highest possible PEGylation degree be desirable. It might well be possible to achieve the same reduction in immunogenicity with lower PEGylation degrees, thus incurring less impairment of antibody function and tumor localization.

In apparent contradiction to the concept of PEGylation as a method of reducing immunogenicity, Brumeanu et al. (1995) used low-degree PEG conjugation (8% modification of primary amines) to enhance the immune response against viral epitopes represented by immunoglobulins, thus obviating the need for an adjuvant in a murine vaccination model. As this in effect is an anti-idiotypic immunization, the question arises if PEGylation may promote selection for responses against the antigen-binding regions of antibodies. It has been demonstrated in the clinic that antibodies recognizing a natural receptor are capable of eliciting anti-idiotypic anti-antibody responses, which then mimic features of the original receptor (Deckert et al., 1996). In the case of the A33 antigen, whose natural ligand is yet to be identified, such a mechanism could carry a potential for autoimmune reactions.

PEGylation caused a modest increase in the circulating dose of huA33 in comparison with the native antibody. A more marked increase in circulation time was observed only at PEGylation degrees that significantly reduced immunoreactivity. The immediate effects of PEG conjugation on circulating dose and tumor localization are determined mainly by 2 factors: protection from enzymatic degradation and reduced diffusion due to increased size (Francis et al., 1996). Protection from degradation should prolong the circulating half-life of a PEGylated molecule independent of its size. The increase in effective diameter, however, is a double-edged effect. On one hand, it prolongs circulating half-life, an effect most prominent with small proteins that pass the renal filter in their native form but are retained after PEGylation. This is the case with antibody fragments, which have shown a marked increase in serum half-life after PEGylation, whereas complete IgG antibodies pass the renal filter neither native nor PEGylated (Delgado et al., 1996; Eno-Amooquaye et al., 1996; Kitamura et al., 1991). On the other hand, an increase in diameter also impedes the diffusion of a protein of any size in perivascular space and tumor tissue, reducing its capability of tumor targeting and penetration. On balance, the effect of the increased diameter on tumor localization is more favorable for smaller antibody fragments, which are excreted rapidly in their non-PEGylated forms, while in larger molecules, such as complete IgG, the impeding effect on tumor targeting prevails.

Furthermore, additionally increasing the already long circulating half-life of native antibodies by PEGylation also reduces the tumor:blood ratio. While a pure immunotherapeutic approach might benefit from an increase in circulation time per se, this effect could considerably impair the efficacy of radioimmunotherapy or antibody-enzyme prodrug therapy. With these therapeutic approaches, a high antibody blood concentration is desirable during the initial phase of tumor penetration. Once the antibody has bound to tumor tissue, rapid clearance of circulating antibody would minimize unwanted systemic effects. Hence it has been suggested to employ clearing antibodies that neutralize the respective tumor-targeting antibody in order to accelerate elimination of unbound antibody from the vascular compartment (Eno-Amooquaye et al., 1996).

The A33 antibody has shown high selectivity for primary and metastatic colon tumor localizations in clinical phase I and phase II studies (Welt et al., 1994, 1996). As the current investigation is limited to an animal model, however, predictions regarding biodistribution in humans have to be made with caution. The human A33 antigen is also expressed in normal colon tissue (Heath et al. 1997; Welt et al., 1994), but the murine equivalent of the human A33 antigen is not recognized by the A33 antibody directed against the human antigen. Therefore, we could not study the effect of PEGylation on antibody localization to normal colon in the mouse model. Only clinical trials will be able to assess if the rapid clearance of A33 antibody from normal colon that has been demonstrated in the clinic will be maintained by a PEG-conjugated preparation.

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Short Communication

A33scFv–cytosine deaminase: a recombinant protein construct for antibody-directed enzyme-prodrug therapy

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A recombinant fusion protein of colon carcinoma binding A33 single chain antibody with cytosine deaminase displayed specific antigen binding and enzyme activity in surface plasmon resonance and is catalytic activity assay. *In vitro*, it selectively increased the toxicity of 5-FC to A33 antigen-positive cells by 300-fold, demonstrating the potency of this ADEPT strategy.
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Keywords: tumour targeting; A33 antibody; antibody directed enzyme-producing therapy (ADEPT); colon carcinoma; recombinant fusion proteins

Antibody-directed enzyme-prodrug therapy (ADEPT) utilises antibody–enzyme constructs for targeted enzyme delivery to tumours and subsequent localised activation of a prodrug. Its potential has been demonstrated in phase I studies (Webley *et al*, 2001).

Monoclonal antibody A33 recognises a cell-surface antigen that is expressed on ~95% of colon cancers (Garin-Chesa *et al*, 1996). In clinical trials, radiolabelled A33 localised specifically to colon cancer cells, where it was retained for several weeks while clearing within days from normal colon (Welt *et al*, 1996).

Cytosine deaminase (CD) converts 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) and has been empolyed in ADEPT (Wallace *et al*, 1994).

Recombinant fusion constructs should overcome the problems of chemical antibody–enzyme conjugation including inhomogeneous products and large protein size. Several recombinant constructs based on F(ab) and $F(ab')_2$ fragments have been described. Constructs based on single-chain variable fragments (scFv) may have favourable diffusion characteristics in solid tumours, but few descriptions of this approach have been published (Bhatia *et al*, 2000). Here, we report on a new ADEPT concept based on the A33 antigen and recombinant scFv–CD constructs.

MATERIALS AND METHODS

A33scFv (Rader *et al*, 2000) and CD (Austin and Huber, 1993) cDNA were PCR-amplified. Primers based on the published sequences were designed to remove start or stop codons and to add flanking restriction sites so that the DNA could be inserted into the pET 25 expression vector (Novagen, Madison, WI, USA) both directly and downstream of the inserted A33scFv DNA, so that the orientation of the fusion protein was 5′-A33scFv-CD-3′ (vector map available upon request).

A33scFv, CD, and A33scFv-CD and the control construct A33scFv-GFP were expressed by a T7-RNA polymerase-controlled bacterial system using BL21 *Escherichia coli* λDE3 lysogens (Novagen, Madison, WI, USA) at 37°C with IPTG induction at an $OD_{600\,nm}$ of 0.5–0.7. Inclusion bodies were retrieved from cell pellets and solubilised using BugBuster™ reagent with 0.3 $\mu l\,ml^{-1}$ Benzonase and Novagen Refolding Kit (both: Novagen, Madison, WI, USA) according to the manufacturer's instructions. Utilizing a C-terminal histidin tag, the protein was purified on sepharose-bound cobalt (Clontech) with imidazole elution.

Plasmon surface resonance assays were performed as described (Catimel *et al*, 1997) with A33 antigen-coated Biosensor chips. After 400 s, sample flow was replaced by buffer solution. The relative refraction at 600 s was compared with buffer flow and positive controls.

Catalytic activity of cytosine deaminase was determined as described (Austin and Huber, 1993).

For cytotoxicity assays, LIM 1215 or HT 29 tumour cells (Ludwig Institute for Cancer Research cell bank) were incubated on 96-well plates to reach 25–33% surface density. Fusion protein or control was added for 60 min, preceded by 90 min of incubation with "A33scFv-GFP" or hu3S193 IgG (1 $mg\,ml^{-1}$) in blocking experiments. After washing, cells were incubated with prodrug or control for 48 h, washed and grown in medium for 72 h, followed by 3 h in 0.5 $mg\,ml^{-1}$ MTT-solution, DMSO-lysis and photometry at 595 nm.

RESULTS

Protein expression and activity

Fusion proteins were expressed as inclusion bodies with a final culture yield of about 100 $\mu g\,l^{-1}$. With metal affinity chromatography purity was >95% by SDS–PAGE.

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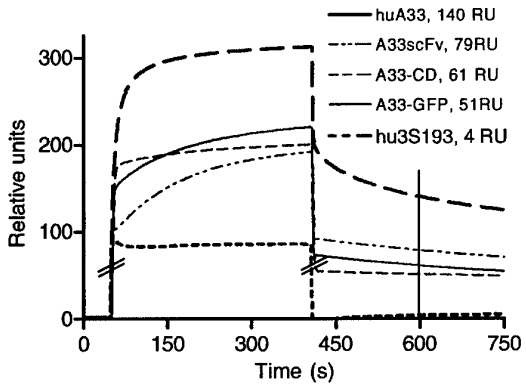

Figure 1 Surface Plasmon resonance. Association and dissociation curves of A33 antibody preparations on an A33 antigen-coated biochip. The chip was exposed to either the complete huA33 IgG antibody (huA33 IgG), the rabbit-derived single chain fragment (A33scFv) or inclusion body preparations of the fusion proteins of A33scFv with either cytosine deaminase (A33scFv–CD IB) or green fluorescent protein (A33scFv–GFP IB). At 400 s, antibody flow was stopped and the chip rinsed with buffer solution. Protein binding is measured by the refraction of a light beam and expressed in relative units (RU) over time. The 600 s time point and the relative units at this point are indicated as approximate correlates of affinity.

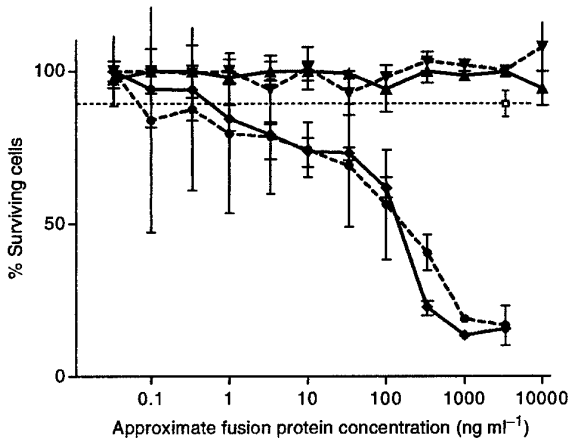

Figure 2 A33scFv–CD-mediated cytotoxicity on A33 antigen-positive *vs* negative cells: LIM1215 cells and HT29 cells were incubated with a dilution series of A33scFv–CD fusion protein and, after washing, with the 5-FC prodrug. Survival was measured by the MTT method as described. A33scFv–CD fusion protein from two different preparations was used on HT29 cells (▲ and ▼) and on LIM1215 cells (● and ◆). As a control, a single, high concentration of A33scFv–GFP (□) was used instead of A33scFv–CD. Mean and s.d. of triplicate samples.

Calculated from the molar extinction coefficient of 1.038 mM^{-1} for 5-FU, the catalytic activity was 2.5 μM min^{-1} for recombinant CD and 0.8 μM min^{-1} for the A33scFv-CD fusion protein.

In surface plasmon resonance, all A33 preparations, but not the 3S193 control, displayed typical association and dissociation curves. Univalent A33scFv showed about half the binding activity of divalent of huA33 IgG (Figure 1), and A33scFv-CD had slightly less binding activity than A33scFv.

ADEPT system *in vitro*

The antigen binding and enzymatic activity of the A33scFv-CD fusion protein was assessed in cytotoxicity assays using complete ADEPT system. The cytotoxicities of 5-FC and 5-FU showed no significant differences between the colon cancer cell lines LIM1215 (A33+) and HT29 (A33–) with an IC_{50} of about 30 mM for 5-FC and 0.3–0.03 mM for 5-FU ($P < 0.05$ for 5-FC *vs* 5-FU, no significant difference between cell lines).

The complete ADEPT system was tested by incubating these two cell lines first with a serial dilution of A33scFv-CD and then, after washing, with the 5-FC prodrug at a fixed concentration. In this assay, crude and purified A33scFv-CD had a dose-dependant cytotoxic effect on A33-positive LIM1215 cells ($IC_{50} \sim 150$ ng ml^{-1}), but not on A33-negative cells ($P = 0.001$ in Wilcoxon rank test). No cytotoxicity was observed with the A33scFv-GFP control (Figure 2).

Without subsequent prodrug incubation, even the highest concentration of fusion protein tested had no cytotoxic effect on A33-positive LIM1215 cells (Figure 3). When binding of A33scFv-CD was blocked by preincubation with "A33scFv-GFP", subsequent 5-FC incubation showed reduced cytotoxicity (IC_{50}, ~30 mM) compared to wells containing the irrelevant isotype control antibody hu3S193 (IC_{50}, <1 mM, $P < 0.01$).

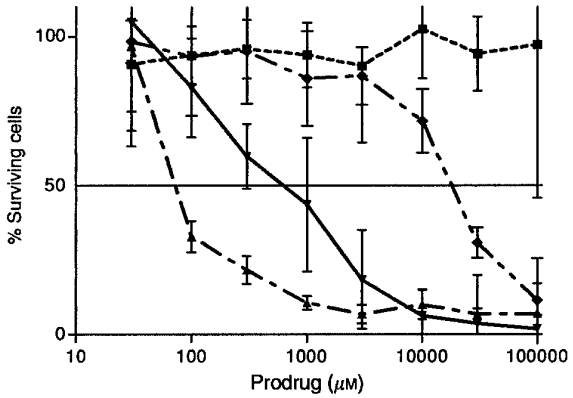

Figure 3 MTT cytotoxicity blocking assay. As a negative control, A33scFv–CD was used without subsequent prodrug incubation (■), and 5-FU alone served as positive control (▲). In the complete ADEPT assay with subsequent 5-FC incubation as described in the text, cells were preincubated either with the "A33scFv-GFP" antibody (◆) or with hu3S193 as an isotypic control antibody (▼) for 1 h before the fusion protein was added. Mean and s.d. of triplicate samples.

DISCUSSION

Two major obstacles have hampered the progress of ADEPT: the needs for specific, accessible antigens and for chemically stable and defined antibody-enzyme constructs of suitable molecular size. The ADEPT system introduced here is novel regarding the targeted antigen and the use of a recombinant scFv-based CD construct.

Incubation of A33-positive tumour cells with this construct increased 5-FC toxicity by about 300-fold, which was selectively blocked by preincubation with "A33scFv-GFP", demonstrating antigen specificity. Neither A33scFv-CD without 5-FC nor a control construct with 5-FC inhibited cell growth, showing that specific enzymatic conversion was necessary for cytotoxicity. Together, these results demonstrate dual (i.e antibody and enzyme) specificity of the construct and functioning of this ADEPT system *in vitro*.

For ADEPT, it is important that CD does not naturally occur in mammalians, making the enzyme construct the exclusive source of

prodrug activation, while allogenic immunogenicity can be addressed by polyethylene-glycol conjugation with preserved A33 binding (Deckert *et al*, 2000).

Only recently has the homohexameric structure of bacterial CD been resolved (Ireton *et al*, 2002). When the described construct showed effective dual function, either its monomer has catalytic activity, or it can form oligomers in solution or after antigen binding. While the published structure supports monomer activity, both hypotheses would explain the lower catalytic activity of A33scFv–CD compared to enzyme alone.

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Specific Tumour Localisation of a huA33 Antibody – Carboxypeptidase-A Conjugate and Activation of Methotrexate-Phenylalanine

Running Title: A33-Carboxypeptidase for ADEPT

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Abstract

In antibody-directed enzyme-prodrug therapy (ADEPT), antibody-enzyme conjugates specifically activate non-toxic prodrugs in tumour tissue. The A33 cognate antigen is a promising target for immunotherapy of gastrointestinal cancers. We have explored A33-based ADEPT with carboxypeptidase A (CPA) and the prodrug, methotrexate-phenylalanine (MTX-Phe).

In A33-positive SW1222 cells, the toxicity of MTX-Phe was about 3 logarithms lower compared to MTX. Preincubation with a huA33-antibody – CPA conjugate (huA33-CPA), but not with an isotypic control conjugate, rendered MTX-Phe equally toxic to MTX. No toxicity was observed in mice receiving MTX-Phe in eightfold the LD_{50} of MTX. Nude mice bearing A33-positive SW1222 colon carcinoma xenografts were injected IV with ^{125}I-labeled huA33-CPA. The conjugate localised to the tumour with a maximum from 6-24 hrs. Pre-treating these mice with excess A33 substantially reduced subsequent conjugate uptake, demonstrating immunologic specificity of tumour-uptake. Total tumour uptake and ratios of tumour over blood or normal tissues, however, were lower than with unconjugated A33. This may explain in part why no significant tumour responses were observed in xenografted mice. In summary, our results demonstrate in principle the feasibility of A33-based enzyme targeting, but they call for small recombinant antibody-enzyme constructs to facilitate tumour penetration and clearance from the bloodstream.

Keywords: Tumour targeting; A33 antibody; methotrexate; prodrug; ADEPT; colon carcinoma

Introduction

Systemic chemotherapy remains the only therapeutic option for metastasised colon cancer, yet its efficacy is low with response rates below 50 percent and limited survival. Strategies for tumour-specific drug delivery such as antibody-directed enzyme-prodrug therapy (ADEPT) are being investigated to reduce toxicity and improve the efficacy [Napier et al., 2000]. In ADEPT, an antibody is used to direct a coupled enzyme to tumour tissue. After clearance of unbound enzyme, a non-toxic prodrug is injected and converted into a cytotoxic metabolite by the tumour-localised enzyme [Begent and Bagshawe, 1996].

A number of ADEPT systems have been proposed that differ in the antigen used for targeting and the enzyme-prodrug pair employed. The group of F. Huennekens introduced methotrexate-alpha-phenylalanine as a prodrug to be converted into methotrexate by bovine carboxypeptidase A [Esswein et al., 1991; Vitols et al., 1995]. Clinical trials on ADEPT have shown that the principle can be applied successfully in man [Martin et al., 1997] [Webley et al., 2001].

Monoclonal antibody (mAb) A33 recognises a recently characterised cell-surface differentiation antigen of approximately 43 kDa that belongs to the immunoglobulin superfamily [Heath et al., 1997]. It is expressed on normal human gastrointestinal epithelium and on ~ 95 % of primary or metastatic colon cancers, but is absent in most other normal tissues [Garin-Chesa et al., 1996]. Upon binding to the A33 antigen, the antibody is internalised into a yet incompletely characterised vesicular compartment, and a significant fraction of the internalised antibody cycles back to the cell surface [Daghighian et al., 1996]. The A33 antigenic system has been the focus of several clinical studies in patients with colon cancer. Phase I/II clinical trials have shown that murine mAb A33 (i) localises with high specificity to colon cancer tissue; (ii) is retained for prolonged periods of up to 6 weeks in the cancer but clears within 5 to 6 days from normal colon; and (iii) has anti-tumour activity as a carrier of ^{125}I or ^{131}I [Welt et al.,

1996]. A humanised version of the A33 antibody (huA33) has been constructed [King et al., 1995] and evaluated in a clinical phase I trial [Welt et al., 2003].

The methotrexate alpha-peptide methotrexate-phenylalanine (MTX-Phe) has been demonstrated to be 2 to 3 logarithms less cytotoxic than methotrexate. Carboxypeptidase-A (CPA) converts this prodrug into MTX with high affinity, thus completely restoring the cytotoxicity of the parent drug [Vitols et al., 1995].

Here we present preclinical investigations into the feasibility of an ADEPT approach based on the A33 antibody and the MTX-Phe– CPA prodrug activation system.

Methods

Animals and cell lines. Forty-four to 49 day old female CD-1-*nu* mice (nude mice; Charles River, Sulzfeld, Germany) were maintained in an enriched environment at the Nuclear Medicine Animal Facility of the Charité – Campus Benjamin Franklin. The animal experiments were performed with strict adherence to animal welfare guidelines under protocol G 0302/01, approved by the animal protection agency of the local government, Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin.

The human colon carcinoma cell lines SW1222 and HT29 were from the cell bank of the Ludwig Institute for Cancer Research at MSKCC. Cells were maintained at 37°C and 5% CO_2 in Eagle's minimum essential medium supplemented with 1 % (v/v) non-essential amino acids and 10 % (v/v) foetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (culture medium), and harvested using 0.1 % (v/v) trypsin and 0.02 % (v/v) EDTA (all reagents: Gibco/BRL, Eggenstein, Germany).

Antibody-enzyme conjugation: Forty nanomoles of huA33 or hu3S193 were derivatised with 1.6 mMol succinimidyl 3-(2-pyridyldithio)propionate (SPDP) in acetonitrile at a molar ratio of 1/6 in 1.2 ml 0.1 M phosphate buffer pH 7.4 for 1 h at 20°C and then centrifuged to remove any precipitate. In parallel, 200 nmol bovine CPA type II (Sigma-Aldrich, Schnelldorf, Germany) was derivatised with 1.6 mM SPDP at a molar ratio of 1/1.1 for 50 min at 20°C. For use in biodistribution experiments, radio-labelled CPA was used (see below). Immediately prior to the conjugation reaction, CPA-SPDP was deprotected by equimolar reduction with dithiothreitol (dTT). The conjugation reaction of antibody-SPDP and deprotected CPA-SPDP to a molar ratio of 1:5 in a total volume of 2.0 ml sterile filtered and degassed 0.02 mM phosphate and 1 M NaCl, pH 8.0, proceeded for 14 hrs at 20°C.

Purification: Fresh conjugate was filtered to remove any precipitate, and loaded onto 5 ml Protein-A Sepharose 4 FF (Pharmacia) in 0.1 M phosphate buffer pH 8.0. Protein was eluted stepwise with 0.1 M sodium citrate of pH 6.0 to pH 3.0. Conjugate and unconjugated antibody formed a single fraction at ~ pH 4.0. Unbound antibody was removed by FPLC anion-exchange chromatography on a WP-PEI 4.6 x 50 mm column (Bakerbond through Fisher Scientific, Schwerte, Germany) in 20 mM Tris-HCl, pH 8.0 with an elution gradient from 0.15 to 1 M NaCl. The conjugate fraction was eluted at 0.2 to 0.25 M NaCl.

Antibody binding activity (mixed hemadsorption assay): Binding of immunoglobulin to SW 1222 tumour cells was detected by erythrocyte-bound protein A (indicator erythrocytes) as previously described [Welt et al., 1994]. The antibody binding titre was defined as the highest dilution that produced unequivocal rosetting of erythrocytes on tumour cells.

Enzyme activity: CPA or the antibody-CPA conjugate to be tested were dissolved in 0.1 M Tris and 0.2 M zinc sulfate at pH 7.3 and mixed with 1 nmol of hippuryl-L-phenylalanine to a final protein concentration of 1 µg/ml in a final total volume of 1 ml and immediately measured photometrically at 254 nm for 10 minutes in a quartz cuvette heated to 37°C.

With an extinction coefficient at 254 nm of 0.36, the specific activity was determined as

U/min = ((Δ mAbs/min) / 0.36) x (1 / protein (µg)) [Vitols et al., 1995].

Stability of the conjugate in vitro: 100 µg of purified conjugate were dissolved in 1 ml of human or murine serum, incubated for up to fourteen days at 37°C, and compared by SDS-PAGE with Coomassie staining.

Synthesis of methotrexate-alpha-phenylalanine (MTX-Phe). MTX-Phe was synthesised by reaction of the p-nitrophenyl ester of 4-amino-4-deoxy-10-methylpteroic acid with L-glutamyl-alpha-L-phenylalanine and purified by high-pressure liquid chromatography to > 99 % purity as described previously [Vitols et al., 1995]. Size and identity of the final product were verified by thin-layer chromatography. Biological

activity after CPA-mediated cleavage was determined by a cytotoxicity assay with unconjugated CPA.

MTT cytotoxicity assay. Suspensions of SW1222 or HT29 cells were adjusted to 50,000 cells/ml in culture medium (see above), plated on 96 well plates to 100 µl per well and incubated at 37°C with 5 % CO_2 until they covered approximately 25 to 33 % of the well surface. At this point, the antibody-enzyme construct or respective control was added in the concentrations indicated (see Results). In blocking experiments, this step was preceded by a pre-incubation of 60 to 120 minutes with the blocking antibody. After additional incubation for 60 min, the plates were washed twice with medium, and the prodrug or control was added. The plates were incubated under the same conditions as before for 48 h, then washed again and incubated with medium alone for another 72 h. Twenty microlitres of 2.5 mg/ml MTT were added per well, and incubation was continued for 3 to 4 hrs. The medium was then replaced by 200 µl DMSO and incubated under mild agitation at 20°C for 30 minutes. Colour intensity correlated with the number of viable cells and was quantified photometrically at 595 nm.

Radio-labelling of huA33 or CPA. CPA prior to conjugation or huA33 were radio-labelled using iodogen beads (Iodo-Beads, Pierce-Perbio, Bonn, Germany), a solid-phase modification of the chloramine T method. Two beads were submersed in 100 µl of sodium iodide 125 (^{125}I, 74 MBq, Amersham Biosciences, Freiburg, Germany). After 5 min, 1.0 mg of protein was added in a volume of 1 ml. After another 7.5 minutes, the reaction was terminated by removing the iodogen beads from the vial, and the product was purified on a sterile Sephadex G25 column (Amersham) preconditioned with 5 % human serum albumin in sterile saline. The protein fractions were pooled and sterile filtered. After conjugation of huA33 with ^{125}I-CPA, specific activity was 19.3 MBq/mg. Immunoreactivity was determined by absorption of 0.1 µg/ml huA33–^{125}I-CPA conjugate with sequential tubes containing 2 x 10^7 antigen-positive cells [Welt et al., 1994]. Background activity was determined by pre-treating cell pellets with a > 100-fold excess

of unlabeled antibody prior to adding radio-labelled antibody. Immunoreactivity was calculated by subtracting background radioactivity (counts per minute, CPM) from cell-bound ^{125}I radioactivity after washing twice in PBS, and dividing the remaining fraction by the total CPM added. Immunoreactivity measured by this method was 37 %.

SW1222 xenografts in nude mice. Nude mice were injected with 10^7 washed SW1222 cells in 150 µl sterile buffer (0.15 M NaCl and 0.1 M sodium phosphate at pH 7.4) into the left thigh muscle. Subsequent experiments were conducted when the tumour had reached a diameter of 0.4 to 0.6 cm, corresponding to a weight of 350 to 400 mg.

Tissue dosimetry of huA33–^{125}I-CPA in mice. SW1222 or HT29 tumour xenograft-bearing nude mice and naive controls were injected IV with protein-equalised doses of 5 to 20 µg (~ 0.24 to 0.97 MBq) radio-labelled conjugate in 100 µl of sterile buffer (0.15 M NaCl, 0.1 M sodium phosphate, pH 7.2).

For organ distribution and tumour uptake studies, mice were sacrificed by neck dislocation after systemic anaesthesia with isoflurane. Lung, liver, spleen, kidneys, and tumour were resected and weighed and a blood sample obtained. The radioactive dose was measured by an automated gamma counter (1277 Gammamaster, LKB Wallac, Erlangen, Germany) and compared to an aliquot of the injected preparation as a standard. Relative doses were calculated as *% injected dose/g = 100 · (sample CPM ÷ sample mass [g]) ÷ injected CPM.*

In-vivo stability of MTX-Phe. Mice were intravenously injected with escalating doses of 12, 24, 36, and 48 mg/kg MTX-Phe. The next higher dose was only applied 48 hrs after the previous dose if no signs of toxicity had been observed thus far.

In vivo application of the ADEPT system. Mice received tumour xenografts as described above and, when the tumour diameter was 4 to 6 mm four to five days later, were injected IV with 20 µg of huA33-CPA conjugate. Another 48 hours later, mice were

injected IV with 12 or 24 mg/kg of MTX-Phe. As the treatment had been tolerated well, the MTX-Phe application was repeated after 24 and 48 hours. The animals were then observed until either the tumour shrank or grew to a diameter greater than 1 cm, or no change occurred for up to 14 days. At these points, or when the tumour appeared to impede the well-being of the animals as judged by their mobility and behaviour, mice were euthanised as described above.

Results

Characterisation of the antibody-enzyme conjugate. CPA and A33 or the isotypic control antibody 3S193 were conjugated using SPDP as a linker. Unbound CPA and, partially, unbound antibody were removed by protein-A affinity and anion exchange chromatography. Polyacrylamide gel electrophoresis (SDS-PAGE) revealed two major bands and a smaller one, corresponding to conjugates with ratios of CPA to antibody between 2 and 5. A band of the size of unreacted antibody was visible, but no unbound CPA could be identified (figure 1a). The yield of total conjugated antibody was 12 to 16 % of the starting material.

The catalytic activity of the enzyme component was determined photometrically using hippuryl-phenylalanine as a substrate. Catalytic activity was 17.3 U/mg of conjugated protein as compared to 38.5 U/mg in the unconjugated enzyme, corresponding to the calculated CPA proportion of the conjugate.

Antibody binding was determined by an established hemadsorption assay with A33 antigen-positive SW1222 colon-carcinoma cells as targets. Cell-bound antibody or conjugate was detected by indicator erythrocytes coated with protein A. The rosetting titre of huA33-CPA was two to four times lower than that of unconjugated huA33. As the distribution of the various conjugation ratios could not be determined exactly, the molar concentration could only be estimated. With this limitation, the binding activity corresponded to the antibody proportion of the conjugate (figure 1b).

No substantial conjugate dissociation in human serum was observed for five days at 37°C as determined by SDS-PAGE (not shown).

CPA renders MTX-Phe cytotoxic *in vitro*. To establish the model ADEPT system *in-vitro*, MTT cytotoxicity assays were performed on SW1222 cells with MTX-Phe in the presence or absence of CPA and compared to incubation with unmodified MTX. The ID_{50} of MTX-Phe was found to be 3×10^{-5} M, while that of MTX was about 1000 times

lower. When CPA was co-incubated with MTX-Phe, the ID_{50} of MTX-Phe was reduced to match that of MTX with 3×10^{-8} M. This was not due to toxicity of the CPA preparation in itself, as a titration series of CPA alone showed no influence on cell growth (figure 2).

huA33-CPA activates MTX-Phe with immunological specificity for A33-positive cells. To test the complete ADEPT system in the described MTT assay, cells were pre-incubated with huA33-CPA or the hu3S193-CPA control conjugate before being washed and then incubated with MTX-Phe. After pre-incubation with 10 µg/ml huA33-CPA or more, a safely non-toxic MTX-Phe dose of 10^{-6} M inhibited cell growth by 50%, whereas pre-incubation with hu3S193-CPA had no effect on cell growth (figure 3).

To investigate further whether this effect was due to immunologically specific antibody binding, cells were incubated with excess unconjugated huA33 prior to the conjugate incubation. This A33-block almost completely removed the effect of huA33-CPA preincubation on MTX-Phe toxicity (figure 3).

Radio-labelled huA33-CPA conjugate localises to A33-positive tumour xenografts in mice. To assess the kinetics and distribution of the huA33-CPA conjugate, SW1222 xenograft-bearing mice were injected IV with ^{125}I-labelled huA33-CPA doses of 20 µg for time course studies or from 5 to 20 µg per animal for biodistribution, corresponding to approximately 0.25 to 1.0 mg/kg of body weight. After 3 to 72 hours or after 24 hours, respectively, mice were sacrificed and tumour, blood, and for biodistribution, key organs were measured in a gamma counter.

The time course showed that after 6 hours the tumour dose had reached its maximum until elimination set in after 24 hours, whereas the blood dose was continuously decreasing from the first measurement at three hours (figure 5). All SW1222 tumours of mice treated with huA33-CPA showed a high radioactive dose compared to blood and most organs. Compared to treatment with radio-labelled unconjugated huA33, however, the dose ratios of tumour to blood and tumour to normal organs were considerably lower.

Especially liver and lung consistently showed high radioactive uptake. In one case, the uptake in spleen was even higher than the tumour dose, but this was not seen in the other animals and may be due to inadvertent contamination (figure 4).

In control mice without tumour, thigh muscle was measured instead, and one group of mice was inoculated with the A33-negative cell line HT29 as a control. In both groups, no significant uptake was observed. One group of mice had been pre-treated with an excess of unconjugated huA33 four hours before receiving 20 µg of radio-labelled huA33-CPA. This pre-treatment lead to a marked reduction in tumour uptake and an over-proportional decrease in the tumour-to-blood ratio.

MTX-Phe is stable in vivo and tolerated well in mice. Mice were treated intravenously with increasing amounts of MTX-Phe up to a dose of 48 µg, which corresponds to eight times the lethal dose of MTX. The prodrug was tolerated well without any physiological or behavioural signs of toxicity, indicating that the prodrug was stable and not activated spontaneously under physiological conditions in mice.

ADEPT in vivo: Tumour-xenografted mice show only little tumour response. Nude mice in groups of three were inoculated with SW1222 xenografts and treated with huA33-CPA as described above, but the conjugate was not radio-labelled and was applied in a uniform dose of 20 µg per animal (1.0 g/kg body weight). After allowing 48 hours for the clearance of unbound conjugate from the circulation, the animals received 12 or 24 mg/kg MTX-Phe. All mice tolerated the treatment well, so after 24 and 48 hours, the same MTX-Phe doses were repeated. Tumour growth appeared to be retarded in the mice with the highest dose of MTX-Phe as compared to controls, but this effect was not significant according to both the Mann-Whitney test and the non-paired t-test with Welsh's correction, and no remissions or stabilisations were observed before the mice had to be euthanised due to the large tumour burden (figure 6).

Discussion

Since antibody-directed enzyme-prodrug therapy (ADEPT) has first been proposed [Philpott et al., 1974], it has met a number of obstacles, yet its general feasibility could be demonstrated in clinical trials [Martin et al., 1997; Webley et al., 2001].

Here we report on an ADEPT system based on the activation of MTX-Phe by bovine CPA as first introduced by the group of F.M. Huennekens [Esswein et al., 1991; Vitols et al., 1995] and on the A33 antigen. This system was functional in vitro with immunological and catalytic specificity: huA33-CPA bound selectively to A33 antigen-positive cells, and only after binding of the conjugate did MTX-Phe inhibit cell growth with the ID_{50} of MTX. An isotypic control conjugate could not induce this effect, and it was completely blocked by pre-incubation with excess huA33.

The antibody-enzyme construct also localised to tumour tissue with immunological specificity in mice bearing A33 antigen-positve tumour xenografts, as demonstrated by its absence in control tumours and by its blocking through pre-treatment with unconjugated A33. Tumour-to-blood ratios, however, were considerably lower than with huA33, and in some cases organs such as lung, liver, and spleen accumulated doses similar to those in tumour.

This latter finding may be due to non-specific uptake by diffusion, to redistribution of tumour-bound antibody during the elimination phase, or to cross-reactivity with yet unknown pulmonary or hepatic antigens. We did not formally prove or reject any of these explanations, but theoretical considerations and some hints in our results make all three likely to play a role: Initial biodistribution of an antibody is in fact by passive diffusion, and only after antigen binding is it retained in tissue whereas unbound antibody moves on and is finally eliminated. Thus, distribution specificity is a matter of the early elimination phase, which is why we chose this time, with (almost) maximum tumour dose but already diminished blood dose, for the biodistribution studies. When a large amount of antibody becomes eliminated after being bound to tumour, it may circulate again and be

redistributed into other capillary beds, which may explain higher doses in organs than in blood. In mice pre-treated with excess unconjugated huA33, however, organ uptake was much lower than in mice receiving huA33-CPA without pre-blocking, hence antibody binding does seem to be involved in organ uptake.

While the very rationale of immunological tumour targeting is specificity, i.e. high tumour-to-blood and tumour-to-organ ratios, the observed organ uptake did not translate into toxicity: None of the mice showed any signs of adverse effects even with the highest prodrug doses. However, the findings of low tumour-to-blood ratios and high organ doses go along with a lack of efficacy in vivo. If at all, only a small difference in tumour progress between treated animals and controls could be observed, which was not statistically significant, and in no case tumour growth was stopped or reversed.

While our results demonstrate that enzyme targeting and prodrug-activation via the A33 antigen are feasible, the proposed ADEPT system was far from being efficient in vivo. Why was that so?

The A33 antigen is expressed in the whole intestine, but radio-iodinated A33 has shown remarkable tumour specificity with tolerable gastrointestinal toxicity in clinical trials [Johnstone et al., 2002; Welt et al., 1994]. The time-dependent specificity described in the Introduction is probably not due to different expression in tumour versus normal cells, but to different internalisation and surface recycling [Daghighian et al., 1996; Heath et al., 1997; Johnstone et al., 2002]. While radio-immunotherapy will profit from internalisation as it brings the radiation source closer to the nucleus, in ADEPT it will reduce the proportion of bound enzyme available on the cell surface. On the other hand, surface recycling may prolong the overall time window for subsequent and repeated prodrug administration, making the kinetics and efficacy of A33-based ADEPT difficult to predict.

Hence the lack of anti-tumour effect in this study does not preclude clinical efficacy of this ADEPT system, but may rather be due to limitations of the mouse xenograft model such as tumour growth kinetics and volume: The SW1222 xenografts grew much

faster than colon cancers in humans do, reaching the equivalent of a 1.6 kg tumour burden in an 80 kg man within four to six days. Thus, even if perfectly efficient tumour targeting were achieved on day 1, administration of the prodrug on day 1+x will find the conjugate diluted by the growth of new tumour cells in the meantime. The absolute tumour volume, too, exceeds that normally treated by chemotherapy rather than surgery in a clinical setting. Thus, our mouse xenograft model may be overly pessimistic. The same applies to details of the ADEPT schedule, which were not investigated extensively: An earlier onset of prodrug administration, more repetitive prodrug injections at closer intervals, and higher prodrug doses – we chose one-half of the safe dose without testing its limit – may all improve efficacy.

Thus, despite the lack of efficacy in a particular mouse model, this study proved the principle of A33-based ADEPT. Nonetheless, there are principal limitations to the enzyme-prodrug system we used. With maximum tumour-to-blood ratios of less than 3, the biodistribution of the antibody-enzyme conjugate showed less tumour specificity than unconjugated huA33. This finding was concordant with a previous study of similar design on PEG-conjugated huA33, which also found increased non-specific organ uptake and reduced tumour-to-blood ratios [Deckert et al., 2000]. PEG-huA33 and CPA-huA33 were of similar size, while these conjugation partners differ fundamentally in their chemical and biological properties, suggesting that the loss of specificity is caused by the increase in size. In addition, the chemical conjugation method does not provide a homogenous product. Even if a uniform conjugation ratio could be obtained, the conjugation sites of the antibody molecule would still be randomly assigned, allowing for undefined variations in antibody affinity.

Together, these limitations of the conjugate call for smaller recombinant constructs, based e.g. on antibody single-chain fragments (scFv) [Deckert et al., 2003]. CPA, however, is expressed as a pro-enzyme and requires post-translational N-terminal trypsin cleavage for activation [Laethem et al., 1996]. This complicates the construction of bifunctional fusion proteins as no N-terminal linkage will be possible and trypsin may

also cleave the antibody fragment. For these reasons, unpublished efforts of our laboratories to produce a recombinant scFv-CPA fusion construct were not successful.

In addition, although the relative toxicity of MTX over MTX-Phe was high, MTX as such is only a moderately toxic drug. The true potential of ADEPT, however, is to be seen in the concentrated local activation of highly toxic drugs that would not be tolerated otherwise [Connors, 1995; Double, 1992].

In summary, while the A33 antigen remains an important target for immunotherapy and ADEPT, other enzyme-effector systems will have to be utilised for the design of new recombinant fusion proteins and "war heads" of higher local toxicity.

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Legends to Figures

Figure 1: Characterisation of the huA33-CPA conjugate: a) SDS-PAGE: lane 1, unconjugated huA33; 2, unconjugated CPA; 3, huA33-CPA conjugate after protein-A purification and, lane 4, after anion-exchange chromatography. b) Hemadsorption assay on SW 1222 cells with huA33 and huA33-CPA conjugate before and after purification: highest dilutions producing rosette formation, linear scale.

Figure 2: In vitro toxicity of drug and prodrug and prodrug activation by CPA. A33 antigen-positive SW1222 cells were incubated with the indicated concentrations of MTX (▲) or MTX-Phe in the absence (Δ) or presence (♦) of a fixed concentration of 10 µg/ml CPA. A 1:3 dilution series of CPA, starting with 100 µg/ml (□), and untreated cells (○) were used as controls.

Figure 3: Immunological specificity of the complete ADEPT system in vitro: SW1222 cells were first incubated with a 1:3 dilution series of either huA33-CPA (♦) or the isotypic control conjugate 3S193-CPA (□) and then with a fixed concentration of 10^{-6} M MTX-Phe. As controls, cells were either left without MTX-Phe (○) or were pre-incubated with an excess amount of unconjugated huA33 (200 µg/ml) prior to huA33-CPA incubation (Δ).

Figure 4: Biokinetics of ^{125}I-labelled huA33-CPA. SW1222 tumour-bearing mice were injected ^{125}I-labelled huA33-CPA and sacrificed at the indicated time points after injection. Blood (○) and tumour doses (●) were measured.

Figure 5: Biodistribution of ^{125}I-labelled huA33-CPA. SW1222 tumour-bearing mice were injected huA33-CPA in doses of 20, 10, or 5 µg. One group of mice each was bearing no tumour or an A33-negative control tumour, one group of mice was pre-treated with an excess amount (100 µg) of unconjugated A33 prior to the huA33-CPA injection, and one group received huA33 instead of A33-CPA conjugate. Bars indicate the average percent injected dose in each tissue and treatment group, error bars show the standard deviation. "T/B" indicates the average dose ratio of tumour over blood for each treatment group. The tissues measured for each group were from top to bottom: blood (empty), lung (large upward hatches), kidney (vertical hatches), liver (large downward hatches), spleen (fine upward hatches), tumour (solid). See text for details.

Figure 6: Tumour growth over time with or without ADEPT treatment. SW1222 tumour-bearing mice were left untreated (○) or received either MTX-Phe 24 mg/kg without huA33-CPA pretreatment (Δ), or huA33-CPA conjugate 20 µg (1.0 mg/kg) and, 48, 72, and 96 hours later, MTX-Phe in doses of 12 µg (◊) or 24 µg (♦). Arrows indicate treatment points.

FIGURE 1

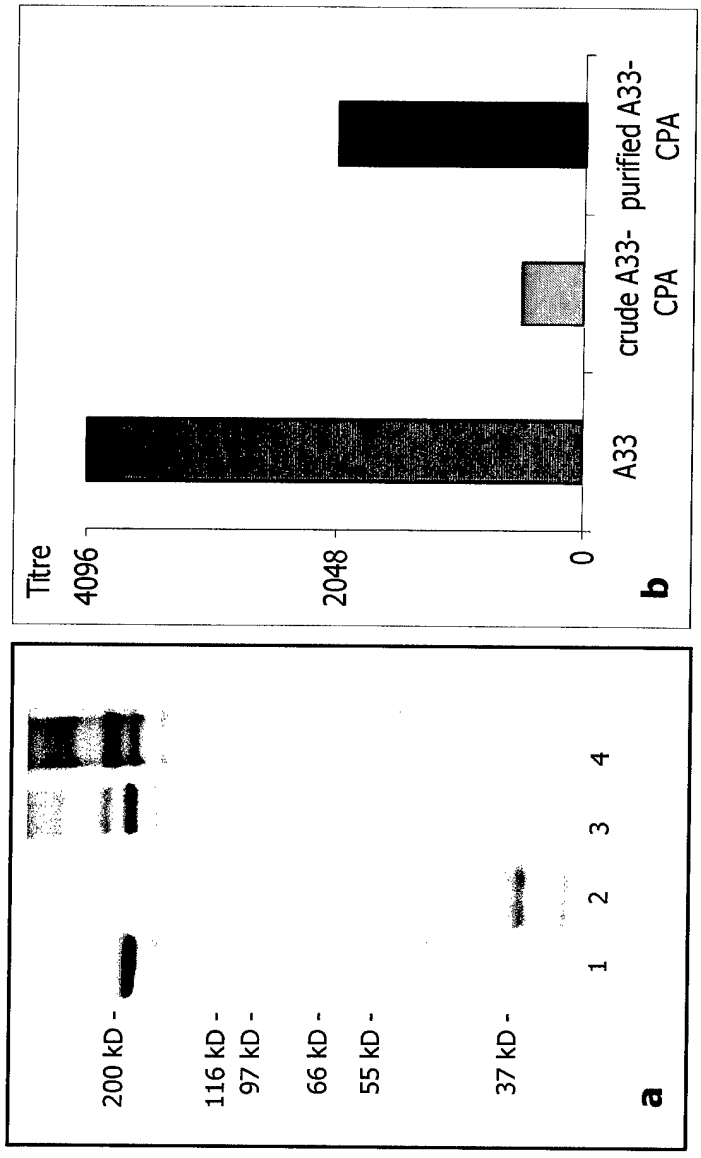
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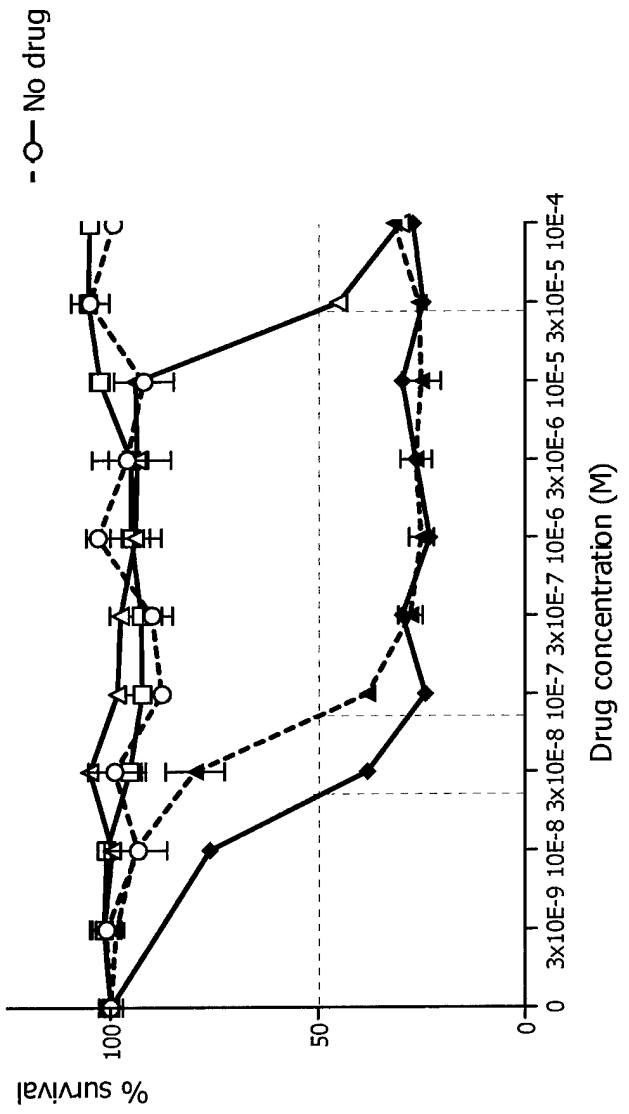
FIGURE 2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Drug concentration (M)

FIGURE 3

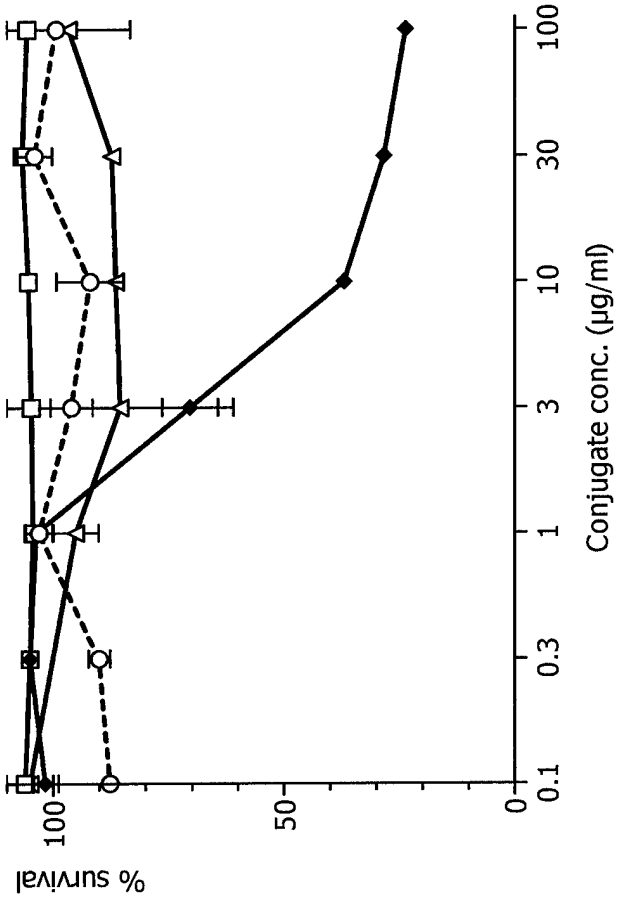

FIGURE 4

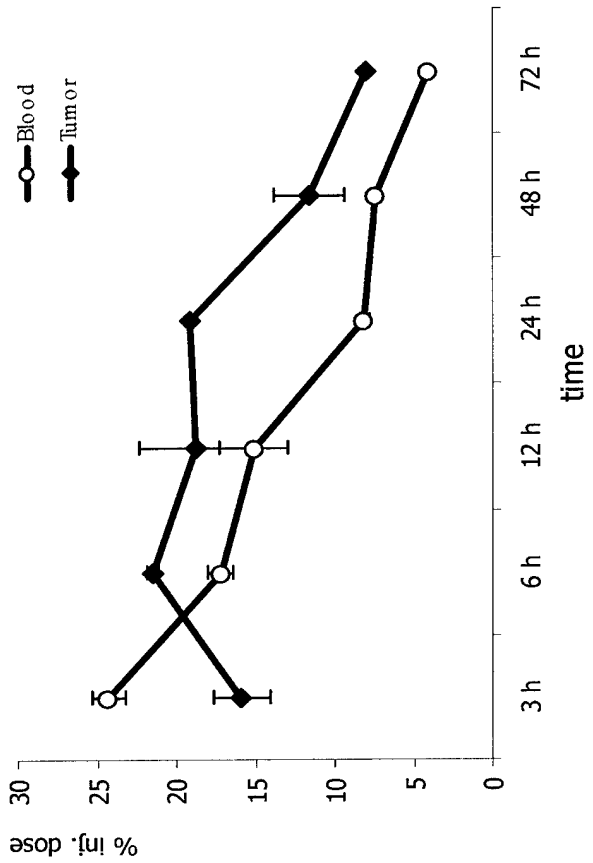

FIGURE 5

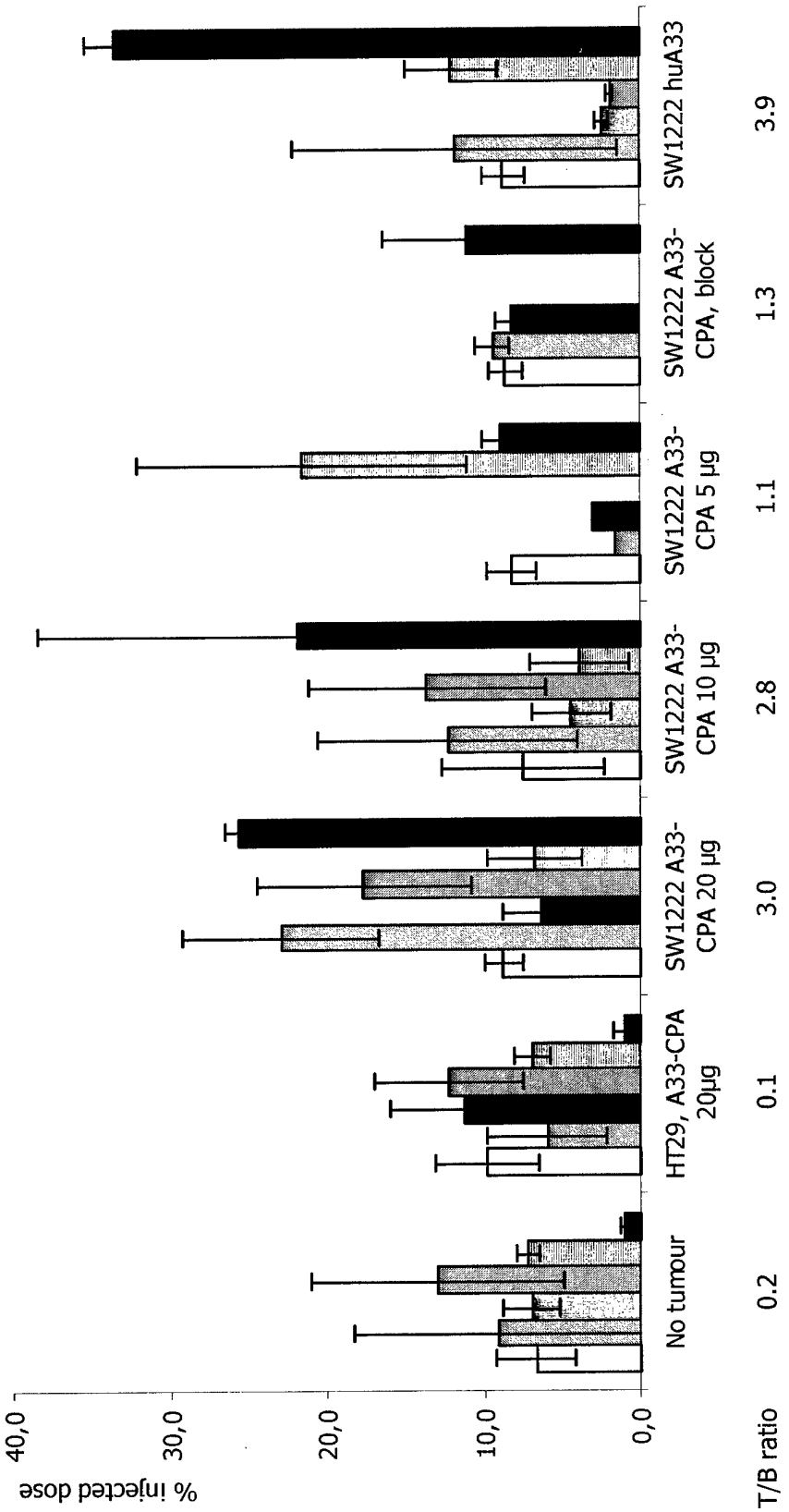
40,0
30,0
20,0
10,0
0,0
% injected dose
No tumour
HT29, A33-CPA 20µg
SW1222 A33-CPA 20 µg
SW1222 A33-CPA 10 µg
SW1222 A33-CPA 5 µg
SW1222 A33-CPA, block
SW1222 huA33
T/B ratio
0.2
0.1
3.0
2.8
1.1
1.3
3.9

FIGURE 6