Cancer Immunol Immunother (2001) 50: 102–108

ORIGINAL ARTICLE

**Christoph Renner · Ingo Stehle · Fook Thean Lee
Cathrine Hall · Bruno Catimel · Edouard C. Nice
Angela Mountain · Angela Rigopoulos
Martin W. Brechbiel · Michael Pfreundschuh
Andrew M. Scott**

Targeting properties of an anti-CD16/anti-CD30 bispecific antibody in an in vivo system

Received: 26 October 2000 / Accepted: 15 December 2000

Abstract Bispecific antibodies are currently being used in clinical trials in increasing numbers in the areas of breast cancer, prostate cancer, non-Hodgkin's lymphoma and Hodgkin's lymphoma. We have previously performed two clinical trials in patients with Hodgkin's disease with an anti-CD30/anti-CD16 bispecific antibody and demonstrated a 30% response rate in a cohort of patients otherwise resistant to standard therapeutic modalities. However, no surrogate marker could be defined in these trials indicative of optimal antibody dosing/scheduling or predictive for favorable response. In order to evaluate accurately the potential biodistribution properties of bispecific antibody in patients, we have performed a detailed analysis of the binding properties and animal model in vivo characteristics of these constructs. For this purpose, the parental antibodies (anti-CD30 and anti-CD16) and the bispecific antibody (anti-CD30/anti-CD16) were radiolabeled with either ^{125}I or ^{111}In. Antibody integrity and binding properties after labeling were confirmed by Scatchard plot and Lindmo analysis. ^{111}In-labeled antibodies revealed superior targeting properties in a standard SCID mouse tumor model. Both the bivalent parental anti-CD30 monoclonal antibody and the monovalent anti-CD30/anti-CD16 bispecific antibody showed excellent uptake in CD30$^+$ tumors which did not differ significantly between the two (maximum uptake 16.5% ± 4.2% vs. 18.4% ± 3.8% injected dose/gram tissue). The equivalent targeting properties of the bispecific antibody compared with the parental anti-CD30 antibody encourages the further clinical development of this bispecific antibody, and might help to explain the clinical responses seen with this antibody so far in patients suffering from Hodgkin's disease.

Key words Bispecific antibodies · Hodgkin's lymphoma · Tumor targeting

C. Renner (✉) · I. Stehle · M. Pfreundschuh
Medical Department I, Saarland University,
Kirrberger Strasse, 66424 Homburg/Saar,
Homburg, Germany
Tel.: +49-6841-163002; Fax: +49-6841-163101

C. Renner · F. T. Lee · C. Hall · B. Catimel · E. C. Nice
A. Mountain · A. Rigopoulos · A. M. Scott
Ludwig Institute for Cancer Research,
Austin & Repatriation Medical Center,
Heidelberg, Victoria, Australia

M. W. Brechbiel
Radioimmune and Inorganic Chemistry Section,
ROB, DCS, National Cancer Institute,
National Institutes of Health,
Bethesda, Maryland, USA

Introduction

With the re-emerging clinical interest for antibody-based approaches in cancer therapy [18, 27], attention is now focusing on strategies to improve the genuine cytotoxic activity of monoclonal antibodies, in order to achieve higher and longer-lasting remission rates. The major handicap of unconjugated monoclonal antibodies (Mabs) is their variable and usually low cytotoxic potential. Therefore, concepts have been developed that take advantage of the high tumor-specificity of Mabs for the specific delivery of cytotoxic drugs, enzymes or radioisotopes [6, 28]. In addition, bispecific antibodies (Bi-Mabs) were generated which are able to redirect potential cytotoxic effector cells to the tumor site [7, 22, 35]. Immunological effector cells that can be activated by such Bi-Mabs include granulocytes, macrophages, natural killer (NK), and T-cells [8, 10, 30]. The advantage of Bi-Mabs is their ability to bridge tumor and effector cells together and to induce local cell destruction subsequently. This concept of Bi-Mabs has proven its efficacy in multiple tumor systems in vitro, and in pre-clinical studies with animal models in vivo [13, 23, 25].

We have performed two clinical trials with an anti-CD30/anti-CD16 Bi-Mab in a total of 31 patients with Hodgkin's disease so far [12, 26]. In both studies, an objective response of 30% following antibody treatment

had been demonstrated. However, neither the first trial which was performed as a dose escalation trial nor the second one where two different infusion regimens were compared, allowed us to design larger phase II/III clinical trials. No surrogate marker emerged in these trials that could be used to optimize antibody dosing and scheduling in order to improve efficacy. This dilemma is not unique to our Hodgkin studies but is typical for almost all antibody-based trials [2, 36, 37].

As a consequence, clinical trials with Bi-Mabs and other biological response modifiers have to be redesigned. Most importantly, a clear distinction between chemotherapy and antibody trials has to be made, as maximum tolerated dose is not an appropriate goal in the field of immunotherapy. The basic fundamental of antibody trials is the fact that the antibody to be tested has to target the tumor specifically with an optimal tumor to normal tissue ratio [29]. Therefore, we initiated the present study to define the binding properties, and conditions needed for radiolabeling of our anti-CD30/anti-CD16 Bi-Mab. We also sought to examine in a SCID mouse model the biodistribution properties of the Bi-Mab in comparison with the parental antibodies.

Material and methods

Antibodies and cell lines

The generation, purification and characterization of the parental anti-CD30 (HRS-3) and anti-CD16 (A9) antibodies and of the bispecific anti-CD16/CD30 antibody have been described previously [12, 13]. A33, a murine antibody directed against a novel antigen found in over 95% of colorectal cancers [16], was used as a sub-class specific control. The recombinant CD30 antigen was generated in our laboratory and has been described previously [24]. Recombinant CD16 antigen was kindly supplied by Dr. C. Sautes, Institut Curie, Paris, France.

The established $CD30^+$ human tumor cell line L540CY has been described elsewhere [4]. SW1222, a CD30-antigen-negative human colonic cancer cell line, was used as a control cell line. All cell lines were cultured under standard conditions in 5% CO_2 atmosphere at 37 °C. RPMI 1640 supplemented with standard antibiotics and 10% FCS (all Gibco, Karlsruhe, Germany) was used, and is referred as complete medium throughout the text. Cell viability in all experiments, as determined by trypan blue exclusion, exceeded 90%.

Plasmon resonance analysis (BIAcore)

Analyses were performed with a BIAcore 2000 biosensor (Pharmacia Biosensor, Uppsala, Sweden) [19]. Carboxymethyldextran-coated sensor chip, CM5 (Research grade) and the amine coupling reagents (*N*-ethyl-*N*′-dimethylaminopropyl-carbodiimide (EDC), hydroxysuccinimide (NHS) and ethanolamine) were obtained from Pharmacia Biosensor. CD30 and CD16 antigens and the related Mabs were re-purified, immediately prior to use in immobilization and kinetic studies, by micropreparative HPLC using a Superose 12 HR 3.2/30 size-exclusion column equilibrated in HBS (10 m*M* HEPES (pH 7.4), 3.4 m*M* EDTA, 150 m*M* NaCl) to ensure homogeneity. The column was connected to a SMART μHPLC system (Pharmacia Biotech, Uppsala, Sweden) and eluted at a flow rate of 100 μl/min. Monoclonal antibody concentrations were determined by ultra-violet absorption at 280 nm using A280 (1 mg/ml) of 1.46. CD16 and CD30 antigen were immobilized onto the biosensor surface using the conventional NHS/EDC chemistry. Purified antibodies were diluted in HBS buffer prior to analysis. Samples (30 μl) were injected over the sensor surface at a flow rate of 10 μl/min. Following completion of the injection phase, dissociation was monitored in HBS buffer for 360 s at the same flow rate. Residual bound antibody was desorbed, and the surface regenerated between injections, using 30 μl of 10 m*M* NaOH. This treatment did not denature the surface, as shown by equivalent signals on re-injection of an antibody-containing sample.

Kinetic analysis of the biosensor data

Binding data were generated by passing varying concentrations of the antibodies 20, 40, 60, 80 and 100 n*M*) over the immobilized CD16 and CD30 antigens. For the Bi-Mab, which should exhibit monovalent binding, the apparent association and dissociation rate constants were calculated from regions of the sensorgrams where 1:1 Langmurien interactions appeared to be operative, with BIAevaluation version 3.0 software that was supplied by the manufacturer.

The goodness of fit between experimental data and fitted curves was estimated by chi-squared analysis using the equation: $\chi^2 = {}_1\Sigma^n(r_f - r_x)^2/n - p$.

Radiolabeling and quality assurance

The parental anti-CD30 antibody HRS-3 and the anti-CD16/anti-CD30 bispecific A9/HRS-3 antibody were labeled with ^{125}I or ^{111}In isotopes, respectively. Isotopes were obtained from Dupont, Life Science Products, Boston, Mass. Radioiodination was performed using a modification of a previously published chloramine-T reaction [14] using a 2.5-fold molar excess of chloramine-T (Merck, Darmstadt, Germany) over antibody, dissolved in 0.5 *M* potassium phosphate buffer (pH 7.2). After a 2-min incubation period the reaction was stopped by the addition of a 10-fold excess of sodium metabisulfite, again dissolved in a 0.5 *M* phosphate buffer, and then purified through a desalting column (P6DG BioRad, Australia) equilibrated with phosphate-buffered saline (PBS).

Antibody labeling with ^{111}In was achieved via a bifunctional metal-ion conjugate, CHX-A″-DTPA [38] using a modification of a previously published method [20]. In brief, a dialysis bag with an Mr cut-off of 10,000 kD (Novex, Sydney, Australia) was treated with 10 m*M* sodium bicarbonate, soaked in deionized water and 1 m*M* EDTA (pH 7.0) to remove heavy metals. Antibodies were dialyzed against a 50 m*M* sodium bicarbonate buffer (pH 8.6) containing 0.15 *M* NaCl for 6 h. CHX-A″-DTPA was added in a molar excess of 5:1 and incubated at room temperature (RT) overnight in the dark. Excess unbound antibody was removed by 8-h dialysis with 20 m*M* sodium acetate buffer containing 0.15 *M* NaCl (pH 6.3). ^{111}In was bound to CHX-A″-DTPA antibody conjugate under acidic conditions (pH 5.5, 20 min), then the pH was raised to 7.0 by the addition of 2.0 *M* sodium acetate followed by 10 m*M* EDTA. The radiolabeled mixture was purified by centrifugal desalting on a Sephadex G50 column equilibrated with PBS.

Radiolabeling was performed on the day of the experiment or of injection into mice, respectively. Prior to use, percentage of unbound radionuclide content was determined by instant thin-layer chromatography (ITLC) [20, 34], and binding ability of the final radiolabeled product was tested by a cell-binding assay. Scatchard analysis was used to determine the binding constant (K_a) and number of antibody molecules bound per cell for ^{125}I- and ^{111}In-labeled antibody. The percentage of antibody-bound isotope was $>95\%$ in all experiments detailed.

Immunoreactivity assay

Immunoreactivity assay (IR) of radiolabeled antibodies to L540CY target cells was determined by linear extrapolation to binding at infinite antigen excess using a 'Lindmo' assay [17]. Twenty nano-

grams of radiolabeled antibodies were added to a range of L540CY cell concentrations and incubated (RT, 45 min) with continuous mixing throughout to keep the cells in suspension. Cells were washed three times, and pellets were measured in a gamma counter (Cobra II, Model 5002, Auto-gamma, Packard Instruments, Canberra, Australia). Three samples of radiolabeled antibody, at the same concentration as that initially added to the cells, were measured at the same time as cell pellets, and percentage binding of antibody to L540CY cells was calculated by the formula: (cpm cell pellet/mean cpm radioactive antibody standards) × 100. Percentage binding was graphed against L540CY cell concentration, and IR calculated as the y intercept of the inverse plot of both values. Cell pellets to which > 100-fold unlabeled antibody was added, prior to radiolabeled antibody, were used as a control to determine background cpm.

Scatchard analysis was used to calculate the apparent association constant (K_a) and number of antibody molecules bound per cell [17]. Unlabelled antibodies at concentrations ranging from 0.625–20 μg/ml were added to 1×10^6/ml L540CY cells and mixed, and subsequently 20 ng of the respective labeled antibody were added. Cells were washed 45 min after incubation, and counted as described above. Calculations were done as described by Collet [3]. When specific bound was plotted against specific bound/free reactive activity, the slope of the straight curve obtained gave the K_a constant of the reaction.

Animal model

In vivo tissue biodistribution was performed in 5 to 6-week old female CB17 SCID mice bred by the SPF Facility, University of South Australia as described [23]. Mice were maintained in autoclaved micro-isolator cages housed in a positive pressure containment rack (Thoren Caging Systems, Hazelton, Pa.).

Suspended in PBS at a volume of 400 μl, 2.5×10^7 L540CY cells were injected subcutaneously into the left inguinal mammary line. SW1222 colon cancer xenografts were established by injection of 5×10^6 cells in the right inguinal area of each mouse, as a control tumor. The growth rates of both tumors differed. To obtain comparable tumor sizes, we injected the more rapidly growing SW1222 colon cancer cells 5 days after inoculation of L540CY tumors. Both tumors were of a size appropriate for use 14 days after this time. Tumor volume was calculated by the formula (length × width^2)/2 where length was the longest axis and width the measurement at right angles to length [32].

Biodistribution studies

Mice were injected intravenously via the retro-orbital plexus with 10 μCi of ^{125}I- or ^{111}In-CHX-A″-DTPA labeled antibody (total 4 μg of antibody, 10 μCi radioactivity) suspended in 150 μl of sterile media. Groups of 4–5 mice, with a mean ± SD tumor volume of 647 mg ± 252, were killed by cervical dislocation following light Ethrane (enflurane) anesthesia at 4, 24, 48, 72 and 96 h (for ^{125}I) or at 4, 24, 48, 72 and 144 h (for ^{111}In) after injection of radiolabeled antibody. Mice were bled via cardiac puncture and the blood collected into heparinized tubes. Tumors and organs (skin, liver, spleen, intestine, stomach, kidneys, brain, bone (femur), lungs, and heart) were immediately removed, blotted dry and weighed (Sartorius Basic Balance, Germany). All samples were counted in a dual chamber gamma scintillation counter (Cobra II, Auto-gamma, Packard Instruments, Canberra, Australia) using a dual tracer program with standard windows set for each isotope. Standards prepared from the injected material were counted each time with tissues and tumors, enabling calculations to be corrected for the isotopes' physical decay. Results of labeled antibody distribution over time were expressed as percent injected dose per gram (%ID/g: (cpm tissue sample/cpm standard) × 100/weight in g) and as tumor to blood ratios. Wilcoxon rank sum tests were performed on the different treatment groups at each time point and between the tissues collected to assess differences observed for statistical significance. Blood clearance kinetics were calculated using a curve fitting program (SAMII; University of Washington, Seattle, USA) and assuming a two-compartment model.

Results

Binding properties of the parental and bispecific antibody

As a first step, the true binding values for the parental antibodies (anti-CD30 and anti-CD16) and the anti-CD30/CD16 Bi-Mab were determined by BIAcore analysis (Fig. 1). The parental CD30 antibody (HRS-3) demonstrated a strong binding, with an affinity constant (K_D) of 4.8×10^{-11} M. The bivalent anti-CD16 antibody (A9) did not bind as strongly to its target antigen, and possessed a more average affinity ($K_D = 1.55 \times 10^{-9}$ M). However, there was a significant loss of affinity for both antibodies when binding monovalently as they do in the Bi-Mab. This effect was more pronounced for the anti-CD30 binding arm. The final affinity in the Bi-Mab construct was $K_D = 2.6 \times 10^{-9}$ M for the anti-CD30, and $K_D = 3.77 \times 10^{-8}$ M for the anti-CD16 site.

Radiolabeling of antibodies

To perform biodistribution experiments in mice, we radiolabeled antibodies with two different radionuclides (^{111}In and ^{125}I), respectively. The proper binding of all antibodies after labeling was proven by Lindmo analysis (data not shown). Scatchard plot experiments revealed that the antibodies had similar affinity constants for their respective targets compared with the initial BIAcore data, indicating that the labeling procedure did not alter significantly the antigen recognition site (data not shown). Inhibition experiments confirmed in a cell-based assay that the binding affinity of the bivalent parental anti-CD30 antibody for the membrane expressed CD30 antigen on Hodgkin cells was higher than the Bi-Mab (Fig. 2). Radiolabeled Bi-Mab was easier to compete-off with unlabeled parental anti-CD30 antibody, and radiolabeled parental anti-CD30 antibody was harder to compete-off with unlabeled Bi-Mab.

Biodistribution analysis in vivo

The ^{125}I/^{111}In-labeled biodistribution study results are presented in Fig. 3. The comparison of %ID/g of ^{125}I- and ^{111}In-labeled antibodies in Hodgkin and control tumors, and at each time point, are detailed. The %ID/g of both isotopes in Hodgkin tumor xenografts peaked for the parental anti-CD30 and the Bi-Mab between 48 and 72 h after antibody injection, with the uptake of ^{111}In-labeled antibodies being up to three times greater than for ^{125}I, and reaching statistical significance

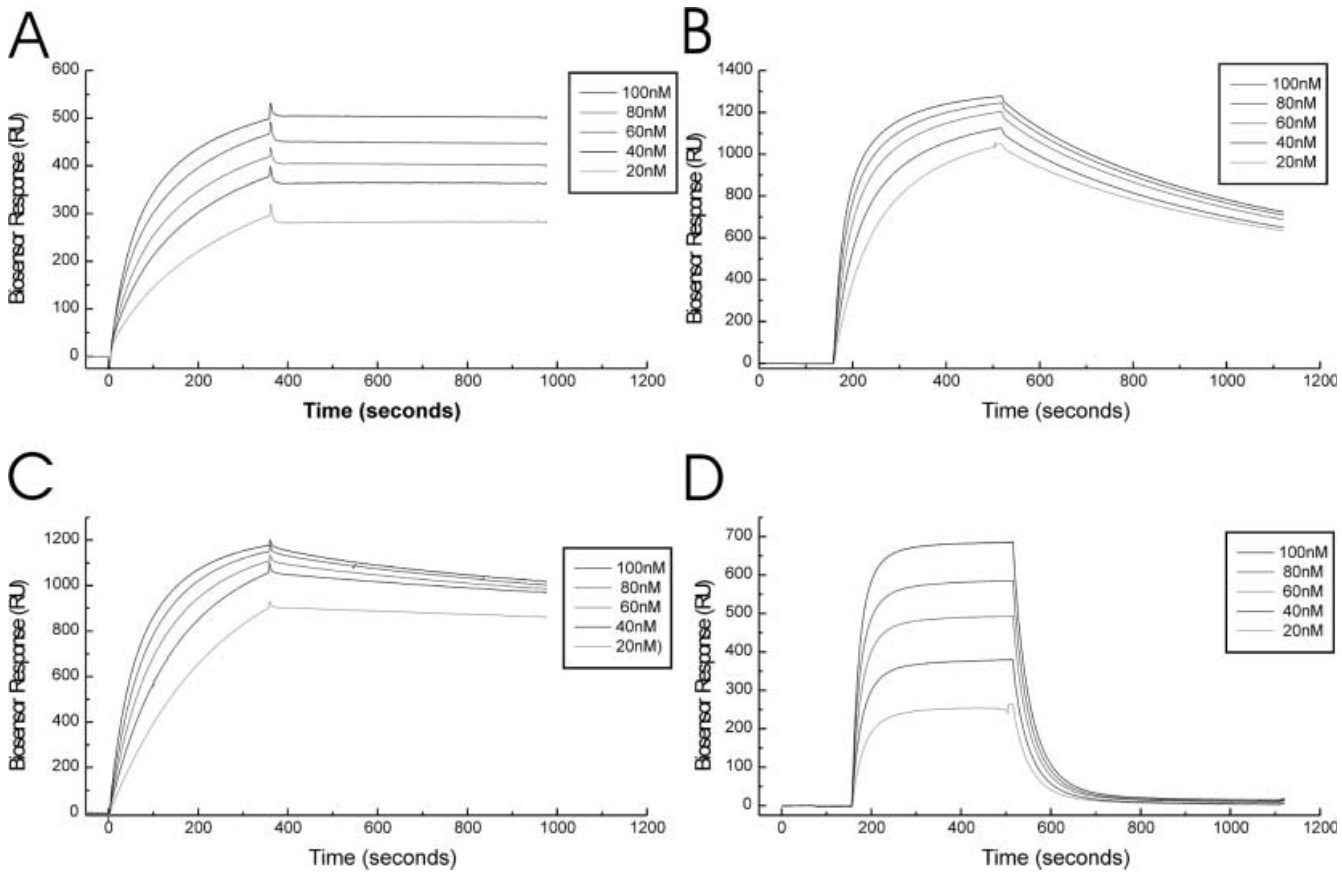

Fig. 1A–D Biosensor analysis of the interaction between anti-CD16, anti-CD30 and bispecific anti-CD16 and anti-30 monoclonal antibodies on immobilized CD16 and CD30 antigens. Varying concentrations of antibodies (20–100 n*M*) were injected (30 µl) at a flow rate of 5 µl/min over immobilized antigen. The sensorgrams shown have been automatically subtracted with the corresponding signal obtained when the same sample was passed over a control blank channel. **A** CD16 IgG over immobilized CD16 antigen; **B** bispecific anti-CD16/anti-30 IgG over immobilized CD30 antigen; **C** CD30 IgG over immobilized CD30 antigen; **D** bispecific anti-CD16/anti-30 over immobilized CD16 antigen

($P = < 0.01$; Fig. 3A). Most importantly, there was no difference in the biodistribution properties of the Bi-Mab when compared with the parental anti-CD30 antibody. The blood levels of both isotopes closely approximated each other throughout the study, declining progressively over time (Fig. 3B). The $T_{1/2\beta}$ as an indicator for clearance of antibody from the body was determined to be 78.84 h for the ^{111}In-labeled Bi-Mab and 85.98 h for the parental anti-CD30 Mab (HRS-3). The tumor to blood ratio for the ^{111}In-labeled parental anti-CD30 and the ^{111}In-labeled Bi-Mab peaked at 1.78 ± 0.1 (6 days) and at 2.05 ± 0.05 (6 days), respectively.

The biodistribution of radiolabel in normal murine tissues was also studied, and results at different time points for different organs are presented in Fig. 4. This detailed analysis was done only with ^{111}In-labeled antibodies, in view of the poor tumor uptake properties of the ^{125}I-labeled antibodies. The ID/g in all normal tissues was less than 15%. Relatively high values in the liver for both antibodies were most likely due to indium metabolism. Antibody uptake (%ID/g) in SW1222 control tumors was less than that seen in CD30 antigen-positive Hodgkin L540 tumors at all time points except at 4 and 24 h. The higher %ID/g at these early stages most likely reflects differences in tumor vascularity between SW1222 and Hodgkin L540 xenografts, with increased blood pool activity being seen in the more vascular SW1222 tumor (data not shown). Both parental anti-CD30 and Bi-Mab uptake in control tumors reflected blood pool activity rather than specific tumor uptake, with low peak uptake and a decline progressively over time.

Discussion

In an attempt to restructure our antibody program on the anti-CD16/CD30 Bi-Mab, we have analyzed the biodistribution pattern of our Bi-Mab in the Hodgkin SCID mouse model established previously [23]. In vitro assays proved that the parental and Bi-Mabs could be labeled efficiently with ^{125}I and ^{111}In. In the SCID mouse system, specific tumor uptake of the parental anti-CD30 and Bi-Mab was observed compared to CD30-antigen-negative control tumor, with the peak tumor (L540) uptake for both antibodies occurring between 48–72 h post-injection. The %ID/g for ^{111}In was significantly

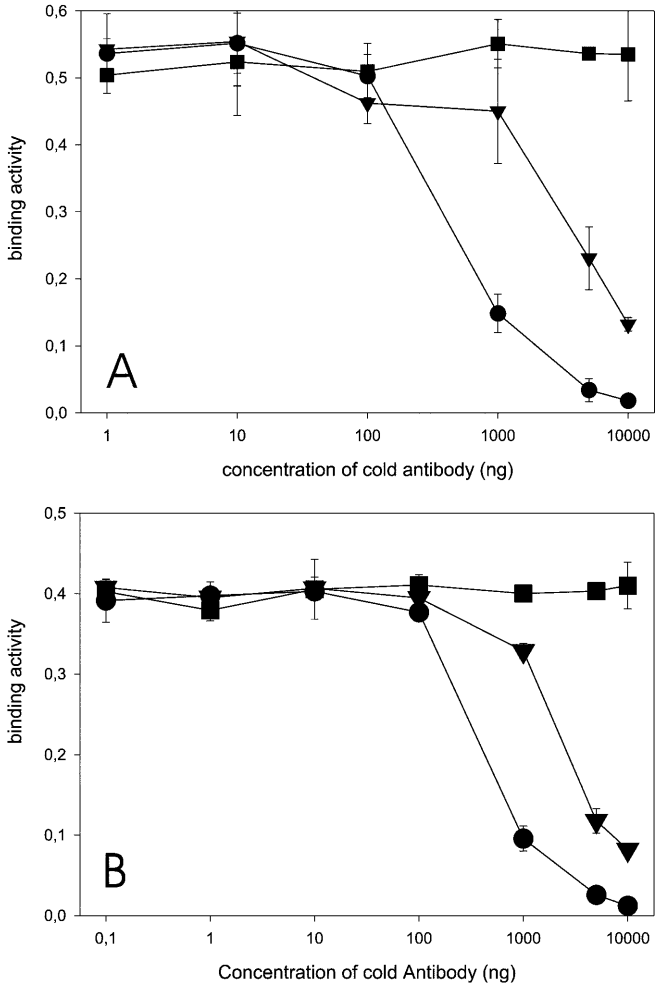

Fig. 2A, B Cross-inhibition of antibodies. Cold parental anti-CD16 (*square*), parental anti-CD30 (*circle*) and anti-CD16/anti-CD30 bispecific antibodies (*Bi-Mab*) (*triangle*) were analyzed for their ability to block antigen binding of non-saturating amounts of either **A** 20 ng parental anti-CD30 monoclonal antibodies (*Mab*) or **B** 20 ng anti-CD16/anti-CD30 Bi-Mab, respectively. The Hodgkin's-derived L540CY cell line was used as target. Standard deviation is indicated by the *bars*

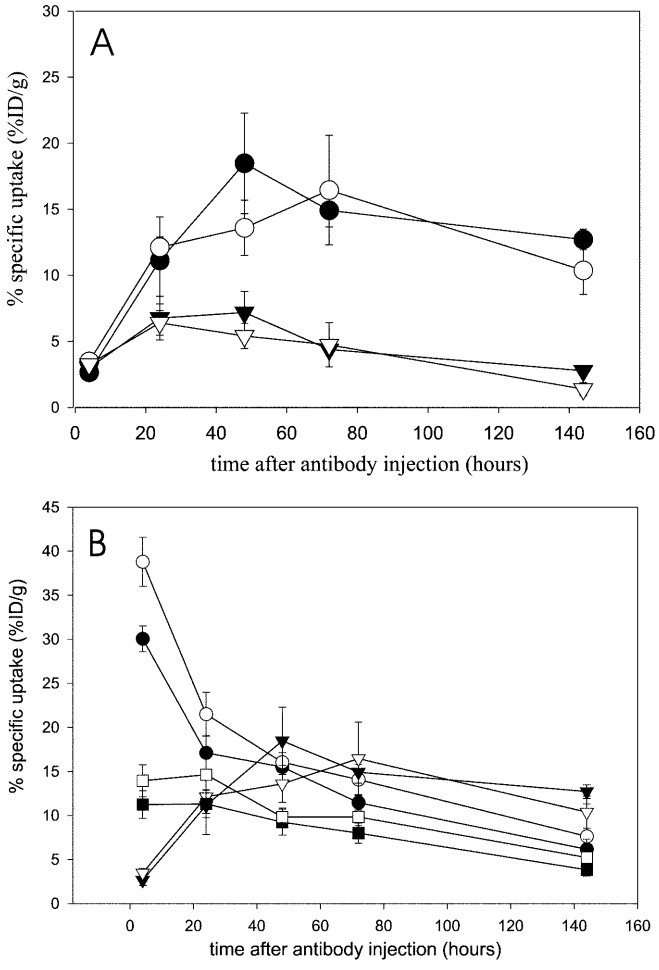

Fig. 3A, B Impact of radionuclide on biodistribution. **A** Specific tumor uptake of ^{111}In-labeled (*circle*) or ^{125}I-labeled (*triangle*) parental anti-CD30 monoclonal antibodies (*Mab*) (*open symbols*) and bispecific anti-CD16/anti-CD30 antibodies (*Bi-Mab*) (*closed symbols*) were analyzed in tumor-bearing SCID mice. Mice were killed at the indicated time points up to 144 h and the percentage of injected dose per gram of Hodgkin's tumor was determined. **B** Enrichment of antibodies in CD30-positive tissues. Biodistribution properties of ^{111}In-labeled parental (*open symbols*) or bispecific antibody (*closed symbols*) was analyzed in blood (*circle*), CD30-antigen-positive Hodgkin's tumors (*triangles*) and CD30-antigen-negative colon cancer (*square*) at the indicated time points. Standard deviation is indicated by the *bars*

higher than for ^{125}I. In addition, ^{111}In was retained within xenografts for a longer period of time than was ^{125}I. Lower absolute levels of iodine uptake than ^{111}In in xenografts have been demonstrated in other studies [16], and this, together with differences in tumor retention of radiolabel, most likely represents differences in cellular processing of radiolabeled ^{125}I and ^{111}In. Catabolism of ^{125}I linked to tyrosine results in the generation of [^{125}I]mono-iodotyrosine within lysosomes, which rapidly leaves the cell via cell-mediated transport systems [21, 34]. In contrast, ^{111}In-DTPA antibodies following endocytosis are delivered to lysosomes and hydrolyzed by lysosomal enzymes into low molecular-weight metabolites which are retained within tumor lysosomes [5, 21]. Within normal tissues, the uptake of ^{111}In in liver was high, which is consistent with known sequestration of radiometals in reticuloendothelial organs [33].

To our surprise, there were no differences in tumor targeting properties between the parental bivalent anti-CD30 antibody and the monovalent Bi-Mab despite in vitro results having revealed a significant difference ($K_D = 4.8 \times 10^{-11}$ M for the parental compared with $K_D = 2.6 \times 10^{-9}$ M for the Bi-Mab) in the binding affinity of these antibodies with the CD30 target antigen. Penetration into the tumor and clearance from the tumor cells was virtually identical for both constructs. In addition, both antibodies were very similar with regard to their biodistribution profile in CD30-antigen-negative tissue and elimination from the body. The impact of affinity on successful targeting retention of antibodies in tumors is still controversial. It has been proposed that

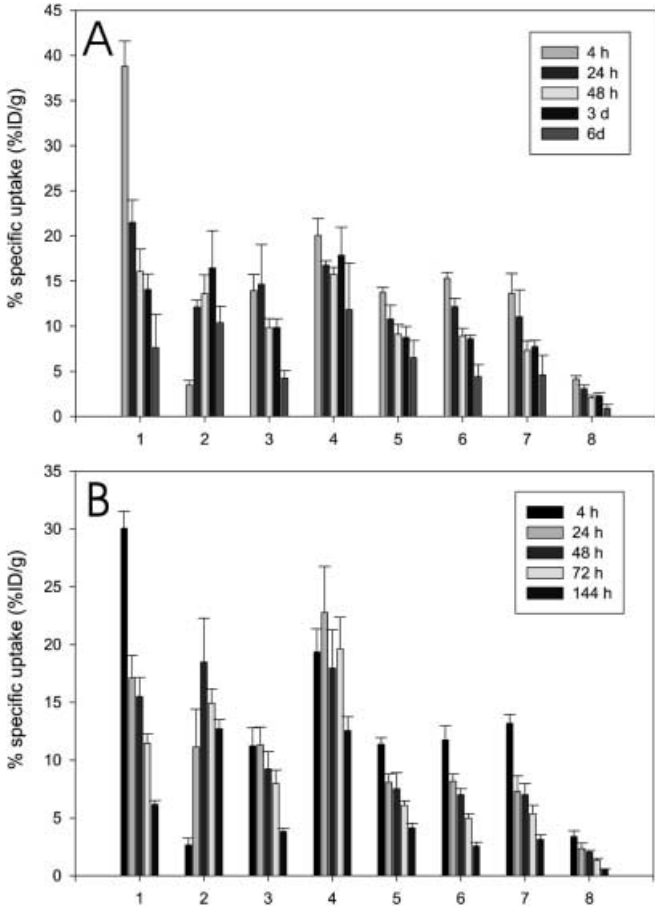

Fig. 4A, B Tissue biodistribution of ^{111}In-labeled antibodies in SCID mice with tumor xenografts. **A** Parental anti-CD30 monoclonal antibody (*Mab*) ($n = 4$–5 mice); **B** bispecific anti-CD16/anti-CD30 antibody ($n = 4$–5 mice). Organs and tissues were resected at indicated time points and analyzed as described. Standard deviation is indicated by the *bars*. Tissues to be analyzed: blood (*1*), Hodgkin's tumor (*2*), colon tumor (*3*), liver (*4*), spleen (*5*), kidney (*6*), lung (*7*) and small bowel (*8*)

high-affinity antibodies do not successfully penetrate deeply into tumors due to a binding site barrier effect. This obstacle has been attributed to interaction with the first antigen encountered at the periphery of the tumor, blocking further diffusion of the antibody into the tumor [1, 9, 15]. However, evidence that increased affinity prolongs tumor retention of radiolabeled antibody comes from studies using TAG-72 specific antibodies. In these studies, high-affinity anti-TAG-72 antibodies localized human tumor xenografts in nude mice better than did the low-affinity ones. Recently, Adams and coworkers [1] published a study where they had generated affinity mutants of a HER2/*neu* specific scFv antibody by site-directed mutagenesis and compared these constructs, with regard to their tumor targeting properties. They demonstrated that the antibody variants used had to cross an affinity threshold to achieve selective tumor penetration and retention. Increasing affinity above a certain threshold did not correlate with a further significant increase in tumor uptake. In the case of our Hodgkin model, the high affinity of the parental anti-CD30 monoclonal antibody seems to be sufficient to achieve good tumor penetration and binding in a bivalent and monovalent format. We could not mimic the situation of the human body, where we have a circulating pool of CD16-antigen-positive cells competing for binding with the Bi-Mab. However, we fortuitously discovered the affinity of the Bi-Mab for the CD16 antigen to be eight times lower than its affinity for the CD30 antigen. This difference in affinity might enable the antibody to penetrate into the CD30-antigen-positive tumor in sufficient amounts, and activate local CD16-antigen-positive effector cells. This theory is supported by our clinical responses seen in Hodgkin's patients after treatment with the Bi-Mab but remains still unproven and is the subject of further investigations.

Based on our pre-clinical and clinical experience with Bi-Mabs in Hodgkin's lymphoma we suggest that Bi-Mab trials should be re-designed. Lacking any accepted predictive markers, clinical studies with Bi-Mabs should focus at the initial stage on in vivo properties such as tumor targeting and antigen accessibility. This proposition is certainly true for native Mabs but can be transferred to Bi-Mabs as well. Bispecific antibodies aim at initiating and perpetuating a local immune response at the tumor site. The most appropriate area for the use of Bi-Mabs (and other biological response modifiers) will be the field of minimal residual disease [31]. As a prerequisite, the Bi-Mab has to be tested and optimized in its structure and application regimen for optimal tumor targeting efficacy. In the second step of a clinical trials program with Bi-Mabs, attention has to focus on the efficient activation of the immune system leading to local tumor destruction and the eventual induction of a systemic immune response [11]. However, the analysis of the proper activation of the immune system has to follow the tumor targeting aspect, as the efficient activation of the immune system without adequate targeting may result in general and non-specific toxicity.

Acknowledgements The study was supported in part by a grant from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie.

References

1. Adams GP, Schier R, Marshall K, Wolf EJ, McCall AM, Marks JD, Weiner LM (1998) Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. Cancer Res 58: 485
2. Canevari S, Mezzanzanica D, Mazzoni A, Negri DR, Ramakrishna V, Bolhuis RL, Colnaghi MI, Bolis G (1995) Bispecific antibody targeted T cell therapy of ovarian cancer: clinical results and future directions. J Hematother 4: 423
3. Collet B, Maros S, Moisan A, Le Cloirec J, Moinereau M, Aumaitre E, Toujas L, Bouguet P (1993) ^{111}Indium-F(ab)′$_2$-NCA 102 monoclonal antibody: in vitro study of a specific agent for the detection of inflammatory foci. Nucl Med Biol 29: 175
4. Diehl V, von Kalle C, Fonatsch C, Tesch H, Juecker M, Schaadt M (1990) The cell of origin in Hodgkin's disease. Semin Oncol 17: 660

5. Duncan JR, Stephenson MT, Wu HP, Anderson CJ (1997) Indium-111-diethylenetriaminepentaacetic acid-octreotide is delivered in vivo to pancreatic, tumor cell, renal, and hepatocyte lysosomes. Cancer Res 57: 659
6. Engert A, Martin G, Amlot P, Wijdenes J, Diehl V, Thorpe P (1991) Immunotoxins constructed with anti-CD25 monoclonal antibodies and deglycosylated ricin A-chain have potent antitumour effects against human Hodgkin cells in vitro and solid Hodgkin tumours in mice. Int J Cancer 49: 450
7. Fanger MW, Graziano RF, Guyre PM (1994) Production and use of anti-FcR bispecific antibodies. Immunomethods 4: 72
8. Ferrini S, Prigione I, Miotti S, Ciccone E, Cantoni C, Chen Q, Colnaghi MI, Moretta L (1991) Bispecific monoclonal antibodies directed to CD16 and to a tumor-associated antigen induce target-cell lysis by resting NK cells and by a subset of NK clones. Int J Cancer 48: 227
9. Fujimori K, Covell DG, Fletcher HJ, Weinstein JN (1990) A modeling analysis of monoclonal antibody perlocation therough tumors: a binding site barrier. J Nucl Med 31: 1191
10. Garrido MA, Perez P, Titus JA, Valdayo MJ, Winkler DF, Barbieri SA, Wunderlich JR, Segal DM (1990) Targeted cytotoxic cells in human peripheral blood lymphocytes. J Immunol 144: 2891
11. Grosse-Hovest L, Brandl M, Dohlsten M, Kalland T, Wilmanns W, Jung G (1999) Tumor-growth inhibition with bispecific antibody fragments in a syngeneic mouse melanoma model: the role of targeted T-cell co-stimulation via CD28. Int J Cancer 80: 138
12. Hartmann F, Renner C, Jung W, Deisting C, Juwana M, Eichentopf B, Kloft M, Pfreundschuh M (1997) Treatment of refractory Hodgkin's disease with an anti-CD16/CD30 bispecific antibody. Blood 89: 2042
13. Hombach A, Jung W, Pohl C, Renner C, Sahin U, Schmits R, Wolf J, Kapp U, Diehl V, Pfreundschuh M (1993) A CD16/CD30 bispecific monoclonal antibody induces lysis of Hodgkin's cells by unstimulated natural killer cells in vitro and in vivo. Int J Cancer 55: 830
14. Hunter WM, Greenwood FC (1962) Preparation of iodine-125 labeled growth hormone of high specific radioactivity. Nature 194: 495
15. Juweid M, Neumann R, Paik C, Perez-Bacete MJ, Sato J, van Osdol W, Weinstein JN (1992) Micropharmacology of monoclonal antibodies in solid tumors: direct experimental evidence for a binding site barrier. Cancer Res 52: 5144
16. King DJ, Antoniw P, Owens RJ, Adair JR, Haines AMR, Farnsworth APH, Finney H, Lawson ADG, Lyons A, Baker TS, Baldock D, Mackintosh J, Gofton C, Yarranton GT, McWilliams W, Shochat D, Leichner PH, Welt S, Old LJ, Mountain A (1995) Preparation and preclinical evaluation of humanised A33 immunoconjugates for radioimmunotherapy. Br J Cancer 72: 1364
17. Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA (1984) Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. J Immunol Methods 72: 77
18. McLaughlin P, Grillo-Lopez AJ, Link BK, Levy R, Czuczman MS, Williams ME, Heyman MR, Bence-Bruckler I, White CA, Cabanillas F, Jain V, Ho AD, Lister J, Wey K, Shen D, Dallaire BK (1998) Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. J Clin Oncol 16: 2825
19. Nice EC, Catimel B (1999) Instrumental biosensors: new perspectives for the analysis of biomolecular interactions. Bioessays 21: 339
20. Nikula TK, Curcio MJ, Brechbiel MW, Gansow OA, Finn RD, Scheinberg DA (1995) A rapid single vessel method for preparation of clinical grade ligand conjugated monoclonal antibodies. Nucl Med Biol 22: 387
21. Press OW, Shan D, Howell-Clark J, Eary J, Appelbaum FR, Matthews D, King DJ, Haines AM, Hamann P, Hinman L, Shochat D, Bernstein ID (1996) Comparative metabolism and retention of iodine-125, yittrium-90 and indium-111 radioimmunoconjugates by cancer cells. Cancer Res 56: 2123
22. Renner C, Pfreundschuh M (1995) Tumor therapy by immune recruitment with bispecific antibodies. Immunol Rev 145: 179
23. Renner C, Jung W, Sahin U, Denfeld R, Pohl C, Trümper L, Hartmann F, Diehl V, van Lier R, Pfreundschuh M (1994) Cure of xenografted human tumors by bispecific monoclonal antibodies and human T cells. Science 264: 833
24. Renner C, Jung W, Sahin U, van Lier R, Pfreundschuh M (1995) The role of lymphocyte subsets and adhesion molecules in T cell-dependent cytotoxicity mediated by CD3 and CD28 bispecific monoclonal antibodies. Eur J Immunol 25: 2027
25. Renner C, Bauer S, Sahin U, Jung W, van Lier R, Jacobs G, Held G, Pfreundschuh M (1996) Cure of disseminated xenografted human Hodgkin's tumors by bispecific monoclonal antibodies and human T cells: the role of human T-cell subsets in a preclinical model. Blood 87: 2930
26. Renner C, Hartmann F, Pfreundschuh M (1997) Treatment of refractory Hodgkin's disease with an anti-CD16/CD30 bispecific antibody. Cancer Immunol Immunother 45: 184
27. Riethmueller G, Holz E, Schlimok G, Schmiegel W, Raab R, Hoffken K, Gruber R, Funke I, Pichlmaier H, Hirche H, Buggisch P, Witte J, Pichlmayr R (1998) Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. J Clin Oncol 16: 1788
28. Sahin U, Hartmann F, Senter P, Pohl C, Engert A, Diehl V, Pfreundschuh M (1990) Specific activation of the prodrug mitomycin phosphate by a bispecific anti-CD30/anti-alkaline phosphatase monoclonal antibody. Cancer Res 50: 6944
29. Scott AM, Welt S (1997) Antibody-based immunological therapies. Curr Opin Immunol 9: 717
30. Segal DM, Qian JH, Mezzanzanica D, Garrido MA, Titus JA, Andrew SM, George AJ, Jost CR, Perez P, Wunderlich JR (1992) Targeting of anti-tumor responses with bispecific antibodies. Immunobiology 185: 390
31. Segal DM, Weiner GJ, Weiner LM (1999) Bispecific antibodies in cancer therapy. Curr Opin Immunol 11: 558
32. Scheiber G, Hellstrom KE, Hellstrom I (1992) An unmodified anticarcinoma antibody, BR96, localizes to and inhibits the outgrowth of human tumors in nude mice. Cancer Res 52: 3262
33. Sharkey RM, Motta-Hennessy C, Pawlyk D, Siegel JA, Goldenberg D (1990) Biodistribution and radiation dose estimates for yttrium- and iodine-labeled monoclonal antibody IgG and fragments in nude mice bearing human colonic tumor xenografts. Cancer Res 50: 2330
34. Shih LB, Thorpe SR, Griffiths GL, Diril H, Ong GL, Hansen HJ, Goldenberg DM, Mattes MJ (1994) The processing and fate of antibodies and their radiolabels bound to the surface of tumor cells in vitro: a comparison of nine radiolabels. J Nucl Med 35: 899
35. Staerz UD, Yewdell JW, Bevan MJ (1987) Hybrid antibody-mediated lysis of virus-infected cells. Eur J Immunol 17: 571
36. Valone FH, Kaufman PA, Guyre PM, Lewis LD, Memoli V, Deo Y, Graziano R, Fisher JL, Meyer L, Mrozek-Orlowski M (1995) Phase Ia/Ib trial of bispecific antibody MDX-210 in patients with advanced breast or ovarian cancer that overexpresses the proto-oncogene HER-2/*neu*. J Clin Oncol 13: 2281
37. Weiner LM, Clark JI, Ring DB, Alpaugh RK (1995) Clinical development of 2B1, a bispecific murine monoclonal antibody targeting c-erbB-2 and Fc gamma RIII. J Hematother 4: 453
38. Wu C, Kobayashi H, Sun B, Yoo TM, Paik CH, Gansow OA, Carrasquillo JA, Pastan I, Brechbiel MW (1997) Stereochemical influence on the stability of radio-metal complexes in vivo. Synthesis and evaluation of the four stereoisomers of 2-(*p*-nitrobenzyl)-trans-CyDTPA. Bioorg Med Chem 5: 1925