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# Bifunctional NHS-BAT Ester for Antibody Conjugation and Stable Technetium-99m Labeling: Conjugation Chemistry, Immunoreactivity and Kit Formulation

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Conjugation chemistry and kit formulated binding of the NHS ester of 6-(4'-(4"-carboxyphenoxy)butyl)-2, 10-dimercapto-2,10-dimethyl-4,8-diazaundecane (NHS-BAT ester) to monoclonal antibodies (MAbs) was investigated. The functionalities of the resulting BAT conjugated and  $^{99\text{mTc}}$ -labeled MAbs BW 431/26, MAb 425 and bispecific MDX210 (fragment construct) were tested by immunoreactivity and immunoscintigraphy. **Methods:** The kinetics and chemistry of the conjugation reaction were monitored by high-performance liquid chromatography, size-exclusion chromatography and positive fast-atom-bombardment mass spectra (FAB-MS). The  $^{99\text{mTc}}$  BAT-MAbs were tested with various immunoreactivity assays. The biodistribution of  $^{99\text{mTc}}$ -BAT-BW 431/26 in rats was compared with directly labeled BW 431/26. **Results:** At pH 8.5 and 25°C, the reactivity of the NHS-BAT ester was high with 90% completion after 30 min. The conjugation yield of 19  $\mu\text{M}$  MAb and 228  $\mu\text{M}$  NHS-BAT ester amounted to 30%. Higher NHS-BAT ester concentrations afforded higher BAT-to-MAb ratios. According to FAB-MS, the conjugation competing hydrolysis surprisingly occurred at the NHS ring. Almost quantitative  $^{99\text{mTc}}$  labeling was achieved after 5 min at 25°C. Immunoreactivity of the  $^{99\text{mTc}}$ -BAT antibodies showed >90% recovery and proved to be insensitive to BAT-to-MAb ratios of up to 10. The  $^{99\text{mTc}}$ -BAT-BW 431/26 showed similar organ distribution but revealed less urinary excretion compared with the directly labeled BW 431/26. Immunoscintigraphy with  $^{99\text{mTc}}$ -labeled and BAT-BW 431/26 and BAT-MAb 425 showed the respective biological function in vivo. **Conclusion:** According to straightforward conjugation chemistry, the ease of  $^{99\text{mTc}}$  labeling and the application of a simple ultrafiltration technique, the NHS-BAT ester represents a nondestructive, universally applicable bifunctional ligand to introduce stable  $^{99\text{mTc}}$  protein binding sites. Kit

formulated conjugation/labeling can be performed with little time requirements and laboratory experience.

**Key Words:** bifunctional ligand; NHS-BAT ester; technetium-99m; radioimmunoscintigraphy; BW 431/26; MAb 425; MDX210

*J Nucl Med* 1996; 37:362–370

The efforts that have been undertaken to improve the binding of complexed  $^{99\text{mTc}}$  to antibodies or antibody fragments resulted in a series of experimental approaches. The techniques that have been described are commonly subdivided into two categories: direct labeling methods in which the metal is complexed by preformed sulfhydryl groups, and indirect methods in which chelating groups are introduced into proteins and used thereafter for complexation with  $^{99\text{mTc}}$  (preconjugation route). Additionally, the latter route was modified by complexing the activated ligand before conjugation (precomplexation route). The current state of research was recently reviewed (1–4).

Because of the favorable complex chemistry of multidentate chelators, the emphasis of our work was focused on indirect methods. The design of heterobifunctional ligands which have the chelating moiety on one side of the molecule and the activated ester on the other is hampered, however, by the intrinsic problem that nucleophilic heteroatoms of the ligand potentially interact with the electrophilic center at the location of the leaving group. In early experiments, this problem was bypassed by applying protection groups at the sulfhydryls and by using amide nitrogens on the chelate, as realized with the activated esters of the S protected  $\text{N}_2\text{S}_2$ -pentanoate (5–8). At ambient temperature and neutral pH, the complexation yields of the respective antibody conjugates proved to be insufficient. The binding of  $^{99\text{mTc}}(\text{V})$  needed elevated temperatures or high

Received Oct. 24, 1994; revision accepted Jun. 29, 1995.

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No reprints are available from the author.

pH conditions less suited for proteins. Thus, before conjugation to antibodies, the activated  $\text{N}_2\text{S}_2$ -pentanoate ligand had to first be transformed to the  $^{99\text{m}}\text{Tc(V)}$  complex (9).

An alternative conjugation agent was introduced in which an N-carboxymethyl substituted diaminodithiol (DADT) ligand was activated through intramolecular thiolactone formation. Resistance of this compound against intramolecular amide formation was furnished by methylating the remaining secondary amino group (10). Conjugation to an antibody at pH 9 using 100:1 molar excess of the activated DADT and the complexation with  $^{99\text{m}}\text{Tc}$  was reported to be successful (11).

The N-hydroxysuccinimide ester of 6-hydrazinonicotinic acid (HYNIC), which was recently introduced as a conjugation agent, forms different  $^{99\text{m}}\text{Tc}$  binding sites. The transfer of preformed  $^{99\text{m}}\text{Tc}$ -gluconate complex to the protein is suggested to proceed not by ligand exchange but through the formation of a Tc-diazenido double bond (12). The labeling was reported to proceed rather slowly (13).

We hoped to develop a bifunctional chelating ligand comprising a *bis*(aminoethanethiol) (BAT) chelator and a reactive NHS ester group both separated by a butylene spacer. This approach was thought to be advantageous since BAT ligands are known to complex  $^{99\text{m}}\text{Tc(V)}$  rapidly at ambient temperature and neutral pH with high yields. The complexes are stable and the NHS group represents an even, at moderate basic pH, rapidly reacting leaving group. This article discusses the chemistry of the NHS-BAT ester conjugation,  $^{99\text{m}}\text{Tc}$  labeling, preservation of immunoreactivity and biological function after NHS-BAT ester conjugation and describes a kit formulated conjugation/labeling procedure.

## MATERIALS AND METHODS

### Chemicals

All commercially available chemicals were of analytical grade and used without further purification. High-performance liquid chromatography (HPLC) was performed on LATEK systems (Latek, Heidelberg, Germany) equipped with variable UV and gamma radiation detectors. Nucleosil C18 (5  $\mu\text{m}$ , 270  $\times$  4 mm) (Macherey and Nagel, Düren, Germany) reverse-phase HPLC columns were used. The solvents used throughout the experiments consisted of MeOH and 0.9% TRIS buffer adjusted to pH 2.6 with  $\text{H}_3\text{PO}_4$ . The detectors were equipped with a dual-channel integrator C-R5A (Shimadzu Europe, Duisburg, Germany). Analytical size-exclusion chromatography (SEC) was performed on one-way columns (100  $\times$  4 mm) filled with Sephadex G 50 (Pharmacia, Freiburg, Germany). Radioactive antibody solutions were placed on top of the support and developed (not eluted) with 0.4 ml 1 M NaCl/0.5 M phosphate buffer (pH 7.4). Thereafter, the tubes were scanned across a collimated NaI(Tl) detector and recorded on a C-R5A integrator (Shimadzu). All data recorded with these integrators were processed with CLASS-UniPac software (Shimadzu) run under Windows 3.1 (Microsoft). Centristart I ultrafiltration tubes (Sartorius, Göttingen) with an exclusion limit of 20 kD were used to separate low molecular contaminants of antibody solutions. At the end of each filtration step, an initial volume of 2.5 ml was reduced to about 250  $\mu\text{l}$ . Ellman's assay on thiol group concentration was performed by mixing 10  $\mu\text{l}$  of a test solution with 100  $\mu\text{l}$  5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (0.1 M  $\text{Na}_2\text{HPO}_4$ , pH 8.0). The absorbance measured at 412 nm was referred to a standard curve obtained with dithiothreitol (DTT). Visible and UV absorbance measurements were recorded with a Perkin Elmer 554 spectrophotometer. Fast-atom-bombardment mass spectra (FAB-MS) were measured with an instrument using glycerol or thioglycerol as a solvent matrix and a 15 keV  $\text{Cs}^+$  ion beam.

### Preparation of N-Hydroxysuccinimide Ester of 6-(4'-(4'-carboxyphenoxy)butyl)-2,10-dimercapto-2,10-dimethyl-4,8-diazaundecane (BAT Acid)

The NHS ester of this BAT ligand was prepared according to a recently published synthesis (14). The NHS-BAT ester was stored in DMF at  $-18^\circ\text{C}$  for months without measurable deterioration (data not shown). Proof of the integrity of the conjugation agent was regularly checked by comparing HPLC peak integrals monitored at 270 nm, an absorbance maximum of the NHS-BAT ester. The NHS-BAT ester eluted after 14.3 min using a methanol gradient starting at 40% and ending at 100% after 30 min. The amount of thiol groups determined with Ellman's reagent served as the absolute measure of the NHS-BAT ester concentration.

### Description of Antibodies

We used BW 431/26, MAb 425 and the bispecific antibody MDX210 for the conjugation experiments. While the former two are intact MABs, the first directed against CEA and the second against EGF receptors, the latter represents an antibody fragment construct, which was prepared according to the procedure described by Glennie et al. (15). Here F(ab') fragments of MAb 520C9 (anti proto oncogene Her2/neu) and MAb M22 (anti type 1 Fc receptor expressed on human cytotoxic effector cells, Fc $\gamma$ RI) were chemically linked to each other. Both the antibodies were produced by in vitro cultivation of respective hybridoma cells and the F(ab') fragments were separately prepared by digestion of the antibodies with pepsin followed by reduction with mercaptoethylamine (MEA). The M22 F(ab') was derivatized with o-phenylenedimaleimide (oPDM) then incubated with 520C9 F(ab') to obtain the bispecific antibody M22 F(ab')-oPDM-520C9 F(ab') or MDX210.

### Preparation of BAT Conjugated Antibody

One milligram of the antibody (6.67 nmole) dissolved in 250  $\mu\text{l}$  0.1 M phosphate buffer (pH 8.5) was mixed with 15  $\mu\text{l}$  4.5 mM NHS-BAT ester dissolved in DMF. The mixture was left for 30 min at ambient temperature and thereafter stopped by adding 300  $\mu\text{l}$  0.1 M  $\text{KH}_2\text{PO}_4$  reducing the pH to 6.5. To determine conjugation yields without losing significant amounts of antibody, 20  $\mu\text{l}$  of this solution was analyzed by reverse-phase HPLC with 50% isocratic MeOH at 270 nm. The antibody did not elute from the column under these conditions. The conjugation yields were calculated from the peak integrals using the following formula:

Conjugation yield (%) = 100

$$\times \frac{[\text{I}_{\text{NHS-BAT ester}}]^0 - [\text{I}_{\text{NHS-BAT ester}}]^t - 2.08 \times [\text{I}_{\text{BAT acid}}]^t - 1.17 \times [\text{I}_{\text{hydrolysis prod.}}]^t}{[\text{I}_{\text{NHS-BAT ester}}]^0}$$

The peak integrals  $[\text{I}]^t$  of unreacted NHS-BAT ester, major hydrolysis product and BAT acid at the end of conjugation were corrected according to their molar absorbances and subtracted from the NHS-BAT ester integral originally started with  $[\text{I}]^0$ . From that point, BAT ligand-to-antibody ratios were calculated according to: (conjugation yield)  $\times$  (moles NHS-BAT ester used for the conjugation)/(moles of antibody).

To remove the hydrolysis products and unreacted NHS-BAT ester, two ultrafiltration steps were used and the BAT-MAB solution was ready for complexation or was stored frozen for later application.

### Reactivity of NHS-BAT Ester with Antibody

Measurements of conjugation kinetics were performed with 1 and 2 mg antibody (BW 431/26) dissolved in 0.1 M phosphate buffer of pH 8.0 and 8.5. The total volume was held constant at 350  $\mu\text{l}$ , including 17.7  $\mu\text{l}$  of 4.5 mM NHS-BAT ester (DMF). Every 12 min, 20- $\mu\text{l}$  samples were injected into the HPLC and analyzed using 50% isocratic MeOH. The conjugation yields were calculated as previously described.

### Technetium-99m Labeling of BAT-Antibody

Technetium-99m labeling was performed by mixing the thawed or freshly prepared BAT-antibody conjugate (1 mg in 0.25 ml 0.1 M phosphate buffer pH 6.5) with 1.5 GBq  $^{99m}\text{TcO}_4^-$  (0.5 ml 0.9% NaCl) and 1  $\mu\text{l}$  5 mM  $\text{SnCl}_2$  (0.1 M tartaric acid). After 5 min standing at ambient temperature, the solution was diluted to 2.5 ml with 0.9% NaCl solution, subjected to one ultrafiltration as described above and evaluated for labeling efficiency. The floater containing <10% of nonreacted  $^{99m}\text{Tc}$  and about 90% of the remaining stannous ions was removed and discarded. The labeling yields are summarized in Table 1. Thereafter,  $^{99m}\text{Tc}$ -BAT MAb was filtered sterile and used for experimental or clinical application.

Stability measurements were performed by mixing  $^{99m}\text{Tc}$ -labeled BAT-BW 431/26 with an equal volume of human serum and by challenging with a 5000-fold molar excess of DTPA (related to MAb).

Adventitious binding of reduced  $^{99m}\text{Tc}$  at MAb sites outside of the conjugated BAT-ligands was tested by reacting mixtures of 1 mg MAb 425 (0.2 ml 0.9% NaCl), 6.67  $\mu\text{l}$  5 mM BAT acid (DMF) and 1.8 GBq pertechnetate (0.5 ml) with 1.3  $\mu\text{l}$  and 13  $\mu\text{l}$  5 mM  $\text{SnCl}_2$  (0.1 M tartaric acid). Analyses of free and protein-bound radioactivity were performed with Sephadex G50 SEC.

The reduction capacity of the BAT ligand with respect to the formation of intramolecular MAb sulfhydryl groups was studied by performing the conjugation steps as described above using the BAT acid instead of the NHS-BAT ester. The contact time of the BAT acid with the MAb 425 at pH 8.5 was 1 hr, and the ultrafiltrated MAb 425 was assayed with Ellman's test on sulfhydryl groups.

### Biodistribution Studies of BAT Conjugated and Directly Labeled BW 431/26

Time course biodistribution studies were performed with Sprague Dawley rats weighing 200–250 g. Technetium-99m-BAT-BW 431/26 (five BAT ligands per MAb) [50 MBq (0.03 mg)] and directly labeled  $^{99m}\text{Tc}$ -BW 431/26 (~10 SH/MAb) were injected into a lateral tail vein. The injectate volume was determined by weighing the syringe before and after injection. At the designated times postinjection, the animals were weighed, killed by cervical dislocation and dissected. The organs were blotted dry when appropriate, weighed and counted along with standards of the injected dose in a gamma counter with the counting window set from 80 to 200 keV. The results were expressed as the percent injected dose per gram (%ID/g) tissue. Urine and feces were collected at 1, 4 and 24 hr postinjection. The samples were counted, referred to the above standards and calculated as the percent injected dose.

### Immunoreactivity of BAT-Antibody

Immunoreactivity of  $^{99m}\text{Tc}$ -BAT BW 431/26 was assessed with an antigen excess cell binding assay (16) using formaline fixed cells of the human colon carcinoma line CoCa<sub>2</sub>. According to a

recently described procedure, the radioactivity bound to the cells was determined 4 hr after incubation at an increasing cell mass until maximum binding was achieved (17).

The immunoreactive integrity of the BAT-MAb 425 was studied with a competitive binding ELISA. In this assay, BAT-MAb 425 and biotin-labeled MAb 425 competed for binding to the human EGF receptor immobilized on MaxiSorp microtiter plates (Nunc, Wiesbaden, Germany). Control experiments were performed with unmodified MAb 425 and the detection of bound biotin-labeled MAb 425 was furnished with streptavidin peroxidase conjugate. From these data (means of two assays each), competition curves were plotted and compared according to their shape and half-maximal binding concentration. The human EGF receptor was isolated from the supernatant of the human cell line A431 (18).

Immunoreactivity of  $^{99m}\text{Tc}$ -labeled BAT-MDX210 was tested by immunofluorescence and cytotoxicity measurements after the radioactivity decayed to a nondetectable level.

**Neutrophil Isolation.** The PMN were isolated from the blood of patients receiving G-CSF as previously described (19). Cytospin preparations were used to assess the purity of isolated cells, which was higher than 95% with few contaminating eosinophils. Cell viability, determined by trypan blue exclusion, exceeded 95%.

**Immunofluorescence.** The PMN and SK-br3 breast cancer cells were incubated with antibodies to Fc $\gamma$ RI [M22, IgG1 (20)] and to Her2/neu [520C9, IgG1 (21)] as well MDX210 or BAT-MDX210. All antibodies were obtained from MEDAREX (Princeton, NJ). During antibody incubation, polyclonal human IgG (4 mg/ml) was added to inhibit nonspecific binding to Fc $\gamma$ RI. After washing, cells were stained with fluorescein isothiocyanate (FITC)-conjugated F(ab')<sub>2</sub> fragments of goat anti-mouse antibody detecting IgA, IgM and IgG heavy- and light-chain. Immunofluorescence was analyzed on a flow cytometer.

Cytotoxicity assays were performed as described recently (19): SK-br3 breast cancer cells were used as target cells, which were grown in RPMI 1640 (Seromed) supplemented with 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 4 mmole/liter L-glutamine and 10% fetal calf serum (RF10<sup>+</sup>). Target cells were labeled with  $^{51}\text{Cr}$  (100  $\mu\text{Ci}$  for 3 h). After washing, cell numbers were adjusted to  $1 \times 10^5/\text{ml}$  in RF10<sup>+</sup>. Neutrophils (50  $\mu\text{l}$ ), target cells (50  $\mu\text{l}$ ), sensitizing antibody (MDX210 or BAT-MDX210) and RF10<sup>+</sup> were added to give a final volume of 200  $\mu\text{l}$ . Concentrations of sensitizing antibodies were tested from 0.02 to 50  $\mu\text{g}/\text{ml}$  and effector-to-target ratios (E/T) ranged from 10:1 to 80:1. For the experiment in Figure 6b, 50 U/ml G-CSF (Behring, Marburg, Germany) were added to give maximal in vitro stimulation. Assay duration was always 3 hr. Release of label from triplicates was measured and analyzed using the formula:

$$\% \text{ specific lysis} = \frac{(\text{experimental cpm} - \text{spontaneous cpm})}{(\text{maximum cpm} - \text{spontaneous cpm})} \times 100.$$

TABLE 1  
Summary of Labeling Data and Immunoreactivity Assays\*

Antibody†	Labeling yield (%)	Specific activity (GBq/ $\mu\text{mole}$ MAb)	Immunoreactivity (% of original)	Method
BAT-BW 431/26	94.1 $\pm$ 3.2	211	94.7 $\pm$ 0.4	Cell binding
BAT-MAb 425	93.3 $\pm$ 2.5	213	92.7 $\pm$ 4.1	ELISA
BAT-MDX 210	93.5 $\pm$ 2.9	140	90.7 $\pm$ 4.5	Cytotoxicity assay

\*Results are expressed as means  $\pm$  s.d.

†BAT-to-MAb ratios ranged between 3.5–4.5.

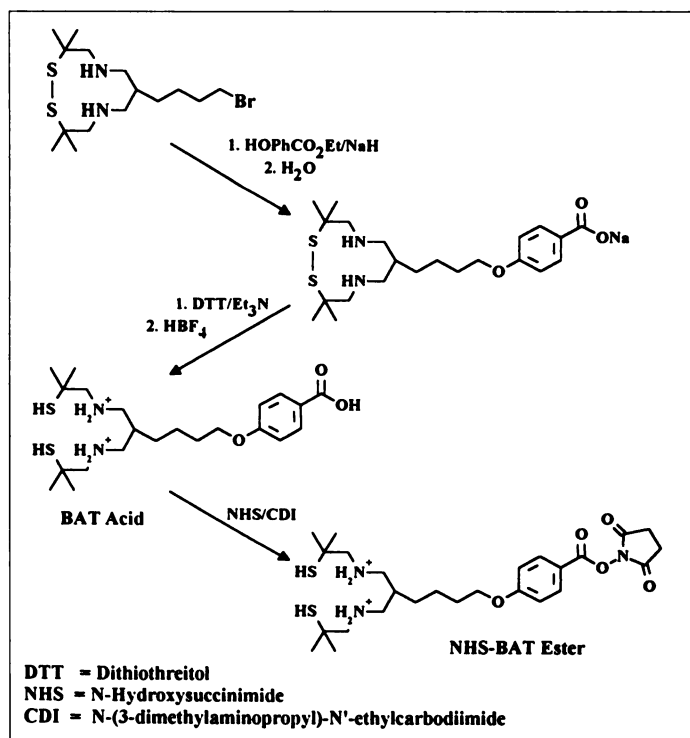


FIGURE 1. Synthesis of NHS-BAT ester.

## RESULTS

### Synthesis of NHS-BAT Ester

The NHS-BAT ester was synthesized according to the reaction scheme outlined in Figure 1 (14). The separation technique of the resulting NHS-BAT ester was accomplished by RP18-TLC with salt-free solvents. The activated ester was isolated as the  $\text{HBF}_4$  salt, with an overall yield of 15.7%.

### Reactivity of NHS-BAT Ester with Antibody

The conjugation yield of the NHS-BAT ester with the BW 431/26 increased with time, increasing pH and antibody concentration. Figure 2 depicts conjugation yields (medians) of the NHS-BAT ester which were obtained from three independent series of measurements. The series were measured at pH 8.0 and 8.5. The antibody concentrations were adjusted to 19  $\mu\text{M}$  (curves with rhombi and squares) and to 38  $\mu\text{M}$  BW 431/26 (curve with triangles) dissolved in phosphate buffers of the indicated pH. The concentration of the NHS-BAT ester was held constant at 228  $\mu\text{M}$ .

While after 60 min at pH 8.0 the reaction with 19  $\mu\text{M}$  BW 431/26 was still incomplete, the curve obtained at pH 8.5 turned into a plateau. Doubling of the antibody concentration from 19 to 38  $\mu\text{M}$  almost doubled the conjugation yield to 52% within 60 min. The BAT ligand-to-MAb ratios attained under these conditions ranged from 3 to 3.6. At pH 8.5, about 90% of this value was already acquired after 30 min. Using an antibody concentration of 38  $\mu\text{M}$  and a NHS-BAT ester concentration of 700  $\mu\text{M}$ , this ratio could be raised to about 10 BAT ligands BW 431/26.

### Chemistry of Conjugation Reaction

During the reaction of the NHS-BAT ester with the antibody molecule, amide bonds are predominantly formed at  $\epsilon$ -amino groups of lysine. As documented in Figure 2, the reaction did not proceed quantitatively. Most of the unconjugated part of the NHS-BAT ester was hydrolyzed. According to comparative HPLC measurements, the retention of the major hydrolysis product did not compare with the retention of the expected free acid. The amount of BAT acid formed by hydrolysis was small.

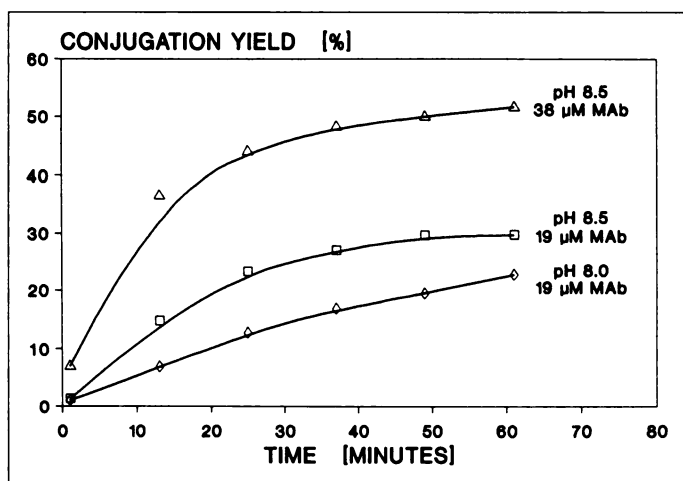


FIGURE 2. Conjugation kinetics of the NHS-BAT ester as a function of MAB concentration and pH.

The major hydrolysis product was isolated by preparative HPLC and analyzed by FAB-MS. Figure 3 shows the positive FAB-MS spectra of the NHS-BAT ester and the major hydrolysis product. The difference of 18 amu indicates the addition of water at the NHS ring of the activated ester.

### Labeling of the BAT-Antibody

Technetium-99m labeling was performed by mixing 1 mg BAT antibody with 1.5 GBq pertechnetate eluate and adding 0.75 mole equivalent  $\text{Sn}^{2+}$  (referred to IgG). The labeling yields were >90% at BAT-to-MAB molar ratios of about 4. Table 1 summarizes the individual labeling results achieved with the two intact MABs (BAT-BW 431/26 and BAT-MAB 425) and one MAB fragment construct (BAT-MDX 210).

Stability measurements concerning the liberation of  $^{99\text{m}}\text{Tc}$  resulting from oxidation and transchelation reactions were performed in human serum and DTPA solutions. Figure 4

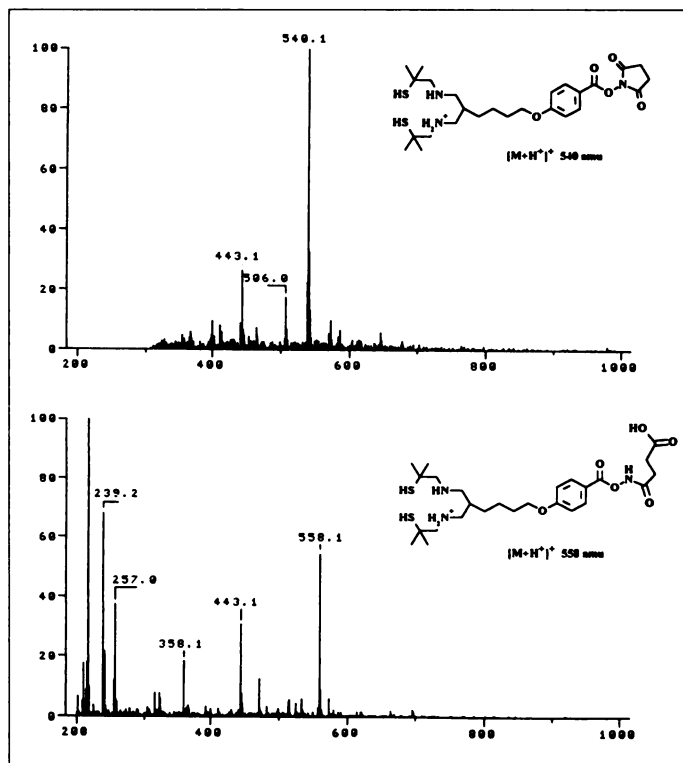
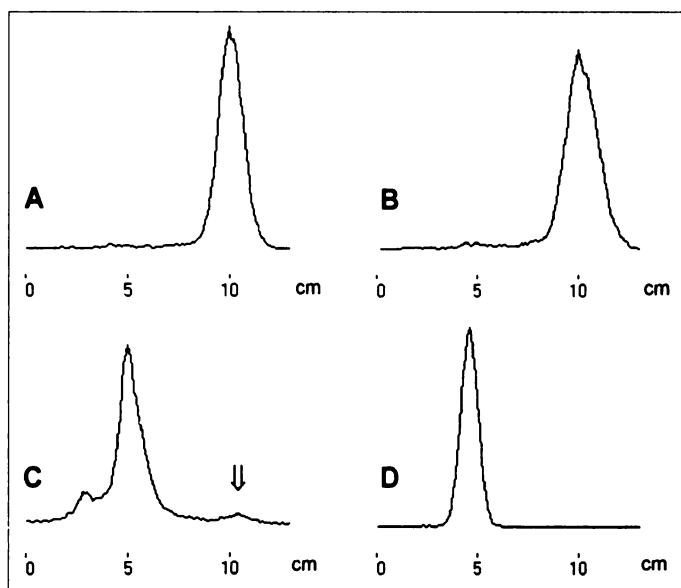


FIGURE 3. Positive FAB-MS of the NHS-BAT ester (upper) and hydrolysis product (lower panel).



**FIGURE 4.** Sephadex G50 SEC  $\gamma$ -scans of (A)  $^{99m}\text{Tc}$ -BAT-BW 431/26, 6 hr after mixing with human serum, (B) 6 hr after mixing with human serum and DTPA, (C) of  $^{99m}\text{Tc}$ -BAT acid formed in the presence of MAb 425 (the arrow points to directly labeled antibody) and (D) [ $^{99m}\text{Tc}$ ]pertechnetate.

illustrates gamma scans of one-way Sephadex G50 SEC developed 6 hr after mixing with human serum (Fig. 4A) and an additional 5000-fold molar DTPA excess (Fig. 4B). The radioactivity appearing between 0–7 cm was <2% (Fig. 4A) and <3% (Fig. 4B). The SEC of [ $^{99m}\text{Tc}$ ]pertechnetate points to the location of low molecular  $^{99m}\text{Tc}$  compounds (Fig. 4D).

The amount of adventitious binding of reduced  $^{99m}\text{Tc}$  to MAb sites was tested by reacting MAb 425 (1 mole equivalent), BAT acid (5 mole equivalents) and [ $^{99m}\text{Tc}$ ]pertechnetate solutions with equimolar stannous salt solutions and a 10-fold molar excess (both referred to MAb). At 5 min reaction time, the amount of protein bound radioactivity was 3.4% and 4.0%, respectively. One hour later, the values increased to 3.8% for equimolar experiment and to 10.6% for the 10-fold molar  $\text{Sn}^{2+}$  excess. Figure 4C shows the SEC gamma scan developed 1 hr after mixing with equimolar  $\text{Sn}^{2+}$ , which indicates 3.8% protein bound radioactivity (arrow). Investigations of the formation of sulfhydryl's potentially resulting from the interaction of the BAT ligand with intramolecular cystine groups of the MAb 425 were negative. This result was proved by performing the conjugation routine with the BAT acid instead of the NHS-BAT ester and analyzing the SH concentration after removal of the BAT acid by ultrafiltration.

## Biodistribution Studies of BAT Conjugated and Directly Labeled BW 431/26

Technetium-99m-labeled BAT-BW 431/26 and directly labeled BW 431/26 were evaluated *in vivo* for differences between the two labeling modalities. The biodistribution data are summarized in Table 2. The blood clearance was slow for both antibodies but little faster for the BAT conjugated BW 431/26. All other organs showed comparable values except the 24-hr kidney value of the directly labeled BW 431/26 were increased. The 24-hr urinary excretion was 26% for the directly labeled BW 431/26 compared to 9% for the  $^{99m}\text{Tc}$ -BAT antibody. At that time, the 24-hr excretion activity was somewhat higher for the latter compound (8.4 versus 4.4). The chemical nature of the radioactivity appearing in the 4-hr urine of  $^{99m}\text{Tc}$  BAT-BW 431/26 animals was determined to be 71% with [ $^{99m}\text{Tc}$ ]pertechnetate and 29% of complexed  $^{99m}\text{Tc}$  which was not identical with the  $^{99m}\text{Tc}$ -BAT acid.

## Immunoreactivity of BAT-MAb and BAT-Fragment

Immunoreactivity of  $^{99m}\text{Tc}$ -BAT-BW 431/26 was tested with the human colon carcinoma line CoCa<sub>2</sub>. The immunoreactive fraction was calculated from double-inverse plots, total-to-specific binding versus 1/cell concentration, extrapolated to infinite cell concentration (20). The mean of three independent measurements yielded  $94.7\% \pm 0.4\%$ .

Immunoreactivity measurements of BAT-MAb 425 (ELISA) revealed virtually no change of the binding characteristics as compared with the original MAb 425. The shape of both curves were about congruent and the half-maximal binding concentration of the BAT-MAb 425 was  $2.7 \pm 0.1$  nmole/liter (control:  $2.5 \pm 0.1$  nmole/liter).

The bispecific antibody MDX210 consisting of two oPDM linked F(ab') fragments, one directed against the high affinity receptor for IgG (Fc $\gamma$ RI) as a cytotoxic trigger molecule on effector cells and the other against the proto-oncogene product Her2/neu on target tumor cells, was tested after introduction of four BAT ligands/MDX210, complexation with 1.5 MBq  $^{99m}\text{Tc}$  and decay of radioactivity. Binding of MDX210 and BAT-MDX210 to both target molecules was compared by immunofluorescence. MDX210 and BAT-MDX210 showed similar binding to Her2/neu, as demonstrated by binding to SK-br3 breast cancer cells, and to Fc $\gamma$ RI, as tested with neutrophils from G-CSF treated patients (Fig. 5).

The biological activity of MDX210 and BAT-MDX210 was compared measuring cytotoxicity of neutrophils against SK-br3 cells. Tumor cell lysis was optimal with 0.4  $\mu\text{g}/\text{ml}$  for both antibodies (Fig. 6A). Also at lower effector-to-target ratios,

**TABLE 2**

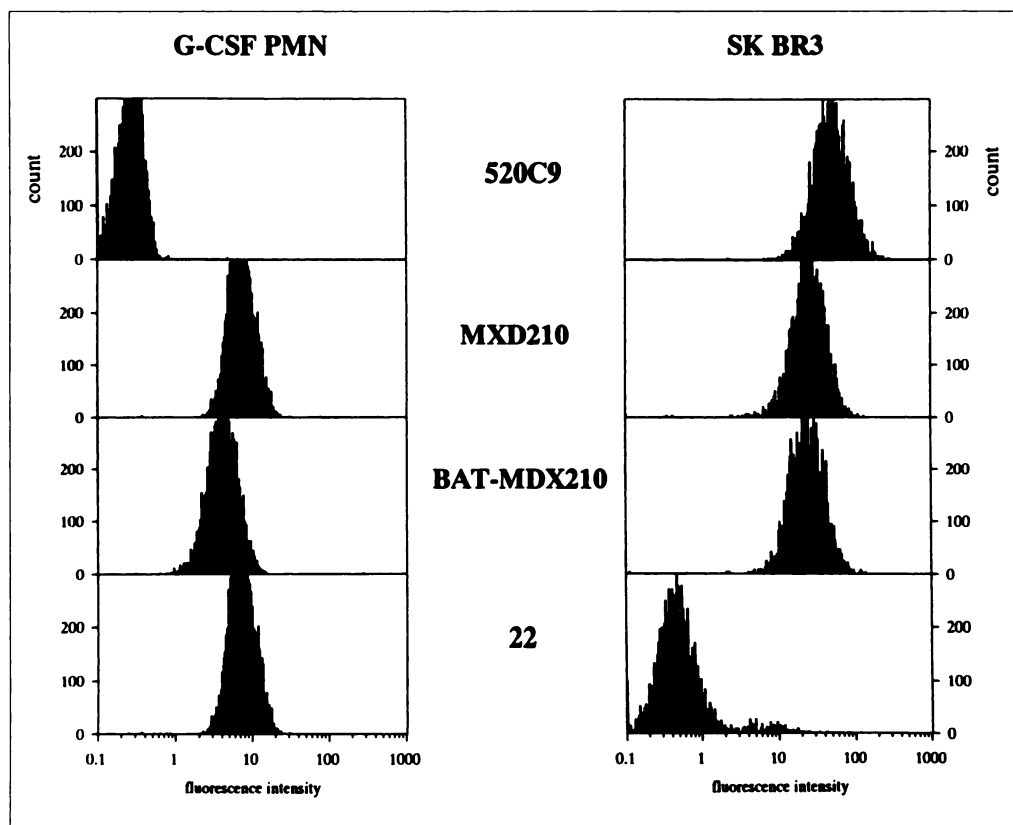
Biodistribution Values (%ID/g) of Rat Tissues after Injection with Technetium-99m-BAT-BW 431/26 and Directly Labeled BW 431/26

Time (hr)	$^{99m}\text{Tc}$ -BAT-BW 431/26*									
	Blood	Heart	Lung	Spleen	Liver	Kidney	Muscle	Thyroid†	Urine†	Feces†
1	1.92	0.69	1.84	1.20	2.18	1.71	0.04	0.23	—	—
4	2.08	0.62	1.22	0.69	1.53	1.39	0.04	0.19	3.6	0.0
24	0.94	0.50	1.14	0.77	1.30	1.70	0.08	0.18	9.5	8.4
Time (hr)	Directly labeled BW 431/26*									
	Blood	Heart	Lung	Spleen	Liver	Kidney	Muscle	Thyroid†	Urine†	Feces†
1	5.56	—	1.93	0.82	1.69	2.03	0.07	0.14	1.2	0.0
24	1.87	—	0.96	0.51	0.63	7.24	0.10	0.06	26.2	4.4

\*Medians of three rats.

†%ID, ranges were omitted for a better overall view.

\*Mean of two rats.



**FIGURE 5.** Binding of BAT-MDX210 and MDX210 to Her2/neu expressing SK-br3 cells and to Fc $\gamma$ RI positive PMN from G-CSF treated patients analyzed by immunofluorescence.

both antibodies gave comparable results (Fig. 6B). In three experiments (antibody concentration 0.4  $\mu$ g/ml, effector to target 80:1), BAT-MDX210 showed  $90.7\% \pm 4.5\%$  of the activity of MDX210 (Table 1).

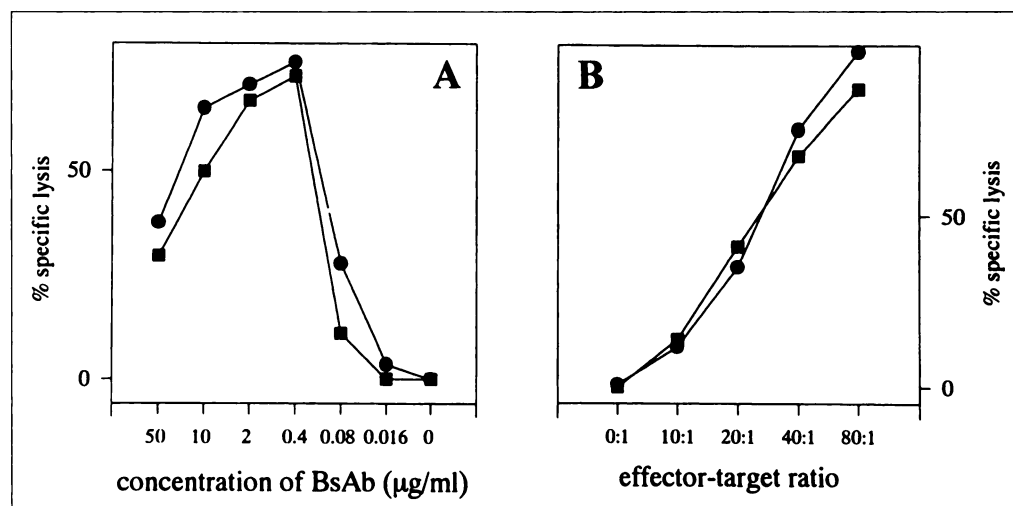
#### Immunoreactivity as a Function of BAT-Ligand/MAB Ratio

The influence of the BAT ligand-to-antibody ratio on antigen binding characteristics was tested with the anti-CEA antibody BW 431/26. Figure 7 illustrates immunoreactive plots obtained with  $^{99m}\text{Tc}$ -BAT-BW 431/26 at BAT-to-MAb ratios of 1.7, 3.4, 5.2 and 9.6. The immunoreactive fractions of all preparations were close to 100% and the slopes of the regression lines showed negligible variations except for the BAT-to-MAb ratio of 9.6 which was slightly increased.

#### Radioimmunoscinigraphy

Radioimmunoscinigrams obtained with two different  $^{99m}\text{Tc}$  labeled BAT-MABs are depicted in Figure 8. They demonstrate

the dependence of the biodistribution of  $^{99m}\text{Tc}$ -BAT antibodies from their biological function rather than from the  $^{99m}\text{Tc}$ -BAT label. Figure 8A shows the biodistribution of 600 MBq  $^{99m}\text{Tc}$ -BAT-BW 431/26 4 hr after injection with the accumulation of radioactivity in a recurrent rectum carcinoma. The tumor could be visualized behind the emptied urinary bladder (arrow). At that time, most of the radioactivity was found in the blood pool and blood-rich organs (heart and lungs), while another part was taken up by the liver and excreted through the kidneys. Figure 8B illustrates a 4-hr image obtained after injecting 700 MBq  $^{99m}\text{Tc}$ -BAT-MAB 425. The high uptake in the liver was due to its high EGF receptor concentration. This sponge-like accumulation produced an extreme reduction of the background activity. Some of the radioactivity was excreted through the liver and kidneys. An EGF receptor-positive pharynx carcinoma was also visualized on the scan (arrow). The faint uptake in the thyroid was due to a known [ $^{99m}\text{Tc}$ ]pertechnetate impurity (4%) which



**FIGURE 6.** Cytotoxic activity of PMN from G-CSF treated patients against SK-br3 tumor cells of BAT-MDX210 (squares) and MDX210 as a function of (A) bispecific antibody (BsAb) concentrations and (B) effector-to-target ratios.

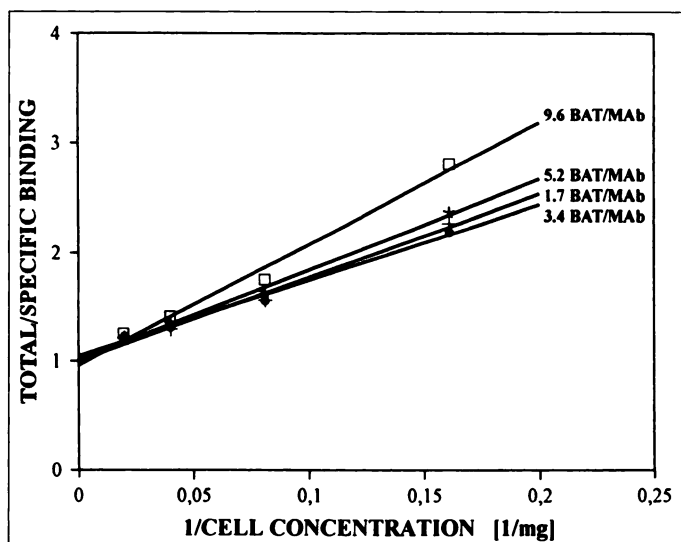


FIGURE 7. Immunoreactive plots of  $^{99m}\text{Tc}$ -BAT-BW 431/26 with various BAT-to-MAb ratios.

was not removed from that preparation. No thyroid activity was seen with pertechnetate-free preparations.

## DISCUSSION

### Conjugation and Labeling Chemistry

The chemistry of the bifunctional NHS-BAT ester demonstrates its value as a preconjugation agent for MAbs. The results supplement existing procedures and indicate improvements in the field of  $^{99m}\text{Tc}$  protein labeling. The benefit of a BAT-type ligand is based on its characteristics to form rapidly and with high yield  $^{99m}\text{Tc}$  complexes at neutral pH and ambient temperature. These characteristics compare well with those of other  $^{99m}\text{Tc(V)}$  chelators.

As previously mentioned, this kind of bifunctional chelating agent needs protection groups against the nucleophilic attack of heteroatoms at the activated ester site. Surprisingly, for the

NHS-BAT ester, protonation of the secondary amino groups was found to be sufficient for stabilization. Upon deprotonation and in aprotic solvents, the NHS-BAT ester reacted with itself, whereas it merely hydrolyzed (v.i.) in aqueous solutions. The tertiary sulfhydryl groups were not found to be involved in the cleavage of the NHS-leaving group. The rather low yield of 23% in the formation of the activated ester (Fig. 1) indicated that the heteroatoms participate in the formation of undesired side products. Although no change could be detected in HPLC after storage for several days at ambient temperature, the DMF solution of the NHS-BAT ester was always stored cold.

During conjugation, the presence of stabilizers like sorbitol should be avoided. Alcohols readily react with the NHS-BAT ester to the corresponding esters and therefore reduce the conjugation yield. It is therefore recommended that the proteins be purified by ultrafiltration to eliminate competing reactants, to obtain the appropriate pH for conjugation and to concentrate the protein solution for sufficient conjugation yields.

Depending on the reaction conditions (pH, NHS-BAT ester and protein concentration), the amount of conjugated BAT ligands varied. As long as the antibody solubility was at or above 6 mg/ml in phosphate buffer of pH 8.5, more than 50% conjugation yield was obtained (Fig. 2). The decrease of pH reduced the NHS-BAT ester reactivity and enhanced the reaction time without improvement in conjugation yield. At pH 8.5, a sufficiently rapid conjugation was achieved. Alkaline sensitive proteins, however, may therefore be conjugated at a reduced pH affording prolonged incubation times.

Hydroxyl ions compete for the nucleophilic attack at the activated ester group. HPLC measurements and disagreement of the major hydrolysis product with the retention of the expected free BAT acid point to an unknown product. The analysis of that compound by preparative HPLC and FAB-MS surprisingly revealed a parent ion at 18 amu higher than NHS-BAT ester (Fig. 3). The only interpretation for the unexpected result is that the hydroxyl ions attack and add to the NHS ring. This result relates to the aminolytic NHS-ring

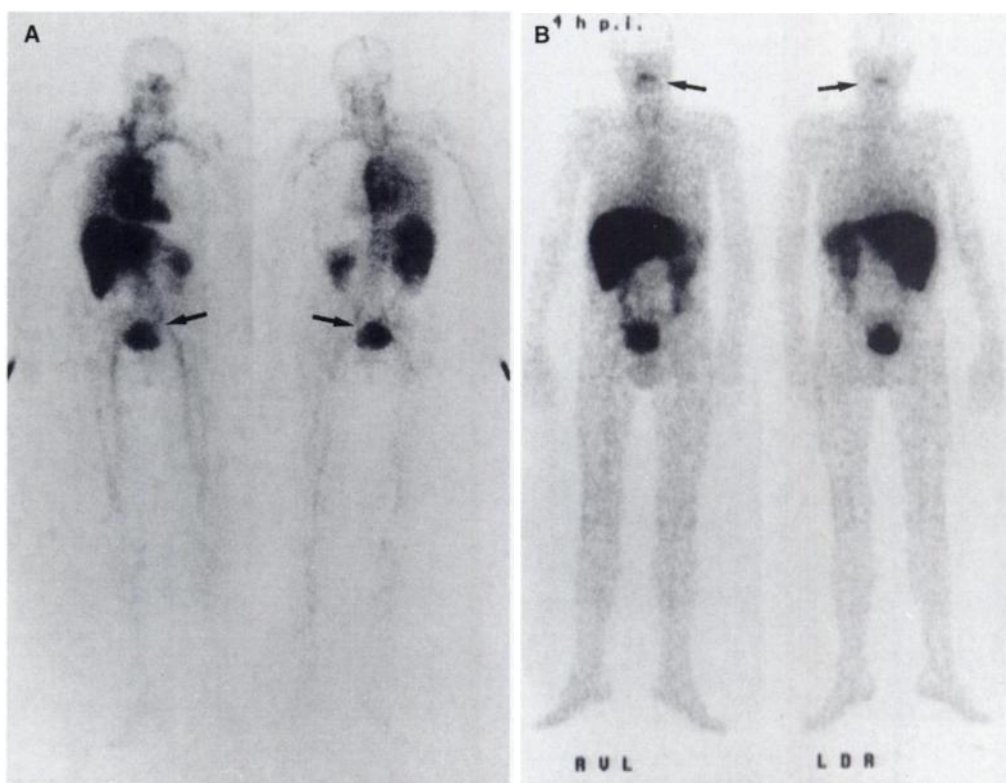


FIGURE 8. Anterior and posterior views of 4-hr radioimmunoscintigrams of (A)  $^{99m}\text{Tc}$ -BAT-BW 431/26 and (B) BAT-MAb 425. Arrows point to tumors.

TABLE 3

Summary of BAT Conjugation and Technetium-99m Labeling Data  
(Kit Formulation)

1.	Add 15 $\mu$ l 4.5 mM NHS-BAT ester solution to 1 mg of the MAb (250 $\mu$ l 0.1 M phosphate buffer, pH 8.5) and leave for 30 min at ambient temperature.
2.	Stop conjugation by adding 300 $\mu$ l 0.1 M $\text{KH}_2\text{PO}_4$ .
3.	Remove hydrolysis products by ultrafiltration (2 $\times$ ).
4.	Store BAT-MAB frozen or,
5.	Label by mixing BAT-MAB with 1.5 GBq pertechnetate (0.5 ml 0.9% NaCl) and adding 1 $\mu$ l 5 mM $\text{SnCl}_2$ (0.1 M tartaric acid).
6.	After 5 min, perform one ultrafiltration and filter sterile.

opening observed during peptide synthesis performed in aprotic solvents (22). Monitoring the reaction for several days with HPLC, the primary hydrolysis product disappeared under formation of the actually expected BAT acid. Other products due to oxidative ring closure- or intra- and intermolecular reactions were not found within the time scale of the conjugation reaction. Thus, conjugation and workup of the conjugation products did not afford an inert atmosphere.

Labeling was performed using minute amounts of stannous tartrate as a reducing agent. Equimolar amounts of BAT-antibody and  $\text{Sn}^{2+}$  were sufficient for labeling yields >90%. The specific activities of the three antibodies summarized in Table 1 are high and may be optimized individually. The method of choice for routine evaluation of protein-bound radioactivity was ultrafiltration instead of the more time-consuming SEC.

The stability of the  $^{99\text{m}}\text{Tc}$  label is demonstrated in Figure 4A, where gamma scans of Sephadex G50 SEC indicated negligible cleavage of protein bound radioactivity after 6 hr contact with human serum. The same result was observed after 6 hr contact with an additional 5000-fold molar excess DTPA (Fig. 4B). The lack of significant amounts of radioactivity associated with low molecular weight contaminants indicated the stability of the  $^{99\text{m}}\text{Tc}$ -labeled BAT-BW 431/26.

The question whether the protein-bound  $^{99\text{m}}\text{Tc}$  is located at the conjugated chelator or at sulfhydryl groups of the antibody, which may have been formed by the action of  $\text{Sn}^{2+}$ , was checked by complexing the unconjugated BAT acid in presence of the MAb 425 under comparable conditions routinely used for labeling BAT-MAB 425. The SEC gamma scan in Figure 4C shows the formation of a small amount of directly labeled  $^{99\text{m}}\text{Tc}$  antibody. The area around the 10-cm mark (arrow) was 3.8% one hour after complexation. This value was also small using even a tenfold  $\text{Sn}^{2+}$  concentration 5 min after the addition of the components. The directly labeled fraction increased, however, to about 11% one hour later, which illustrates the unfavorable influence of a high stannous salt concentration on the labeling of pre-conjugated antibody (1–3). On the other hand, undesired influences of stannous ions on the protein may be attenuated by applying ultrafiltration just after  $^{99\text{m}}\text{Tc}$  complexation as outlined in Table 3.

The formation of intramolecular SH-groups by the BAT ligand itself could not be detected. Under the conditions used for the NHS-BAT ester conjugation, ultrafiltered MAb 425 solutions were negative according to Ellman's test on sulfhydryl groups.

### Biological Evaluation

Organ distribution studies of  $^{99\text{m}}\text{Tc}$ -BAT-BW 431/26 and directly labeled BW 431/26 (23) in rats provided data about differences between the metabolic fate of  $^{99\text{m}}\text{Tc}$  either bound to the tetradentate BAT chelator or complexed to SH groups

liberated from reduced cystines. Obvious divergences were noticed in the slightly faster blood clearance of  $^{99\text{m}}\text{Tc}$ -BAT-BW 431/26 and in an enhanced kidney value of directly labeled BW 431/26 at 24 hr postinjection. This observation was in accordance with the high urinary excretion of the latter antibody. On the other hand, the gut excreta of the directly labeled antibody was half the amount of  $^{99\text{m}}\text{Tc}$ -BAT-BW 431/26. The totally cleared radioactivity amounted to 17.9% for  $^{99\text{m}}\text{Tc}$ -BAT-BW 431/26 and 30.6% for directly labeled BW 431/26 at 24 hr postinjection. This difference in excretion may be explained by less stability of the partially reduced antibody. Because of its altered structure, it seems to be more susceptible to biodegradation compared to the intact protein carrying covalently bound BAT ligands. Radioactivity in the urine was, in both cases, mostly [ $^{99\text{m}}\text{Tc}$ ]pertechnetate and to a lesser extent complexed to unknown ligands.

The conservation of the antibody function after conjugating about four BAT ligands per antibody was studied with various immunoreactive measurements. The results summarized in Table 1 indicate that little destruction occurred with the three antibodies after using the NHS-BAT ester conjugation method. This result was especially important for bifunctional antibody fragment construct MDX210 in which BAT ligand conjugation and  $^{99\text{m}}\text{Tc}$  labeling should have no influence on both biological functions. Figure 5 demonstrates positive immunofluorescence of the whole antibodies M22 and 520C9 on G-CSF stimulated PMN and SK-br3 cells, respectively, while BAT-MDX210 showed both functions, resulting in comparable fluorescence intensities as the bispecific MDX210. The SK-br3 cells express the proto-oncogene product Her2/neu and PMN of G-CSF treated patients stain positive for Fc $\gamma$ RI (24).

Since the bispecific antibody MDX210 can mediate cytotoxicity of Fc $\gamma$ RI expressing neutrophils from G-CSF treated patients (19), the biological activity of MDX210 and BAT-MDX210 was also compared by measuring this effect against SK-br3 cells. Figure 6 illustrates the biological activity of BAT-MDX210 and MDX210 on the cytotoxic action of neutrophils isolated from G-CSF treated patients against SK-br3 tumor cells, which reveals comparable results when either antibody concentrations (Fig. 6A) or effector-to-target ratios (Fig. 6B) were varied (Table 1).

Additionally important for the scope of the method was whether there is a limitation in respect to the BAT-ligand concentration on the antibody molecule. The immunoreactivity of  $^{99\text{m}}\text{Tc}$ -BAT-BW 431/26 was therefore studied as a function of the BAT-to-MAB ratio. Figure 7 demonstrates little deviation of the plots at BAT-to-MAB ratios of up to 5.2. Both the intercepts with the ordinate (immunoreactive fraction) and the slopes were similar. At a ratio of 9.6 BAT-to-MAB, however, the slope of the regression line was increased. In respect to the theory to which this method relates (16), this effect may be interpreted with a reduction of the association constant  $K_a$ . Our understanding is that the ligands at ratios of ten BAT ligands-to-antibody start to interfere by sterical and/or hydrophobic interactions with the antigen. Lipophilic characteristics of a series of  $^{99\text{m}}\text{Tc}$ -BAT complexes have been published (25). Ratios of 3.5–4.5 BAT-ligands-to-MAB, which were used to achieve high labeling yields of >92% with 1.5 GBq  $^{99\text{m}}\text{Tc}$ /mg antibody, are therefore considered a sufficient number of ligands for preservation of immunoreactivity and  $^{99\text{m}}\text{Tc}$  complexation capacity.

The comparison of the  $^{99\text{m}}\text{Tc}$ -BAT versus  $^{125}\text{I}$  label for immunoreactivity of an anti-rat sarcoma antibody (A2.6) was investigated in an earlier study (26). Here the A2.6 was assayed

with a cell binding assay, which revealed a slightly better result for the BAT conjugate.

Human whole-body distributions 4 hr after injection of  $^{99m}\text{Tc}$ -BAT-BW 431/26 and BAT-MAb 425 are depicted in Figure 8. The radioimmunoscintigrams reveal significant biological differences which are in accordance with specific binding sites of the respective antibodies (27,28). While the former is retained mostly in the blood pool and blood-rich organs, the latter was, to a high extent, taken up by the EGF receptor-rich liver which reduces the background activity of the other organs. This result was due to antibody characteristics that are not significantly influenced by the  $^{99m}\text{Tc}$ -BAT label. Additional visualization of tumors expressing the respective antigens (arrows) indicates the clinical value of the method.

The conjugation and labeling procedure of a bifunctional chelating agent is summarized in Table 3. Because of its simplicity, we termed the summary of the manipulations "a kit formulation." The steps were not particularly labor-intensive and can be performed by inexperienced personnel. For routine applications, steps 1–3 were performed in advance. The frozen aliquots of the BAT antibody were thawed at the time of application and  $^{99m}\text{Tc}$  labeling was performed afterwards by the addition of pertechnetate eluate and  $\text{Sn(II)}$  tartrate which reduced the preparation time to minutes.

## CONCLUSION

As demonstrated, the application of the NHS-BAT ester as a conjugation agent for rapid introduction of stable  $^{99m}\text{Tc(V)}$  binding sites is not restricted to intact antibodies. Proteins of the molecular size of HSA were also successfully conjugated and labeled by this method,  $\text{F(ab')}_2$  and Fab fragments as well as recombinant products, in particular, represent interesting proteins for this labeling technique. Here the protein destructing direct labeling technique reaches the border of its scope because reduction of cystine groups within the protein may lead to loss of biological function.

## ACKNOWLEDGMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG). We thank Drs. Bremer, Kuhlmann and Haase (Hoechst AG, Frankfurt/M), and Dr. Matzku (E. Merck, Darmstadt) for supplying the unmodified BW 431/26 and MAb 425 antibody, respectively.

Presented in part at the 9th and 10th International Symposium on Radiopharmaceutical Chemistry (Paris 1992, Kyoto 1993) and the 40th Annual Meeting, Society of Nuclear Medicine, 1993, Toronto, Canada.

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