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Short communication

Site-specific conjugation of bifunctional chelator BAT to mouse IgG_1 Fab' fragment

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Key words

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Abstract

Aim: To perform a site-specific conjugation of Fab' fragments of a mouse monoclonal antibody(MoAb) B43(of IgG_1 subtype) to a bifunctional chelator 6-[*p*-(bromoacetamido) benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N*',*N*'',*N*'''-tetraacetic acid (BAT) via the thiol groups in the hinge distal to the antigen-binding site of the Fab'. **Methods:** B43 was cleaved using a simple 2-step method. First, stable $F(ab')_2$ was produced by pepsin treatment. Fab' with free thiol in the hinge region was then obtained by cysteine reduction of $F(ab')_2$. Second, a site-specific conjugation of Fab' to thiol-specific BAT was performed in a one-step reaction. **Results:** The Fab' fragment had approximately 1.8 free thiol groups per molecule after cysteine reduction. The conjugation efficiency and the chemical yield were approximately 1.28 moles chelator/Fab' and 74% of the initial concentration of Fab', respectively. The $F(ab')_2$, Fab' and Fab'-BAT all maintained reasonable antigen-binding properties. ^{67}Cu labeling of the conjugate under standard conditions did not impair the immunoreactivity of Fab'-BAT. **Conclusion:** This is a simple and efficient method for producing immunoreactive conjugates of Fab'-BAT, which can be used to make radiometal-labeled conjugates for further diagnostic and therapeutic applications.

Introduction

Monovalent antibody fragments are known to have major advantages over intact immunoglobulin G (IgG) in immunohistochemistry and nuclear scintigraphy[1,2]. $F(ab')_2$ fragments prepared by pepsin digestion can be separated by mild reduction into free thiol-containing, univalent Fab' fragments. An advantage of having such Fab' fragments with free thiol groups in the hinge region is that they can be conjugated directly with other thiol-specific molecules for site-specific conjugation[3,4]. Another desirable feature is that the thiol groups in the hinge of Fab' are remote from the antigen-binding site and therefore antigen-binding is not sterically impaired by the conjugation. This method of site-specific conjugation has been widely used for conjugating antibodies with enzymes such as peroxidase and glucosidase with minimal polymerization and without impairing the activity of Fab'[5,6]. The labeling of antibodies with thiol-specific bifunctional chelating agents for metal ions has a number of potential new applications, including *in vivo* diagnosis and therapy and *in vitro* immunoassays[7–9]. In the past linking agents such as 2-iminothiolane(2-IT), which generates a linkage containing a disulfide bond and an amidinium bond, were always used as "bridges" to connect bifunctional chelating agents, such as 6-[*p*-(bromoacetamido) benzyl]-1,4,8,11-tetraazacyclotetradecane-*N*,*N*',*N*'',*N*'''-tetraacetic acid (BAT) and antibodies containing no free thiol groups[10,11]. This kind of linking agents was able to offer antibody the free thiol groups, which may be used for further conjugation, whereas free thiol-containing Fab' fragments do not need this step. We are not aware of any previous reports in the literature on the site-specific conjugation of bifunctional chelating agents with Fab' fragments directly using their thiol groups in the hinge region.

In the present study, we prepared $F(ab')_2$ from mouse MoAb B43 by using a modified pepsin digestion method, and reduced the divalent fragment with cysteine to generate

free thiol-containing Fab' in the hinge region. The Fab' was then directly conjugated with BAT, a thiol-specific bifunctional chelator for Cu, Co and similar metals without a linking agent. The emphasis of the present report is on the detailed description of the preparation of the conjugate molecule and its antigen-binding properties compared with those of the parent B43 molecule.

Materials and methods

Preparation of B43 F(ab')$_2$ and Fab' fragments Purified B43 directed against ovarian carcinoma-associated antigen CA125 was from AltaRex (Edmonton, Canada). Stock solutions of the B43 were at a concentration of 4.7–5.0 mg/mL in pH 7.5 phosphate buffered saline (PBS). The pH value of an aliquot of the stock solution was adjusted by 1 mol/L pH 3.5 ammonium citrate buffer (Sigma, St Louis, USA) to pH 3.6. The aliquot was then mixed with bovine pepsin (2660 U/mg, Sigma) at a molar enzyme:B43 ratio of 1:5 . The mixture was incubated at 37 ºC for 6 h with continuous shaking. The reaction was terminated by increasing the pH of the mixture to 7.5 with the addition of 3 mol/L pH 8.3 Tris buffer. The products were diluted 200 times with pH 7.5 PBS and spun in 30 kDa concentrator tubes (Amicon, Beverly, USA). The final concentration of protein as measured by UV absorbance at 280 nm was approximately 5 mg/mL on a model SP8-400 UV/VIS Spectrophotometer (Pye Unicam, Cambridge, UK). The purity of F(ab')$_2$ was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Fab' fragments were obtained by the reduction of F(ab')$_2$ with 10 mmol/L fresh cysteine (Sigma, St Louis, USA) solution for 30 min at room temperature at pH 7.5. The reaction mixture was diluted 1000 times with pH 5.0 citrate buffer containing 1.0 mmol/L ethylenediamine tetraacetic acid (EDTA) that had been purged with nitrogen, loaded on a 30 kDa concentrator tube and spun in a centrifuge to remove residual cysteine and small fragments of the Fc portion. The concentration of the final sample was approximately 5.0 mg/mL according to UV absorbance at 280 nm. The purity of Fab' was analyzed by size exclusion high-performance liquid chromatography (SEC-HPLC) (Waters, Milford, USA) and SDS-PAGE.

Determination of concentration of thiol groups in Fab' fragment Ellman's reagent (Boehringer-Mannheim, Mannheim, Germany) was employed to determine the concentration of thiol groups[12]. Five microliters of Ellman's solution (4.0 mg/mL in distilled water) and 25 mL each of 6 cysteine standard solutions (0, 31.5, 62.5, 125, 250, 500 μmol/L) were added to each well of a 96-well plate. Reaction buffer (200 μL; 0.1 mol/L sodium phosphate; pH 8.0) was then added to each well. The reaction was allowed to proceed at room temperature for 15 min. Absorbance of the wells at 405 nm was measured on an OTC 400 96-well plate reader (Organon Teknika, Durham, USA). The values obtained for the standards were plotted to give a standard curve. The concentration of the unknown sample was inferred from the curve.

Preparation of B43 Fab'-BAT The production of milligram quantities of thiolated Fab' fragments of B43 prepared as described earlier has allowed the design and implementation of a new conjugation methodology for labeling Fab' with radiometals. The central strategy here was to exploit the high chemical reactivity of the hinge thiol groups on the Fab' fragment as attachment sites for BAT and the thiol reactive bromoacetamido linking chemistry. To a solution of 150 μmol/L Fab' in sodium citrate buffer (pH 5.0) containing 1 mmol/L EDTA was added a freshly diluted solution of BAT in 0.2 mol/L Na_2HPO_4 (pH 8.5) buffer to afford final concentrations of Fab' and BAT of 72 μmol/L and 11 mmol/L, respectively, at pH 7.4. After various time periods at 37 ºC, the reaction was fractionated according to molecular weight by SEC-HPLC. Thirty minutes reaction time was found to be optimal. The monovalent conjugate fraction was collected and concentrated using a 30 kDa concentrator. The conjugate was stored at -70 ºC until immediately before use.

Preparation of ^{67}Cu- BAT-Fab' The specific activity of the $^{67}CuCl_2$ in 2 mol/L HCl (Nordion International, Kanata, Canada) varied from 0.8 to 6 mCi/μg copper. ^{67}Cu in dilute HCl was dried on a 70 ºC heat block under a gentle stream of nitrogen gas. B43 Fab'-BAT in 0.1 mol/L ammonium citrate (pH 5) was added to the dried ^{67}Cu and incubated for 30 min at room temperature. Free ^{67}Cu was scavenged by the addition of 100 mmol/L EDTA to a final concentration of 10 mmol/L. The immunoconjugate was separated from ^{67}Cu-EDTA by SEC-HPLC. The specific activity of ^{67}Cu-BAT-Fab' was 4.5–6 mCi/μg. The purified ^{67}Cu-BAT-Fab' was formulated at 1 mCi/mL in 4% human serum Alb/saline for future uses[13].

Comparison of the immunoreactivities of intact B43 with its Fab' fragment and Fab'-BAT Enzyme-linked immunosorbent assay (ELISA) was used. OVCAR, a human ovarian carcinoma cell line positive for the CA125 antigen was obtained from AltaRex. Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum (FBS) supplemented with 2 mmol/L L-glutamine and 100 U/mL penicillin was used to maintain the cells. One day before each experiment, 1×10^5 OVCAR cells were added to each well of a 96-well flat-bottom tissue culture plate and incubated at 37 ºC overnight in a sterile 5% CO_2 humidified incubator. The attached cells were washed with 37 ºC DMEM

medium without FBS 3 times and were incubated with DMEM medium containing various concentrations of the antibody preparations in a 37 ºC incubator for 2 h. MOPC21 (Sigma), a mouse IgG_1 MoAb, was used as a negative control. The cells were then washed with 37 ºC DMEM medium without FBS 3 times. Peroxidase-conjugated rabbit anti-mouse IgG (1/4000 diluted; Dako, Carpenteria, USA) was added to the wells for 1.0 h incubation at 37 ºC. After 3 washings with 37 ºC DMEM medium without FBS, 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Bio-Rad, Hercules, CA, USA) was added to the wells for approximately 20 min incubation at room temperature with continuous shaking. The reaction was quenched with 1.0 mol/L HCl. Optical intensity at 450 nm in each well was determined using a 96-well microplate reader.

Comparison of the immunoreactivities of Fab'-BAT with ^{67}Cu- BAT-Fab' As described earlier, the serial-diluted Fab'-BAT and ^{67}Cu-BAT-Fab' were used to determine the loss of immunoreactivity.

Statistical analysis Data are presented as mean±SD. Statistical differences between groups were determined by using Student's *t*-test. For all analyses, $P<0.05$ was considered statistically significant.

Results

Preparation of B43 F(ab')$_2$ by pepsin digestion The products of digestion were analyzed by SDS-PAGE under non-reducing conditions. Intact B43 and F(ab')$_2$ fragments yielded single bands of apparent molecular weights of 150 kD and 100 kDa, respectively (Figure 1). Most of the intact B43 (>90%) was converted to F(ab')$_2$ after 6 h digestion, according to densitometer readings (Bio-Rad). There was no indication of further degradation of F(ab')$_2$ after longer digestion times, and no further purification of F(ab')$_2$ was deemed necessary.

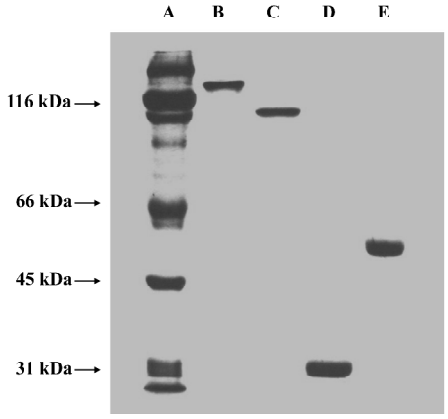


Figure 1. Analysis of B43 and its fragments by SDS-PAGE. Lane A: marker. Lane B: intact B43 monoclonal antibody. Lane C: F(ab')$_2$ fragment. Lane D: Fab' fragment under reducing condition. Lane E: Fab' fragment under non-reducing conditions.

Analysis of B43 Fab' fragments SEC-HPLC analysis indicated that approximately 96% of the F(ab')$_2$ fragments of B43 were cleaved to form Fab' after 30 min reduction with 10 mmol/L cysteine. The number of thiol groups in each Fab' was determined to be 1.8 by the use of Ellman's reagent. The products of reduction were analyzed by SDS-PAGE under reducing and non-reducing conditions. The Fab' fragments yielded single bands of apparent molecular weights of 25 kDa under reducing conditions and 50 kDa under non-reducing conditions, respectively (Figure 1). The free thiol groups in the hinge-region of Fab' can be used for conjugation with other molecules.

Determination of immunoreactivities of Fab'-BAT The ELISA results (Figure 2) showed that the binding of the Fab' fragment to antigen CA125 expressed on OVCAR cells was similar to that of intact B43 ($P>0.05$), indicating that the immunoreactivity of the antibody had not been destroyed during pepsin digestion and cysteine reduction. The immunoreactivity of the conjugate Fab'-BAT was reduced by less than 2 orders of magnitude relative to that of the intact B4 ($P<0.05$).

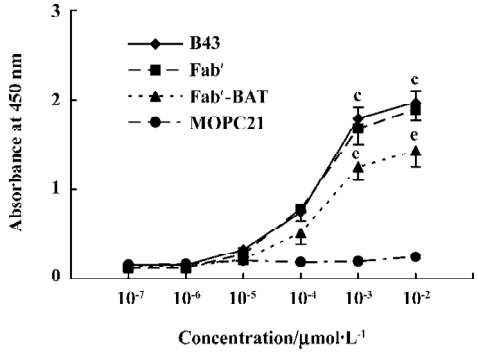
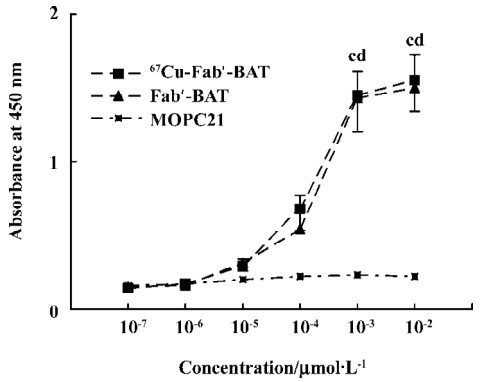


Figure 2. Comparison of B43 immunoreactivity with Fab' fragments and the Fab'-BAT conjugate. Fab' retained virtually identical immunoreactivity to the intact B43, and Fab'-BAT had a less than 2 orders of magnitude reduction in immunoreactivity of the intact antibody. n=3, from 3 separate experiments. Mean±SD. $^cP<0.01$ *vs* normal mouse IgG MOPC21. $^eP<0.05$ *vs* intact B43 and Fab'.

Determination of immunoreactivities of ^{67}Cu-BAT-Fab' The ELISA results (Figure 3) showed that the binding of the ^{67}Cu-BAT-Fab' fragment to antigen CA125 expressed on OVCAR cells was similar to that of BAT-Fab' ($P>0.05$), indicating that the immunoreactivity of the antibody had not been destroyed during ^{67}Cu labeling.



Figrue 3. Comparison of the immunoreactivity of ^{67}Cu-Fab'-BAT with the Fab'-BAT conjugate. The ^{67}Cu labeling did not impair the immunoreactivity of Fab'-BAT. ^{67}Cu-Fab'-BAT retained identical immunoreactivity to the Fab'-BAT. n=3, from 3 separate experiments. Mean±SD. ^{c}P<0.01 *vs* normal mouse IgG MOPC21. ^{d}P>0.05 *vs* ^{67}Cu-Fab'-BAT.

Discussion

Antibodies labeled with longer-lived, positron-emitting isotopes such as ^{67}Cu have advantages over traditionally labeled antibodies because of their improved sensitivity for detecting tumors and small metastases. MoAb Fab' fragments have a shorter biological half-life than F(ab')$_2$ fragments or whole IgG molecules, and are rapidly cleared through both the liver and kidneys. The use of Fab' fragments yields a lower blood-pool activity and improves image contrast compared with intact MoAb and F(ab')$_2$ fragments. ^{67}Cu-labeled Fab' fragments may be more sensitive agents for detecting tumors, and cause the formation of fewer human anti-mouse antibodies (HAMA)[9,14,15].

Current techniques for preparing Fab' fragments necessitate purification steps that may cause irreversible damage to the antigen-binding sites due to denaturation of the antibody[16,17]. For several conjugation methods of antibody fragments and other molecules, such as glutaraldehyde and periodate methods, some active groups or linking agents have to be introduced to the Fab' fragments before they are conjugated to the other molecules[9,18–20]. These steps may further destroy the immunoreactivities of the Fab' fragments. However, some bifunctional chelators, such as BAT, can also be attached to amine, sulfhydryl, imidazole, or thiol groups on the amino acid side chains of the Fab'. The thiol group has been found to be the most reactive among these[21]. The direct use of the thiol group for conjugation may be beneficial for preserving the bioactivities of conjugates during the purification process.

In the present study we introduced a simple, 2-step approach for site-specific conjugation of B43 Fab' fragments with BAT directly using the thiol groups in the hinge-region. This method in principle ensures that the coupling sites between BAT and Fab' are distal to the hypervariable region. The method should dramatically reduce the risk of inactivation of Fab' due to modification of amino acid residues that are essential to the spatial organization of the antigen-binding sites. In practice, site-specific conjugation has shown a high yield and an acceptable loss of immunoreactivity of the product.

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