Alterations of Specific and Nonspecific Binding of Monoclonal Antibody by Introduction of Acidic and Hydrophobic Groups

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AB-6 monoclonal antibody (mAb) against bovine serum albumin (BSA) was coupled with β-alanine or n-propylamine using a heterobifunctional cross-linking reagent, N-(ε-maleimidocaproyloxy)succinimide (EMCS) and a linker, poly-L-cysteine to introduce acidic or hydrophobic groups into the mAb. The mAb molecules coupled with β-alanine at lower than 1:16 molar ratios and those coupled with n-propylamine at 1:14 retained the original reactivity to BSA but modification with higher amounts of β-alanine or n-propylamine decreased the reactivity. In contrast, nonspecific bindings of AB-6 mAb to a plastic culture plate and tumor (MDA-MB-453) cells were decreased by modification with β-alanine and increased by n-propylamine. These results suggested that introduction of acidic groups, and hydrophobic groups to antibody leads to fluctuations in the non-specific binding of antibody.

Keywords bovine serum albumin (BSA); monoclonal antibody (mAb); acidic monoclonal antibody; hydrophobic monoclonal antibody; antigen specificity; nonspecific binding

Mammalian cells possess a zeta potential which results from membrane-associated acidic residues and imparts a global negative charge to the cell surface.1–3 Antibodies (Abs) bind to a corresponding antigen molecule by virtue of their ionic, hydrophobic and stereochemical attractions, and the binding to cell surface antigens is regulated by net charge of cell surface membrane. Since Abs generally have a positive charge, this contributes in part not only to immunologically specific but also to nonspecific binding with cells.4

In the present work, anti-bovine serum albumin (anti-BSA) monoclonal antibody (mAb) was modified with polycysteine and then conjugated with an acidic (β-alanine) or hydrophobic (n-propylamine) reagent by using a heterobifunctional cross-linker, N-(ε-maleimidocaproyloxy)succinimide (EMCS).5,6 We report herein that coupling of a relatively large amount of β-alanine with mAb lowered the isoelectric point (pI) of mAb, that the modification did not abolish the antigen binding activity but lowered immunologically nonspecific binding to cells and that introduction of n-propylamine increased hydrophobicity of mAb, leading to an increase in nonspecific binding to the antigen and cells.

MATERIALS AND METHODS

Chemicals Poly-S-carbobenzoxy-L-cysteine (poly-S-CBZ-L-cysteine, M.W. 5000-15000) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co., St. Louis, MO., U.S.A.; a heterobifunctional reagent, EMCS, from Dojindo Lab., Kumamoto, and β-alanine and n-propylamine from Wako Pure Chemical Industries, Ltd., Tokyo. All the reagents used were of reagent grade.

Cells MDA-MB-453 human breast cancer cells maintained in culture were used. When cultured in fetal calf serum (FCS)-containing medium, MDA-MB-453 cells are found to firmly bind bovine serum albumin (BSA) on the surface but they are freed from BSA by culturing for 24 h in Hymedium 606 serum-free medium. The cells with bound BSA and BSA-free cells were used in the experiments.

mAb Anti-BSA mAb, AB-6, which specifically reacts with BSA, was produced in BALB/c mice by transplantation of the corresponding hybridomas prepared in our laboratories. mAb was purified from the ascitic fluids by 50% saturated ammonium sulfate precipitation followed by DEAE column chromatography. AB-6 mAb was used throughout the present work.

Chemical Modification of mAb mAb was modified by the following procedures.

Preparation of Poly-L-cysteine Solution: One mg (0.67 × 10⁻⁸ mol) of poly-S-carbobenzoxy-L-cysteine was dissolved in 250 µl of dimethyl sulfoxide. S-Carbobenzoxy group of the compound was alcoholized by adding 5 µl of sodium methydate and incubated for 30 min at 30 °C in an atmosphere of nitrogen to yield poly-L-cysteine.7 After reaction, the precipitated material was dissolved in 250 µl of PBS and pH of the solution was adjusted to 7.0 with 0.5 N H₃PO₄.

Coupling of β-Alanine and n-Propylamine with EMCS: β-Alanine or n-propylamine in phosphate buffered saline (PBS, 0.67 × 10⁻⁸ mol/µl) was added to the same volume of EMCS solution in tetrahydrofuran (THF, 0.67 × 10⁻⁸ mol/µl) and incubated for 30 min at 30 °C with occasional agitation. After reaction, THF in the solvent was removed by flushing with nitrogen.

Coupling of mAb with EMCS: mAb (6.0 × 10⁻⁸ mol) in 2.25 ml of PBS was incubated with the same molar amount of EMCS in THF for 30 min at 30 °C with occasional agitation. The EMCS–mAb conjugate was used without further purification.

Coupling of EMCS–β-Alanine or EMCS–n-Propylamine with mAbs through Poly-L-cysteine: Poly-L-cysteine solution (50 µl) was added to a mixture of 250 µl of mAb and various amounts of EMCS–β-alanine or EMCS–n-propylamine, and the solution was incubated for 2 h at 25 °C with stirring. After the reaction, the solution was applied to a G-50 column (6 ml of gel) and products eluted with PBS at 200 µl of a fraction
volume were monitored spectrophotometrically at 280 nm. EMCS–mAb treated only with poly-L-cysteine was purified as above and used as a control.

Determination of the Amount of β-Alanine or n-Propylamine Introduced to mAb  Molar ratios of β-alanine or n-propylamine to mAb in the final products were assessed by determining sulphydryl content in the modified Ab. A stock solution (0.01 M, pH 6.65) was prepared by dissolving DTNB in 0.037 M phosphate buffer, pH 8.0. Solution for the reagent blank was prepared by mixing 40 μL of a stock DTNB solution with 10 μL of 0.025 M EDTA (disodium salt) and 150 μL of PBS, pH 7.4. For the reaction mixture, 150 μL of mAb solution in PBS (0.8 – 1.0 mg/mL) was added to the above reagent blank solution. After standing for 45 min, the absorbance of the reaction mixture was read against that of the reagent blank on a spectrophotometer at 412 nm. The amount of β-alanine or n-propylamine introduced into mAb was calculated by subtracting the amount of thiol groups in the modified mAb from that of mAb coupled only with poly-L-cysteine.

Isoelectric Focusing (IEF) IEF was performed in the regular manner using gels prepared from acrylamide, bisacrylamide, Bio-lyte (pH 3.0 – 10.0) and glycerol. An aliquot (20 μL) of a sample solution (1.0 – 1.5 mg/mL) was diluted with a mixture of 4% Bio-lyte and 60% glycerol and loaded on a gel sheet. It was overlaid with 20 μL of the Bio-lyte solution (2% Bio-lyte and 15% glycerol) and the upper and lower reservoirs were filled with 0.02 M NaOH and 0.02 M phosphoric acid solution containing 0.1 mg glutamic acid, respectively. Gels were subjected to electrophoresis for 1 h at 100 V, 4 – 6 h at 200 V and 1 h at 300 V. At the end of the run, the gels were removed and fixed in 25% trichloroacetic acid for 45 min. After equilibration with 30% methanol/10% acetic acid, the gels were stained with 0.25% Coomasie brilliant blue G250 in 25% methanol/10% acetic acid.

The pH gradient formed during electrophoresis was measured using a pH electrode (Toa Co., Tokyo). Immediately after electrophoresis, the gels were sliced into 5 mm sections, which were extracted with distilled water at room temperature for 2 h and pH of the extracts was measured.

Enzyme-Linked Immunosorbent Assay (ELISA) Polyvinylchloride (PVC) 96-well microtiter plates (type E, Sumitomo Bakelite Co., Tokyo) were coated with 50 μL of BSA (50 μg/mL) in PBS at 4°C overnight. Residual unsaturated sites on each well were blocked with 0.5% (w/v) skimmed milk (Yukijirushi Co., Tokyo) in PBS at 37°C for 2 h. A solution containing modified or unmodified mAb was diluted with 0.05% Tween–PBS and an aliquot (50 μL) of the solution was added to each well. After incubation for 1 h at 37°C, wells were washed 5 times with 0.05% Tween–PBS, and BSA–mAb complex in each well was treated with rabbit anti-mouse immunoglobulin serum (Dako Japan Co., Kyoto) for 1 h and then reacted with horse-radish peroxidase-conjugated protein A for 30 min at 37°C. After washing, peroxidase activity was determined colorimetrically after addition of 100 μL of 3,3',5,5'-tetramethylbenzidine solution (0.1 mg/ml) in 0.1 M acetate-citrate buffer (pH 6.0) containing 0.01% H₂O₂. Reaction was stopped with 0.5 M H₂SO₄ and the developed color was measured at 450 nm with an InterMed. ImmunoReader NJ2000. For determination of nonspecific binding of mAb, a PVC plate was used without BSA-coating.

Flow Cytometry BSA-bearing or BSA-free MDA-MB-453 cells were incubated with 100 μL of a solution containing unmodified or chemically modified mAb for 1 h at 4°C and washed with PBS. The cells were then stained with 100 μL of 1:200 dilution fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (Dako) for 1 h at 4°C. After two washes, cells were analyzed for cell-surface fluorescence using a FACSscan (Becton Dickinson, U.S.A.).

RESULTS

Alteration of Properties of mAb by Chemical Modification The scheme for the introduction of acidic and hydrophobic groups to mAb is summarized in Fig. 1. The products of a reaction mixture of EMCS–mAb, EMCS-β-alanine (or EMCS-α-propylamine) and poly-L-

![Fig. 1. Scheme for Preparation of mAb Coupled with Acidic (β-Alanine) or Hydrophobic Reagent (n-Propylamine)](image-url)
cysteine were separated by Sephadex G-50 column chromatography. As the products from EMCS-β-alanine and EMCS-n-propylamine showed almost the same chromatograms, only a profile of the products from EMCS-β-alanine is depicted in Fig. 2. Irrespective of the molar ratios of EMCS-mAb to EMCS-β-alanine or EMCS-n-propylamine in the reaction, mAb couple with β-alanine or n-propylamine through poly-L-cysteine was eluted into the first peak fractions (fractions 4–7) as determined by the protein and SH contents. The material obtained from the first peak fractions was used for further characterization of the conjugates. Henceforce, these materials are called A-8, H-14 and so on, where the character and figure represent the coupled reagent (A, β-alanine; H, n-propylamine) and the average molar ratio to mAb (see Table I), respectively. The second peak fractions (fractions 8–18) did not contain protein but contained unreacted EMCS-β-alanine or EMCS-n-propylamine, both of which showed UV \( \lambda_{\text{max}} \) at 255 nm \( (\varepsilon = 15700) \) and \( \varepsilon = 6590 \) at 280 nm.

The dependence of pH values of the conjugated products on the amounts of mAb coupled with β-alanine and n-propylamine are shown in Table I, and the electrophoresis profiles are shown in Fig. 3. The pH value was lowered by increased amount of β-alanine in mAb. Concomitantly, IEF patterns showed the differences between β-alanine-modified mAb (Fig. 3A lanes 3–5) and unmodified mAb or mAb coupled with poly-L-cysteine alone (Fig. 3A, lanes 1 and 2). Although Sephadex G-50 column chromatography allowed the separation of the reaction mixture to free β-alanine, free poly-L-cysteine and β-alanine-modified mAb fractions, the last fraction contained mAb molecules coupled with different amount of β-alanine as observed by the IEF profile (see Fig. 3).

As to the modification with n-propylamine, pH of the modified mAb was decreased by introduction of n-propylamine to mAb (Fig. 3B, lanes 3–5). The decrease of pH in the n-propylamine-modified mAb might be due to the increased hydrophobicity but not to free SH groups present in the mAb, because blocking of free SH with iodoacetamide did not affect the pH. In this case too, the chromatography did not allow the separation of mAb molecules coupled with different amounts of n-propylamine. mAbs modified with poly-L-cysteine alone or EMCS alone showed the same IEF patterns as unmodified mAb which exhibited 3 to 4 main protein bands (lanes 1 and

<p>| Table I. Isoelectric Point (pH) of AB-6mAb Coupled with Various Amounts of β-Alanine or n-Propylamine |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th>Name of modified mAb</th>
<th>Molar ratio (mAb : reagent)</th>
<th>Reagent bound with mAb (mol)</th>
<th>pH</th>
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<tbody>
<tr>
<td>U (unmodified)</td>
<td></td>
<td></td>
<td>7.40</td>
</tr>
<tr>
<td>Cβ</td>
<td></td>
<td></td>
<td>7.40</td>
</tr>
<tr>
<td>β-Alanine</td>
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<td></td>
<td>8.4</td>
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<td></td>
<td>15.5</td>
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<td>28.6</td>
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<tr>
<td>A-34</td>
<td>1:128</td>
<td></td>
<td>34.0</td>
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<tr>
<td>n-Propylamine</td>
<td></td>
<td></td>
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<tr>
<td>H-14</td>
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<tr>
<td>H-46</td>
<td>1:128</td>
<td></td>
<td>46.2</td>
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</table>

a) AB-6mAb was treated with a reagent (EMCS-β-alanine or EMCS-n-propylamine) at the indicated molar ratios in the presence of poly-L-cysteine.
b) Amount (molar ratio) of β-alanine or n-propylamine coupled to mAb (see Materials and Methods). c) Mean value of replicate samples. d) AB-6mAb treated with poly-L-cysteine alone. e) The products were named by the type of reagent used for mAb modification (A, β-alanine; H, n-propylamine) and the average molar ratio of the reagents coupled to mAb (see third column).
Fig. 4. Effect of Chemical Modification on the Reactivity of AB-6 mAb to BSA

Materials obtained from the first peak fraction in Sephadex G-50 chromatography (see Fig. 2) were adjusted to a concentration of 1.0 µg/ml and then diluted with PBS. The binding of the materials to BSA was assessed by ELISA as described in Materials and Methods. Figure 4A, β-ala-modified AB-6 mAb; ○, unmodified mAb (U); ▲, treated with poly-L-cysteine alone (C); □, A-8; ●, A-16; △, A-29; ■, A-34. Figure 4B, n-propylamine-modified AB-6 mAb: ○, U; ▲, C; □, H-14; ●, H-23; △, H-30; ■, H-46 (for the abbreviated name of materials, refer to Table I). The data represent mean ± S.D. of the mean of triplicate samples.

2). Binding Activity of Chemically Modified mAbs to BSA

Binding activities of chemically modified and unmodified mAbs to BSA were assessed by ELISA. The results are depicted in Fig. 4. The reactivity of A-8 and A-16 to BSA was not significantly reduced as compared with unmodified Ab (Fig. 4A), whereas both A-29 and A-34 were decreased in binding capacity to 68.5% of unmodified mAb, as observed at the concentration of 0.1 µg/ml of mAb.

When mAb was coupled with n-propylamine, H-14 maintained its original binding capacity to BSA (Fig. 4B), but mAb preparations coupled with larger amounts of n-propylamine decreased in binding capacity to BSA; the activity of both H-23 and H-30 decreased to 80% of unmodified mAb and H-46 to 57.7%, as observed at the mAb concentration of 0.1 µg/ml. Coupling of poly-L-cysteine alone did not affect the immunoreactivity of mAb.

Immunologically Nonspecific Binding of Chemically Modified mAb

As assessed by the ELISA, the immunoreactivity of A-8, A-16 and H-14 did not change as compared with the unmodified mAb. We examined the immunologically nonspecific reactivity of these chemically modified mAbs using a PVC plate and MDA-MB-453 cells. The results are shown in Figs. 5 and 6. Binding to a PVC plate was significantly smaller in A-16 mAb and larger in H-14 mAb than unmodified mAb, as determined by ELISA (Fig. 5). The binding differed little between A-8 and A-16, but A-34 showed significantly lower PCV-binding capacity than A-16.

As for the binding to cells, both A-16 and H-14 bound to BSA-bearing MDA-MB-453 cells as did unmodified mAb (Fig. 6A), whereas the binding to BSA-free cells was significantly smaller in A-16 and larger in H-14 than unmodified mAb (Fig. 6B).
DISCUSSION

Although Ab specifically recognizes a corresponding antigen, it often shows nonspecific binding to a macromolecule or to mammalian cells through hydrophobic and/or ionic interactions. Thus, introduction of anionic groups into Ab may decrease the nonspecific binding of Ab. For instance, when negatively charged mAbs coupled with diethylenetriamine pentaacetic acid (DTPA)-succinylated polylysine\textsuperscript{9–11} are given to mice, they show lower nonspecific accumulation in tissues or organs.\textsuperscript{12–15}

To examine the effect of anionic charge in mAb on the immunoreactivity, we devised a new technique of introducing acidic groups to mAb. Although DTPA has frequently been used to introduce acidic groups to Ab,\textsuperscript{9–12} we used β-alanine as the reagent. In our method, both mAb and β-alanine were modified first with a heterobifunctional cross-linking reagent, EMCS, and then EMCS–mAb and EMCS–β-alanine were entailed through poly-l-cysteine at various ratios. The use of EMCS is appropriate to carry out the coupling under aqueous and mild conditions.\textsuperscript{16,17}

Modification of AB-6 mAb with β-alanine at lower than 1:16 (A-8 and A-16) did not alter the binding capacity of mAb to BSA. In order to determine their nonspecific binding activity, we used PVC plates and tumor cells. As expected, the modification of mAb with β-alanine decreased the binding of mAb to a plastic plate and tumor cells.

In addition to electrostatic property, hydrophilic or hydrophobic property also influences the immunoreactivity of mAbs. Therefore, we introduced n-propyamine to mAb employing the same coupling method as above, and examined the change in immunoreactivity of the mAb. In contrast to the acidic modification, the modification with n-propyamine increased the binding of mAb to a plastic plate and tumor cells. Because β-alanine-modified (A-8 and A-16) and n-propyamine-modified (H-14) mAbs showed the same binding capacity to BSA, the introduction of acidic and hydrophobic groups to mAb would account for the alteration of nonspecific binding capacities of mAb. We have applied the present method to another mAb, HBJ127 mAb which recognizes a proliferation-associated antigen, gp125,\textsuperscript{18} and obtained results similar to those above (data not shown).

We recently reported the enhanced binding of mAbs modified with cross-linker.\textsuperscript{19,20} In the present work, we provided a new method for coupling of a variety of reagents to mAbs using a heterobifunctional cross-linking reagent and poly-l-cysteine, and examined the alteration to an anti-BSA mAb in its antigen specific and nonspecific bindings in vitro by introducing acidic or hydrophobic groups to the mAb.

REFERENCES