Effect of trastuzumab interchain disulfide bond cleavage on Fc γ receptor binding and antibody-dependent tumour cell phagocytosis

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The Fc domain of human IgG1 binds to Fc γ receptors (Fc γ Rs) to induce effector functions such as phagocytosis. There are four interchain disulfide bonds between the H and L chains. In this study, the disulfide bonds within the IgG1 trastuzumab (TRA), which is specific for HER2, were cleaved by mild S-sulfonation or by mild reduction followed by S-alkylation with three different reagents. The cleavage did not change the binding activities of TRA to HER2-bearing SK-BR-3 cells. The binding activities of TRA to Fc γ RIIA and Fc γ RIIB were greatly enhanced by modification with mild reduction and S-alkylation with ICH₂CONH₂ or N-(4-aminophenyl) maleimide, while the binding activities of TRA to Fc γ RI and Fc γ RIIIA were decreased by any of the four modifications. However, the interchain disulfide bond cleavage by the different modifications did not change the antibody-dependent cell-mediated phagocytosis (ADCP) of SK-BR-3 cells by activated THP-1 cells. The order of Fc γ R expression levels on the THP-1 cells was Fc γ RII > Fc γ RI > Fc γ RIII and ADCP was inhibited by blocking antibodies against Fc γ RI and Fc γ RII. These results imply that the effect of the interchain disulfide bond cleavage on Fc γ Rs binding and ADCP is dependent on modifications of the cysteine residues and the Fc γ R isotypes.

Keywords: Fc γ receptors/IgG1/interchain disulfide bonds/phagocytosis/trastuzumab.

Abbreviations: ADCC, Ab-dependent cellular cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; Fc γ R, Fc γ receptors; HER2, human epidermal growth factor receptor 2; IVIG, human intravenous immunoglobulin; mAb, monoclonal antibody; S–S bond, disulfide bond; TRA, trastuzumab; TRA-S-A-NH₃⁺, N-(2-aminoethyl) maleimidyl TRA; TRA-S-B-NH₃⁺, N-(4-aminophenyl) maleimidyl TRA; TRA-S-CH₂CONH₂, S-carbamidomethyl TRA; TRA-S-SO₃[−], S-sulfonated TRA.

Over 30 monoclonal antibody (mAb) therapies have been approved by the United States Food and Drug Administration for the treatment of cancer, autoimmune diseases and infectious diseases and approximately 600 clinical trials of mAbs are currently being conducted (1, 2). To decrease their immunogenicity in clinical use, mouse mAbs have been modified to chimeric mAbs or humanized mAbs, or fully human mAbs have been developed by means of transgenic mice or phage displays expressing human H and L chains (3). Most therapeutic mAbs are of the human IgG1 isotype (1–3).

Antibodies (Abs) not only bind to the corresponding antigens resulting in inhibiting or inducing their biological activities but also exert effector functions such as Ab-dependent cell-mediated phagocytosis (ADCP) and Ab-dependent cellular cytotoxicity (ADCC) through interaction between the Fc domains of Abs and Fc γ receptors (Fc γ Rs) on the surface of effector cells (4). There are several different kinds of Fc γ Rs, *i.e.* Fc γ RI (CD64), Fc γ RII (CD32) and Fc γ RIII (CD16). Fc γ RI has a high affinity for human IgG1 while other Fc γ Rs have low affinities (5). Fc γ RIIB suppresses effector functions, while other Fc γ Rs including Fc γ RIIA activate effector functions (4–6).

Trastuzumab (TRA) is a humanized monoclonal Ab (mAb) that specifically binds to human epidermal growth factor receptor 2 (HER2) and has been clinically used for the treatment of HER2-positive breast cancer (7). The doses of TRA, rituximab and bevacizumab that are widely used for cancer therapy are as large as a few hundred milligrams per patient. Therefore, increased potency (in terms of tumour regression) is needed for therapeutic mAbs. There are many different approaches to augment the effector functions of therapeutic mAbs. Ado-trastuzumab emtansine, a conjugate of TRA and mertansine (a cytotoxic agent that binds tubulin), has been approved by the FDA for the treatment of HER2-positive metastatic breast cancer (8). Mogamulizumab, which is an anti-CC chemokine receptor 4 mAb that is defucosylated resulting in high affinity for Fc γ RIIIA, is anticipated to exert enhanced ADCC to kill tumour cells (9). Since the clinical effects of TRA involve ADCP as well as ADCC (10, 11), modified TRA with enhanced effector functions is desired.

In human IgG1, there are 4 interchain disulfide (S–S) bonds between H and L chains and 12 intrachain S–S bonds, each of which is located in a separate domain of IgG1. The interchain S–S bonds are located

in the hinge and the upper C_H2 domain that seems more flexible than other domains in the protein conformation (12, 13). Therefore, the interchain S–S bonds are much more sensitive to cleavage by S-sulfonation or by mild reduction followed by S-alkylation (reduction/alkylation) than are the intrachain S–S bonds (14, 15).

The interchain S–S bonds help maintain the conformation of the hinge and the C_H2 domain of IgG1 Abs, which makes contact with FcγRs (12, 13, 16). Therefore, S-sulfonation and reduction/alkylation of mAbs are expected to modify their effector functions. In the present study, we cleaved the interchain S–S bonds of TRA by 4 different methods, and the modified TRAs were compared with the original TRA in terms of binding affinities for FcγR isotypes and ADCC activities.

Materials and Methods

Cells, chemicals and antibodies

SK-BR-3 and Ramos cells were kindly provided by the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). THP-1 cells (human monocytic leukaemia cell line) were purchased from the American Type Culture Collection (Rockville, MD). SK-BR-3, Ramos and THP-1 cells were cultured in RPMI1640 (Wako Pure Chemical Industries Ltd., Osaka) containing 10% FBS (Equitech-Bio, Inc., Kerrville, TX), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich Co., Saint Louis, MO).

Trastuzumab (anti-HER2, Herceptin) and Rituximab (anti-CD20, Rituxan) were purchased from Chugai Pharmaceutical Co., Ltd (Tokyo) and Zenyaku Kogyo Co., Ltd (Tokyo), respectively. They were dialyzed against phosphate-buffered saline containing 0.2% polyethylene glycol 4000 (PBS/PEG) to remove additives of small molecules. PEG prevents aggregation of IgG. N-(2-Aminoethyl) maleimide trifluoroacetate salt and N-(4-Aminophenyl) maleimide were purchased from Sigma-Aldrich Co. (Saint Louis) and Tokyo Chemical Industry Co., Ltd., respectively.

Mild S-sulfonation or mild reduction followed by S-alkylation of trastuzumab

Mild S-sulfonated TRA (TRA-S-SO₃[−]) was prepared based on the method of Masuho *et al.* (14), with some modifications. TRA (0.5 mg/ml), 54 mM Na₂SO₃ (Wako Pure Chemical Industries Ltd) and 21 mM Na₂S₄O₆ (Sigma-Aldrich Co., Saint Louis, MO) were mixed in 50 mM Tris–HCl buffer (pH 8.2) containing 0.14 M NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA) and 0.2% polyethylene glycol 4000 (modification buffer). The reaction mixture was incubated at 37°C for 4 h to cleave the interchain S–S bonds and then dialyzed against PBS/PEG.

TRA (0.5 mg/ml) and 10 mM dithiothreitol (Sigma-Aldrich Co.) were mixed in modification buffer. The reaction mixture was incubated at 37°C for 1 h to reduce the interchain S–S bonds. After removing dithiothreitol by dialysis, one third of the reduced TRA was allowed to react with 3 mM N-(2-aminoethyl) maleimide at room temperature for 6 h, another one third was allowed to react with 3 mM N-(4-aminophenyl) maleimide at room temperature for 6 h, and another one third was allowed to react with 50 mM ICH₂CONH₂ at 37°C for 30 min. These reaction mixtures were dialyzed against PBS/PEG.

Biochemical analyses of the original and modified TRAs

The interchain S–S bonds between H and L chains were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions with a 12% polyacrylamide gel. Samples (1 µg protein/lane) were subjected to SDS-PAGE, and the protein bands were stained with Bio-Safe Coomassie (Bio-Rad Laboratories, Inc., Hercules, CA).

TRAs were also analysed by gel filtration high-performance liquid chromatography (HPLC) on a Protein Pak G3000SWXL column (Tosoh Corp., Tokyo) at a flow rate of 1 ml/min in 0.1 M sodium

phosphate buffer (pH 6.8). Samples containing 10 µg of protein were injected into the column, and the elution profiles were analysed by using HPLC ChromNAV software (Jasco Corporation, Tokyo).

Binding of TRAs to the HER2 antigen on SK-BR-3 cells

SK-BR-3 cells were used to evaluate the binding activities of TRAs to the HER2 antigen on the cell surface. SK-BR-3 cells (1.0 × 10⁶ cells/ml) were blocked in FACS buffer (PBS containing 0.1% BSA and 0.02% NaN₃) at 4°C for 30 min. After centrifugation at 2,000 rpm for 5 min at 4°C, the supernatants were discarded and the cells were allowed to react with serially diluted TRAs in 0.1 ml of FACS buffer at 37°C for 1 h. The cells were washed twice with 0.5 ml of FACS buffer and then allowed to react with 10 µg/ml fluorescein isothiocyanate-conjugated goat anti-human κ chain (Millipore, Billerica, MA) in 0.1 ml of FACS buffer on ice for 30 min. The cells were again washed twice and suspended in 0.5 ml of FACS buffer. The fluorescence intensities of individual samples were measured by using a FACS LSR flow cytometer equipped with CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

FcγRs-binding assay by ELISA

Recombinant proteins consisting of the extracellular domains of human FcγRs fused with glutathione S-transferase were prepared as described previously (17). There are two genotypes in FcγRIIIA, FcγRIIIA-Val158 and FcγRIIIA-Phe158. In the present study, FcγRIIIA-Val158 was used since its binding affinity to Fc is higher than FcγRIIIA-Phe158. Microtiter plates (Becton-Dickinson Co., Franklin Lakes, NJ) were coated with 4 µg/ml of the FcγR proteins in PBS at 4°C overnight and blocked with 25 mM Tris HCl (pH 7.4) containing 0.14 M NaCl, 0.5% BSA, 2 mM EDTA and 0.05% Tween 20 (ELISA buffer) at 37°C for 2 h. TRAs were serially diluted in ELISA buffer and dispensed into individual wells of the FcγR-coated plates, which were then incubated at 37°C for 2 h. The plates were washed three times with ELISA buffer, and then 0.25 µg/ml horseradish peroxidase (HRP)-conjugated goat anti-human IgG (H + L) affinity-purified F(ab')₂ (Chemicon International, Inc, Temecula, CA) in ELISA buffer was dispensed into the wells, and the plates were incubated at 37°C for 1 h. After washing again in the same manner, 0.1 ml of 0.4% *o*-phenylenediamine dihydrochloride and 0.01% hydrogen peroxide in 0.1 M citrate buffer (pH 5.0) were added to the wells, and the plates were allowed to stand at room temperature for 20 min. Absorbance at 450 nm was measured by using a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA).

Ab-dependent cell-mediated phagocytosis (ADCP) assay

SK-BR-3 cells, or the target cells, were suspended in Hank's balanced salt solution (Gibco, Grand Island, NY) containing 10 mM HEPES (HBSS-F), and the cell suspension was centrifuged at 1000 rpm at 4°C for 5 min. The supernatant was discarded, and the cells were resuspended in 950 µl of HBSS-F. Then, 50 µl of 10 µM Calcein-AM (Dojindo Molecular Technologies Inc., Kumamoto, Japan) was added to the cell suspension, and the cells were incubated at 37°C for 30 min. The cells were washed twice with ADCP buffer (RPMI1640 containing 1% FBS, 10 mM HEPES at pH 7.2, 100 U/ml penicillin and 100 µg/ml streptomycin) and suspended in ADCP buffer at 4 × 10⁵ cells/ml. Aliquots of 0.1 ml of the SK-BR-3 cell suspension (0.4 × 10⁵ cells/tube) were mixed with 40 µl of serially diluted TRAs in 5-ml polystyrene round-bottom tubes (BD Biosciences, San Jose, CA) and were incubated at 37°C for 1 h.

THP-1 cells or the effector cells were activated by culturing in a CO₂ incubator for 48 h in RPMI1640 medium containing 100 nM phorbol 12-myristate 13-acetate (PMA; Wako Pure Chemical Industries). The activated THP-1 cells were mixed with SK-BR-3 cells opsonized with TRAs at the effector cell to target cell ratio (E/T) of 4/1, and the cell suspension was incubated at 37°C for 2.5 h. Then, the cells were washed twice with FACS buffer and stained with 1.5 µg of phycoerythrin (PE)-labelled anti-CD89 mAb (Santa Cruz Biotechnology, Inc., CA) in 0.4 ml of FACS buffer at room temperature for 30 min. The cells were washed twice, resuspended in 0.5 ml of FACS buffer, and analysed by using a FACS LSR flow cytometer.

The stained cells were irradiated by using a laser that emitted argon ions at 488 nm. Calcein-AM-labelled target cells were detected in the FL1 channel (530 nm), and PE-labelled effector cells were detected in the FL2 channel (575 nm). Cells detected only in the

Fig. 1 Illustration of TRA (human IgG1) and four differently modified TRAs in which the interchain S–S bonds were cleaved. The disulfide bonds within TRA, which is specific for HER2, were cleaved by mild S-sulfonation or by mild reduction followed by S-alkylation with three different reagents.

FL1 channel (FL1-positive cells) were judged as non-phagocytosed, and cells detected in both FL1 and FL2 (FL1 and FL2 double-positive cells) were judged as phagocytosed. ADCP was calculated according to the following formula: $\text{ADCP} (\%) = (100 \times \text{FL1 and FL2 double-positive}) / \text{FL1-positive}$. ADCP values were measured in triplicate, and the results are expressed as the means $\pm$ SD.

The THP-1 cells were analysed by flow cytometry with 1 $\mu\text{g}/\text{ml}$ of anti-Fc γ RI mAb (10.1), anti-Fc γ RII mAb (7.3) or anti-Fc γ RIII mAb (3G8) on ice for 45 min. The THP-1 cells were stained with 1 $\mu\text{g}/\text{ml}$ of FITC-labelled goat anti-mouse IgG Abs on ice for 30 min.

For Fc γ R-blocking experiments, 7 nM each of anti-Fc γ RI (clone 10.1), anti-Fc γ RII (clone 7.3), and anti-Fc γ RIII (clone 3G8) mAbs (Ancell Corporation, Bayport, MN) were added to the activated THP-1 cells and incubated at room temperature for 30 min before mixing them with SK-BR-3 cells opsonized with TRAs.

Statistical analyses

Experiments were performed in triplicate. The results are expressed as means $\pm$ SD, and the Student's *t*-test was performed as compared with the original TRA. The differences were considered statistically significant when the *P* value was less than 0.05.

Results

Cleavage of the interchain S–S bonds in TRA

The interchain S–S bonds in TRA were cleaved by one of four methods—mild S-sulfonation or mild reduction followed by S-alkylation with three different reagents, giving TRA-S-SO₃⁻, TRA-S-A-NH₃⁺, TRA-S-B-NH₃⁺ and TRA-S-CH₂CONH₂ (Fig. 1). Since there are four interchain S–S bonds in human IgG1, TRA-S-SO₃⁻ has about eight additional negative charges, and both TRA-S-A-NH₃⁺ and TRA-S-B-NH₃⁺ have about eight additional positive charges as compared with the original TRA and TRA-S-CH₂CONH₂.

The cleavage of the interchain S–S bonds was analysed by SDS-PAGE under non-reducing conditions (Fig. 2). TRA-S-SO₃⁻ (lane 2) and TRA-S-CH₂CONH₂

Fig. 2 SDS-PAGE analysis of TRAs. Lanes 1, 2, 3, 4 and 5 are the original TRA, TRA-S-SO₃⁻, TRA-S-CH₂CONH₂, TRA-S-A-NH₃⁺ and TRA-S-B-NH₃⁺, respectively. Those proteins were electrophoresed under non-reducing conditions on 12% gels and stained with Bio-Safe Coomassie. This result was reproduced in three independent experiments.

(lane 3) each comprised mostly H and L chains, unlike the original TRA (lane 1). In these two preparations, more than 90% of the interchain S–S bonds were cleaved by the treatment. In the cases of TRA-S-A-NH₃⁺ and TRA-S-B-NH₃⁺, HL, H₂, H₂L and H₂L₂ bands were found in addition to H and L bands (lanes 4 and 5). The results show that about 80% of H–H and 85% of H–L interchain S–S bonds were cleaved.

Fig. 3 Gel filtration analysis by HPLC. The molecular weight of each TRA and its contamination of aggregated TRA were analysed by gel filtration HPLC on a Protein Pak G3000SWXL column in 0.1 M sodium phosphate buffer (pH 6.8). These profiles were reproduced in three independent experiments.

Gel filtration HPLC revealed that each of the modified TRAs retains the H₂L₂ form without any separated H and L chains in the absence of detergent like SDS and that more than 95% of TRAs were monomers (not aggregates) without any differences among their elution profiles (Fig. 3).

Binding activities of TRAs to the HER2 antigen on SK-BR-3 cells

TRA binds to the HER2 antigen on the surface of SK-BR-3 cells. Binding activities of differently modified TRAs were compared with that of the original TRA by using flow cytometry with SK-BR-3 cells. There was a semi-logarithmic increase in binding between 1 and 100 nM of TRA. The binding profiles of the modified TRAs were very close to that of TRA, although significant differences were found at some concentrations and some modifications (Fig. 4). Irrelevant mAb rituximab did not bind to SK-BR-3 cells. These results suggest that neither the cleavage of the S–S bonds nor different modifications of the sulfhydryl residues affected the binding affinity to HER2 antigen on the cell surface.

Effect of the S–S bond cleavage on the binding of TRAs to FcγR isotypes

The binding to FcγR isotypes was compared among unmodified TRA and modified TRAs by performing ELISA with extracellular fragments of FcγR isotypes (Fig. 5). The binding to FcγRIA was apparently decreased by the cleavage of interchain S–S bonds, irrespective of the difference of cysteine residue modification. In regard to FcγRIIA, unexpectedly, the binding affinities were found to be unchanged or largely increased by the S–S bond cleavage. The affinities of TRA-S-CH₂CONH₂ and TRA-S-B-NH₃⁺ were much higher than TRA-S-A-NH₃⁺, TRA-S-SO₃⁻ and TRA. The binding affinities of TRAs for FcγRIIB were very similar to those for FcγRIIA, and the affinities of TRA-S-CH₂CONH₂ and TRA-S-B-NH₃⁺ were much higher than unmodified TRA. The binding affinities with FcγRIIA were decreased by interchain S–S bond cleavage, like those for FcγRIA. The binding affinities were in the order of TRA > TRA-S-B-NH₃⁺ >> TRA-S-CH₂CONH₂ > TRA-S-A-NH₃⁺ > TRA-S-SO₃⁻.

These results suggest that the binding between IgG molecules without the interchain S–S bonds and

Fig. 4 Binding activities of TRAs to the HER2 antigens on SK-BR-3 cells. SK-BR-3 cells were incubated with serially diluted TRAs and then stained with FITC-labelled goat anti-human IgG κ chain Abs. Mean Fluorescence intensities (MFI) of the stained cells were measured by flow cytometry. The flow cytometry experiments were performed in triplicate, and the mean fluorescence intensities are shown as the means $\pm$ SD. The differences compared with the original TRA were evaluated by the Student's *t*-test, and the *P* values are indicated with ** and *** for $P < 0.01$ and $P < 0.001$, respectively.

Fig. 5 Effect of the interchain S-S bond cleavage of TRA on its binding affinities for Fc γ R. The original TRA and modified TRAs were allowed to react with the extracellular domain of Fc γ RIA, Fc γ RIIA, Fc γ RIIB or Fc γ RIIIA coated on microtiter plates. The bound TRAs were measured with HRP-labelled goat anti-human IgG (H + L) Abs. The explanatory notes in the figures indicate which symbols are for individual TRAs, respectively. The binding activities were assessed in triplicate, and the absorbance at 450 nm is shown as the means $\pm$ S.D.

Fig. 6 ADCP assay by flow cytometry. THP-1 cells were activated by 100 nM PMA for 48 h. SK-BR-3 cells were labelled with Calcein-AM and allowed to stand without TRA (A) or to react with 4 nM TRA (B). THP-1 cells were incubated with the opsonized SK-BR-3 cells at the E/T ratio of 4:1 and then stained with PE-labelled anti-CD89 mAb. Numbers of the cells stained with both Calcein-AM and PE were analysed by flow cytometry. The reproducibility was confirmed by performing the experiments in triplicate.

cell-surface Fc γ R_s differs based on cysteine modification and Fc γ R isotype.

Effect of the S–S bond cleavage on ADCP activities

ADCP was assessed by flow cytometry. The target cells were SK-BR-3 cells that were stained with Calcein-AM and opsonized with different TRAs. The effector cells were THP-1 cells that had differentiated into macrophage-like cells and were stained with PE-anti-CD89 mAb. These two cell types were mixed and incubated at the E/T ratio of 4:1. Calcein-AM-positive cells and PE-anti-CD89-positive cells were measured by flow cytometry (Fig. 6). FL1-H-positive cells are SK-BR-3 cells, and the cells positive in both FL1-H and FL2-H are SK-BR-3 cells that were phagocytosed by THP-1 cells. THP-1 cells phagocytosed 26.2% of SK-BR-3 cells that were allowed to react with 4 nM TRA (Fig. 6B), while they phagocytosed 11.5% of SK-BR-3 cells without TRA (Fig. 6A).

ADCP activities of unmodified and modified TRAs were compared at concentrations between 0.16 and 20 nM (Fig. 7). Phagocytosis was increased with increased TRA concentration, while rituximab, which has the human Fc domain and irrelevant antigen specificity, did not exert any phagocytosis. Four different types of modified TRAs showed almost identical curves of phagocytosis as compared with that of the original TRA. As for TRA-S-SO₃[−] and TRA-S-CH₂CONH₂, their ADCP activities were not significantly different from that of the original TRA. The phagocytosis with TRA-S-A-NH₃⁺ and TRA-S-B-NH₃⁺ was slightly weaker than that of the original TRA at concentrations of 0.16 and 0.8 nM, although they were as potent or more potent than that of TRA at the higher concentrations. Therefore, despite the large difference in the binding affinities of unmodified and modified TRAs for Fc γ R_s, there was not a large difference in ADCP activities.

Fc γ R isotypes involved in ADCP by TRAs

THP-1 cells were differentiated into macrophages by PMA treatment, and the binding of mAbs specific for

Fig. 7 Comparison among the ADCP activities of unmodified and modified TRAs. The activities were determined by flow cytometry as shown in Fig. 6. The numbers of cells that were phagocytosed by PMA-activated THP-1 cells were assessed in triplicate, and the results are expressed as the means $\pm$ SD. The differences between TRA and modified TRAs were assessed by the Student's *t*-test, and the *P* values are indicated with *, ** and *** for $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

individual Fc γ R isotypes to the cells was assessed by flow cytometry. The order of the fluorescence was Fc γ R_{II} > Fc γ R_I > Fc γ R_{III} (Fig. 8). The results suggest the amounts of those Fc γ R isotypes expressed on the activated THP-1 cells.

ADCP by TRAs was blocked by mAbs specific for Fc γ R_I, Fc γ R_{II} and Fc γ R_{III} by adding the mAbs to the co-culturing medium of SK-BR-3 cells opsonized with TRAs and THP-1 cells. As a result, blocking by an anti-Fc γ R_I mAb significantly suppressed ADCP of all TRAs (Fig. 9). Blocking by an anti-Fc γ R_{II} mAb also showed suppression of ADCP, but the extent was smaller than that by an anti-Fc γ R_I mAb. Blocking by an anti-Fc γ R_{III} mAb did not provide significant suppression except for unmodified TRA. Taken together with the expression of individual Fc γ R_s, these results suggest that both Fc γ R_I and Fc γ R_{II} would be involved in ADCP by TRAs.

Fig. 8 Flow cytometry analysis of Fc γ R isotypes on the surface of PMA-activated THP-1 cells. The cells were analysed by flow cytometry with an anti-Fc γ RI mAb (10.1), anti-Fc γ RII mAb (7.3) or anti-Fc γ RIII mAb (3G8). The THP-1 cells were allowed to react with anti-Fc γ R mAbs or an isotype control mAb (M2, mouse IgG1) and stained with FITC-labelled goat anti-mouse IgG Abs. These histograms were reproduced by three flow cytometry experiments.

Fig. 9 Blocking of individual Fc γ R with anti-receptor mAbs and its effect on ADCP by PMA-activated THP-1 cells. Flow cytometry as shown in Fig. 6 was used to measure the percentage of SK-BR-3 cells engulfed by PMA-activated THP-1 cells in the presence of 4 nM of individual TRAs. An anti-Fc γ RI mAb (10.1), anti-Fc γ RII mAb (7.3) or anti-Fc γ RIII mAb (3G8) at the mAb concentration of 7 nM was used to block the Fc γ R isotypes on the THP-1 cell surface. The phagocytosis was measured in triplicate, and the results are expressed as the means $\pm$ SD. Asterisks indicate significant differences from phagocytosis without any blocking mAb (** P < 0.01, * P < 0.05).

Discussion

ADCP results from the binding of Abs to antigens on the surface of target cells and the binding of their Fc moieties to Fc γ Rs on the surface of effector cells (11, 18). There are more than five different isotypes of Fc γ Rs, and the individual isotypes are expressed at various amounts on different effector cells involved in different effector functions (4–6). We cleaved the S–S bonds located at the hinge region and upper

C_H2 domain of TRA, which is humanized IgG1. The modified TRAs as compared with the original TRA were studied on their binding affinities for four major Fc γ R isotypes and their ADCP abilities by using a combination of SK-BR-3 cells and PMA-activated THP-1 cells. The original TRA and four modified TRAs showed different binding affinities for individual Fc γ R isotypes. Unexpectedly, the S–S bond cleavage did not always decrease their affinities; rather, the S–S bond cleavage also increased the affinities for some of the receptor isotypes. However, the modified TRAs exerted ADCP activities as much as the original TRA.

The interchain S–S bonds were cleaved by two different methods, mild S-sulfonation and mild reduction with dithiothreitol. The free cysteine residues generated by reduction were modified with three different compounds (Fig. 1). Mild S-sulfonation under the conditions applied here cleaves interchain but not intrachain S–S bonds because the H and L chains of S-sulfonated IgG1 have 3 and 1 cysteine-SO₃Na residues, respectively (14, 15). Liu and co-workers showed that the interchain S–S bonds were much more susceptible than intrachain S–S bonds to reduction and that the bonds between H and H chains were slightly more susceptible than the bond between H and L chains (15). In the present study, the interchain S–S bonds in TRA were almost totally cleaved in TRA-S-SO₃[−] and TRA-S-CH₂CONH₂, while 20% of the bonds between H and H chains and 15% of the bonds between H and L chains remained in TRA-S-A-NH₃⁺ and TRA-S-B-NH₃⁺ (Fig. 2). However, all of the modified TRAs retained the H₂L₂ structure without any free H or L chains, and the monomers in those TRAs were more than 95% like the original TRA (Fig. 3).

The cleavage of the interchain S–S bonds did not affect the binding of TRA to HER2 antigens on SK-BR-3 cells (Fig. 4). The concentration-dependent curves for individual TRAs were not significantly different, although the binding values at some concentrations of TRAs were significantly different from those of the original TRA. The interchain S–S bond cleavage also did not affect the antigen-binding activity of rituximab (19).

THP-1 cells differentiate into macrophages and express their particular ratios of different Fc γ R isotypes by PMA activation (20, 21). The THP-1 cells used in the present study expressed Fc γ RI, Fc γ RII and Fc γ RIII, as shown by flow cytometry with mAbs against those individual receptors (Fig. 8). Their MFIs were in the order of Fc γ RII > Fc γ RI > Fc γ RIII, suggesting their expression levels on THP-1 cells. The binding affinities of TRAs for 4 Fc γ R isotypes were assessed by ELISA with recombinant extracellular fragments of those receptors. Unexpectedly, TRA-S-CH₂CONH₂ and TRA-S-B-NH₃⁺ were much higher than the original TRA in terms of binding affinities for both Fc γ RIIA and Fc γ RIIB, although all four modified TRAs were lower than the original TRA in binding affinities for both Fc γ RIA and Fc γ RIIIA (Fig. 5). The amino acid identities between Fc γ RIIA (accession no. [NP_001129691.1](#)) and Fc γ RIIB ([NP_003992.3](#)) were as high as 200/226 (88%), while the identities between Fc γ RIA

(NP 000557.1) and Fc γ RII, the identities between Fc γ RII and Fc γ RIIIA (NP 000560.5) and the identities between Fc γ RIA and Fc γ RIIIA were as low as 40% to 50%. In addition, crystallographic study of the complex of Fc and Fc γ Rs suggested that Fc γ RII and Fc γ RIII interact with different sites of Fc (12, 22). Therefore, the affinities of TRAs for Fc γ RIIA were very similar to those for Fc γ RIIB and different from those for Fc γ RIA and Fc γ RIIIA.

The amounts of aggregated molecules were less than 5% of the modified TRAs and as small as that of the original TRA (Fig. 3). In addition, aggregated IgG showed augmented binding affinities for Fc γ RIIIA as well as both Fc γ RIIA and Fc γ RIIB (22). Therefore, the differences of their affinities for Fc γ R isotypes results from the differences of the affinities of TRA monomers, but not aggregates, for Fc γ R isotypes.

Human intravenous immunoglobulin (IVIG) is composed of IgG1 (54–58%), IgG2 (37–43%), IgG3 (2–3%) and IgG4 (0.6–1.0%). IVIG has similar binding affinities for Fc γ Rs; cleavage of the S–S bonds decreased the affinities for Fc γ RIA and Fc γ RIIIA and only slightly increased those for Fc γ RIIA and Fc γ RIIB (22). In the case of rituximab, however, the S–S bond cleavage enhanced the binding affinity for Fc γ RIIIA, although the effects on the affinities of rituximab for other Fc γ Rs were consistent with those of TRAs (19). According to the DrugBank, TRA and rituximab are different from naturally occurring IgG1 (UniProtKB accession no. P01857). In TRA (DrugBank accession number DB00072), proline is inserted between amino acids 217 and 218, and in rituximab (DB00073), 215 valine is exchanged for alanine (23). These differences in amino acid sequences might account for the difference in the effect on binding affinities for Fc γ RIIIA.

Since there are four interchain S–S bonds in the IgG1 molecule, TRA-S-A-NH₃⁺ and TRA-S-B-NH₃⁺ have about eight additional cations as compared with the parental TRA at the hinge region and the upper C_H2 domain where Fc γ Rs interact with IgG1 molecules (12, 13, 24). TRA-S-SO₃[−] has eight additional anions, while the electrical charge of TRA-S-CH₂CONH₂ is equivalent to that of TRA. These differences in electrical charge must result in different conformations. Actually, TRA-S-A-NH₃⁺ and TRA-S-B-NH₃⁺ showed very similar binding affinities for Fc γ RIA. However, their affinities were very different for other Fc γ Rs (Fig. 5). The binding affinities of TRA-S-B-NH₃⁺ and TRA-S-CH₂CONH₂ were apparently higher than those of the parental TRA for Fc γ RIIA and Fc γ RIIB. TRA-S-SO₃[−] showed binding affinities equal to or lower than other TRAs in all 4 Fc γ R isotypes. The large difference in the binding affinities of the two cationic TRAs (TRA-S-A-NH₃⁺ and TRA-S-B-NH₃⁺) results from the difference in their conformations at the hinge and the upper C_H2 regions. The conformational difference is caused by the difference in the molecular size of their modification groups and in their hydrophobic and hydrogen-bond interactions with Fc γ Rs, although their conformations were not analysed. Despite the remarkable differences among the binding affinities between individual TRAs

and Fc γ R isotypes, the concentration-dependent curves of ADCP activities determined with PMA-activated THP-1 cells and HER2-bearing SK-BR-3 cells were extremely close, implying that the interchain S–S bond cleavage does not affect ADCP activity (Fig. 7). Blocking experiments using mAbs against Fc γ RI, Fc γ RII and Fc γ RIII suggest that involvement of Fc γ RI in ADCP would be largest irrespective of the original TRA and modified TRAs and that Fc γ RII would also be involved in ADCP (Fig. 9). Blocking of Fc γ RIII decreased ADCP only slightly with the 4 kinds of TRAs, except for TRA-S-B-NH₃⁺.

ADCP with THP-1 cells and rituximab was also blocked with anti-Fc γ RI and anti-Fc γ RII mAbs but not with an anti-Fc γ RIII mAb (25). THP-1 cells express Fc γ RIIB as well as Fc γ RIIA (26), and Fc γ RIIB inhibits Fc γ RIA/IIA-mediated phagocytosis (27). However, it is unknown how the inhibitory Fc γ RIIB is involved in our ADCP system as compared with Fc γ RIA and Fc γ RIIA. In addition, Fc γ RIII was reported not only to exert phagocytosis but also to induce inhibitory signalling (28). In our previous study, interchain S–S bond cleavage of rituximab resulted in increased binding affinity for Fc γ RIIIA and decreased ADCC (19), suggesting inhibitory signalling through Fc γ RIIIA. The observation that the effect of an anti-Fc γ RIII mAb on ADCP by THP-1 cells was small might be due to the balance of the activating and inhibitory signalling through Fc γ RIIIA. Another possible explanation is the low expression level of Fc γ RIII on THP-1 cells (Fig. 8).

In conclusion, the cleavage of the interchain S–S bonds located at the hinge region and upper C_H2 domain not only decreases but also increases binding affinities of TRA for some Fc γ R isotypes. ADCP by PMA-activated THP-1 cells was not affected by modification of the S–S bonds. However, the expression of Fc γ R isotypes is different in the differentiation of macrophages and other cells such as neutrophils (29) and NK cells (30). Kruspe and colleagues showed that anti-Rh Abs that were reduced and modified with ICH₂CONH₂ had enhanced blocking against Fc γ Rs, resulting in an augmented therapeutic effect for idiopathic thrombocytopenic purpura (31). This previous study together with our present study indicate that some mAbs that are modified at interchain S–S bonds would have greater therapeutic effects than the corresponding unmodified mAbs.

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Conflict of Interest

None declared.

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