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Cleavage of the interchain disulfide bonds in rituximab increases its affinity for FcγRIIIA

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ABSTRACT

The Fc region of human IgG1 mediates effector function via binding to Fcγ receptors and complement activation. The H and L chains of IgG1 antibodies are joined by four interchain disulfide bonds. In this study, these bonds within the therapeutic IgG1 rituximab (RTX) were cleaved either by mild reduction followed by alkylation or by mild S-sulfonation; consequently, two modified RTXs – A-RTX (alkylated) and S-RTX (S-sulfonated) – were formed, and both were almost as potent as unmodified RTX when binding CD20 antigen. Unexpectedly, each modified RTX had a higher binding affinity for FcγRIIIA (CD16A) than did unmodified RTX. However, S-RTX and A-RTX were each less potent than RTX in an assay of antibody-dependent cellular cytotoxicity (ADCC). In this ADCC assay, each modified RTX showed decreased secretion of granzyme B, but no change in perforin secretion, from effector cells. These results provide significant information on the structures within IgG1 that are involved in binding FcγRIIIA, and they may be useful in the development of therapeutic antagonists for FcγRIIIA.

1. Introduction

More than 25 monoclonal antibodies (mAbs) have been developed for treatment of cancer or of immunological and inflammatory diseases [1]. Most of them are chimeric mAbs, humanized mAbs, or human-type mAbs. Antibodies not only bind to antigens, but also exert effector functions – including phagocytosis, complement-dependent cytotoxicity, and antibody-dependent cellular cytotoxicity (ADCC) – via binding to Fcγ receptors (FcγRs) [2,3].

FcγRs play important roles in the activation and suppression of immune responses [4]. FcγRI (CD64) has a high affinity for IgG1, and FcγRII (CD32) and FcγRIII (CD16) each have low affinity for IgG1. IgG1 binds to an FcγR via the hinge and the upper region of the C_H2 domain within the IgG1 [5]. There are 2 disulfide bonds between the two H chains and 1 disulfide bond between each H chain and an L chain; therefore, 4 disulfide bonds join the four chains in the region of the hinge and the upper C_H2 domain. In addition to those interchain disulfide bonds, there are 12 intrachain disulfide bonds, each of which is located in a separate domain of IgG1. The interchain disulfide bonds are much more sensitive to cleavage by S-sulfonation or by reduction followed by alkylation (reduction/alkylation) than are the intrachain bonds [6,7].

The interchain disulfide bonds help maintain the conformation of the hinge region and of the upper C_H2 region of IgG1 antibodies; therefore, we hypothesized that cleavage of all four bonds would change the conformation of IgG1 and thereby result in altered effector function. In fact, reduction of the disulfide bonds reportedly breaks down the open hinge structure and decreases the apparent molecular size of IgG3 [8]; moreover, missense mutations in this region decrease complement-dependent cytotoxic activity [2]. Mutant forms of the middle and/or upper hinge within mAbs decreased or increased binding activity to FcγRIIIA and the suppression or activation, respectively, of ADCC activity [9,10]. Finally, removal of fucose from polysaccharides that are attached to the upper hinge region enhances binding with FcγRIIIA and, therefore, ADCC activity [11].

Rituximab (RTX) is a chimeric IgG1 antibody that specifically recognizes CD20; RTX has been used to treat non-Hodgkin lymphoma [12]. One clinical study of RTX indicated that ADCC was the major mechanism of therapeutic action [13]. ADCC occurs via binding of antibodies to FcγRIIIA on natural killer (NK) cells

Abbreviations: RTX, rituximab; S-RTX, S-sulfonated rituximab; A-RTX, reduced and then alkylated rituximab; mAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; FcγR, Fcγ receptor; KHYG-1/FcγRIIIA, KHYG-1 cells stably expressing FcγRIIIA; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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[3–5]. Here, we mildly S-sulfonated or mildly reduced/alkylated RTX to cleave the interchain disulfide bonds, and the affinity for FcγRIIIA and ADCC activity of each modified RTX were evaluated to assess the importance of the interchain disulfide bonds within IgG1.

2. Materials and methods

2.1. Cell culture and reagents

Human Burkitt's lymphoma Ramos, Raji, or Daudi cells were cultured in RPMI1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/mL streptomycin; KHYG-1 cells were also cultured in this medium with an additional 10 ng/ml IL-2 (Wako Pure Chemical Industries). The KHYG-1 cell lines that stably expressed FcγRIIIA (KHYG-1/FcγRIIIA) were developed by cloning a FcγRIIIA-Val158 cDNA into the pMXs-puro retrovial vector, transfecting this construct into Plat-E packaging cells, and co-culturing the transfected Plat-E cells with KHYG-1 cells (E. Kobayashi et al., unpublished results).

RTX and trastuzumab were purchased from Zenyaku Kogyo Co., Ltd. (Tokyo, Japan) and Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Trastuzumab was used as the non-specific IgG1 control.

2.2. S-sulfonation and reduction/alkylation of RTX

S-sulfonated RTX (S-RTX) and reduced/alkylated RTX (A-RTX) were prepared as described previously [14]. Briefly, 0.5 mg/mL of RTX was allowed to react with 270 mM sodium sulfite and 70 mM sodium tetrathionate at 37 °C for 4 h to generate S-RTX; the solution was then dialyzed against phosphate-buffered saline (PBS). To generate A-RTX, 0.5 mg/mL of RTX was reduced with 10 mM dithiothreitol at 37 °C for 1 h and then treated with 50 mM iodoacetamide at 37 °C for 30 min; this solution was then dialyzed against PBS.

2.3. SDS–PAGE and gel filtration HPLC

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and gel filtration HPLC were performed as described previously [14,15]. SDS–PAGE was performed under non-reducing conditions on 12% acrylamide gel, and the protein bands were stained with Coomassie. The contamination of polymeric and dimeric RTX was assessed by gel filtration HPLC on a Protein Pak 300SW column (Waters Corporation, Milford, MA).

2.4. CD20-binding assay by flow cytometry

Flow cytometry for CD20-binding assay was described previously [15]. Ramos, Raji, and Daudi cells were allowed to react with RTX, S-RTX, or A-RTX and then stained with FITC-conjugated goat anti-human κ chain (Millipore, Billerica, MA). The mean fluorescence intensities were measured using a FACS LSR flow cytometer equipped with CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

2.5. FcRIIIA-binding assay

Enzyme-linked immunosorbent assays (ELISAs) were performed with the extracellular fragments of either of two FcγRIIIA variants, FcγRIIIA Val158 or FcγRIIIA Phe158 [15]. Each variant was used to coat wells of separate 96-well plates, and RTX samples were allowed to react with separately with each variant. The bound RTXs were detected with horseradish peroxidase (HRP)-conjugated goat $F(ab')_2$ anti-human IgG (Chemicon International Inc., Temecula, CA). KHYG-1/FcγRIIIA cells were suspended at 1.0×10^6 cells/mL in FACS buffer (PBS containing 0.1% bovine serum albumin) and then allowed to stand on ice for 30 min. After being washed with FACS buffer, the cells were allowed to react with 670 nM RTX, S-RTX or A-RTX at 4 °C for 30 min. After another wash, cells were stained with FITC-labeled goat anti-human κ chain antibodies on ice for 30 min. After yet another wash, the cells were suspended in 0.5 mL FACS buffer and passed through filters with 59-μm meshes. Fluorescence intensities of cells were measured using a FACS LSR flow cytometer equipped with CellQuest software.

2.6. ADCC assay

ADCC activity was measured using human NK cell line KHYG-1/FcγRIIIA as the effector cells, three types of target cells (Ramos, Raji, or Daudi), and assay methods described previously [15]. Briefly, target cells were stained with 25 μM Calcein-AM (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) at 37 °C for 30 min. KHYG-1/FcγRIIIA cells and the target cells were mixed at an effector/target (E/T) ratio of 25/1 and incubated at 37 °C for 4 h. The fluorescence intensity of calcein released from the target cells was measured with an excitation wavelength of 485 nm and emission wavelength of 538 nm.

2.7. Assays of granzyme B and perforin

Ramos cells were suspended in RPMI1640 containing 2 mM L-glutamine, 1% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 2×10^5 cells/mL and allowed to react separately with each one of five concentrations of modified or unmodified RTX at room temperature with gentle rotation for 1 h. Ramos cells (0.1 mL of 1×10^5 cells/mL) were then mixed with KHYG-1/FcγRIIIA cells (0.1 mL of 25×10^5 cells/mL). Each cell suspension was incubated at 37 °C in 5% CO_2 for 4 h.

ELISA kits for granzyme B and perforin were purchased from Mabtech AB (Nacka Strand, Sweden); granzyme B and perforin were measured according to the manufacturer's instructions. Briefly, an anti-granzyme B mAb (GB10) was used to coat 96-well plates, and bound granzyme B was measured with biotinylated mAb (GB11) and HRP-streptavidin. Similarly, an anti-perforin mAb (Pf-80/164) was used to coat 96-well plates, and bound perforin was detected with a biotinylated anti-perforin mAb (Pf-344).

3. Results

3.1. Cleavage of the interchain disulfide bonds and effect on RTX-CD20 binding

The interchain disulfide bonds within mature RTX were cleaved by one of two methods – mild S-sulfonation or mild reduction/alkylation. SDS–PAGE with non-reducing conditions was then used to analyze RTX and two RTX variants, S-RTX and A-RTX, which were generated by S-sulfonation and reduction/alkylation, respectively (Fig. 1A). S-RTX and A-RTX preparations each comprised mostly H and L chain and only small amounts of H_2, whereas RTX preparations comprised H_2L_2; these findings indicated that either S-sulfonation or reduction/alkylation cleaved the interchain disulfide bonds; more than 90% of interchain disulfide bonds were cleaved in the S-RTX and in the A-RTX preparations. Aggregation of IgG generally increases binding affinity to FcγRs [14]; therefore, the molecular weights of proteins within the RTX, S-RTX, or A-RTX preparations were examined by gel filtration HPLC. Only

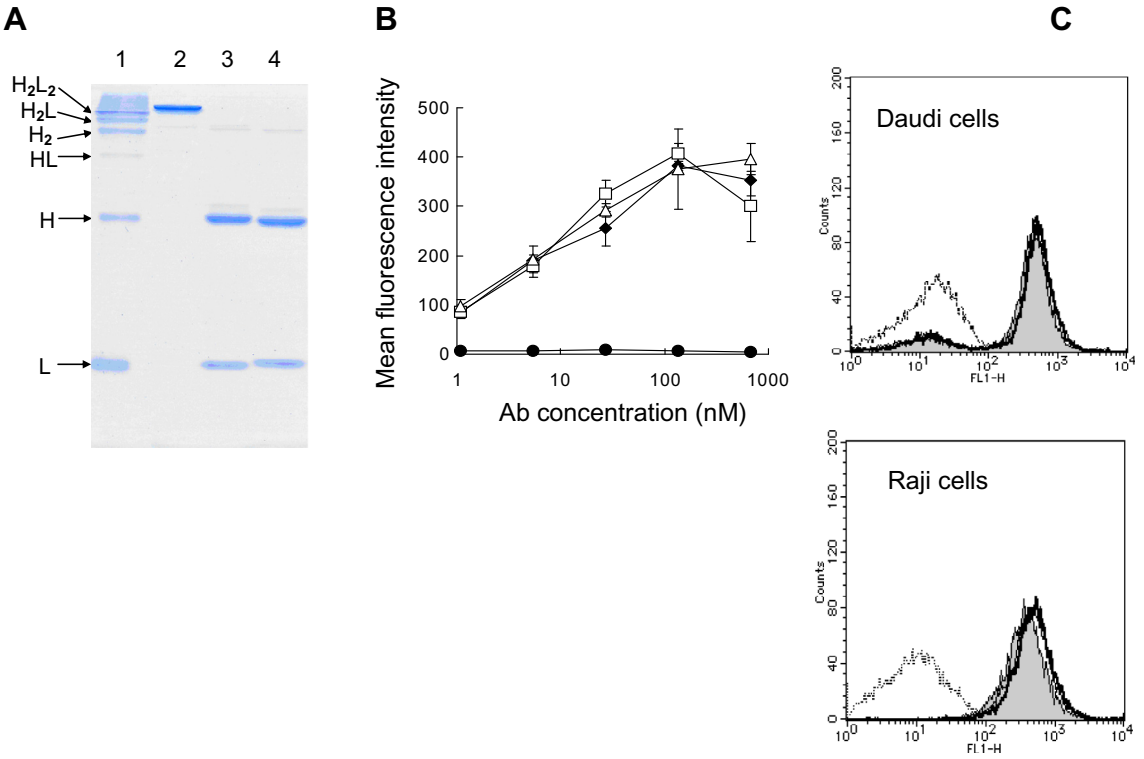

Fig. 1. Cleavage of the interchain disulfide bonds and its effect on antigen-binding activity. (A) SDS–PAGE under non-reducing conditions on 12% gel, protein bands stained with Coomassie. Lane 1: molecular weight markers (partially reduced and then alkylated IgG), lane 2: RTX, lane 3: S-RTX, and lane 4: A-RTX. (B) Flow cytometry of Ramos cells. RTX (♦), S-RTX (□), A-RTX (△), and non-specific IgG1 control (●). The experiments were done in triplicate, and the values are expressed as means ± SD. (C) Flow cytometry of Daudi and Raji cells stained with 30 μg/mL RTXs and then the second antibodies. Shaded histograms indicate the fluorescence with RTX, and those drawn by thin lines, thick lines and dotted lines represent the fluorescence with S-RTX, A-RTX and non-specific IgG1 control, respectively.

monomers, no polymers or dimers, were evident in any preparation of RTX, S-RTX, or A-RTX (data not shown).

Flow cytometry and CD20-positive Ramos, Raji, and Daudi cells were used to assess binding between each mAb and the CD20 antigen. RTX, S-RTX, and A-RTX each bound to Ramos cells in a dose-dependent manner, and each modified RTX was as potent as or slightly more potent than unmodified RTX in binding to this cell-surface antigen (Fig. 1B and C); this finding indicated that interchain disulfide bond cleavage did not affect or slightly increased the affinity of the antibody for CD20.

3.2. Effect of the cleavage on the binding to FcRIIIA

ELISA was used to examine IgG-FcγRIIIA binding between extracellular fragments of FcγRIIIA-Val158 or FcγRIIIA-Phe158 and each RTX variant. Unexpectedly, both S-RTX and A-RTX, which each lacked any interchain disulfide bonds, had much higher binding affinities for each type of FcγRIIIA than did parental RTX (Fig. 2A). The order of the binding affinity was A-RTX > S-RTX > RTX; moreover, A-RTX and S-RTX were as active as RTX in binding to FcγRIIIA-Val158 at approximately one-fiftieth and one-thirtieth, respectively, of the RTX concentration. The possibility remained that results of the ELISA, which assayed only the extracellular fragments of FcγRIIIA, would differ from results of a whole-cell assay. Therefore, KHYG-1/FcγRIIIA cells that expressed the entire FcγRIIIA-Val158 molecule on the cell surface were used for whole-cell assays. RTX, S-RTX, or A-RTX was allowed to react with KHYG-1/FcγRIIIA cells, and bound antibodies were measured with FITC-labeled secondary antibodies. In this system, S-RTX and A-RTX again had higher binding affinities for FcγRIIIA than did RTX (Fig. 2B). Therefore, these results indicated that cleavage of the interchain disulfide bond enhanced binding affinity between an RTX mAb and FcγRIIIA.

3.3. Effect of the cleavage on ADCC activity

The ADCC activity of each RTX variant was assessed using the human NK cell line or KHYG-1 cells transfected with FcγRIIIA-Val158 (KHYG-1/FcγRIIIA) as effector cells and Ramos cells stained with Calcein-AM as target cells at an E/T ratio of 25/1. RTX exerted cytotoxicity in a dose-dependent manner and caused the release of 37% of the calcein; in contrast, the non-specific IgG1 control caused minimal cytotoxicity (Fig. 3A). Both S-RTX and A-RTX were substantially less potent than RTX in this ADCC assay, but each was more potent than the control. The differences between cytotoxicity of each modified RTX and that of RTX were significant ($p \leqslant 0.01$) at RTX concentrations between 0.67 and 67 nM (Fig. 3A). At E/T ratios of 12.5 and 6.3, ADCC activity of each modified RTX was lower than that of unmodified RTX (data not shown). The finding that the modified RTXs were less potent than RTX in ADCC activity was confirmed using two other Burkitt's lymphoma cell lines, the Raji and Daudi lines, as target cells (Fig. 3B).

3.4. Effect of the cleavage on the secretion of granzyme B and perforin from effector cells

Granzyme B and perforin were expressed in the KHYG-1/FcγRIIIA cells; therefore, we hypothesized these proteins were involved in the ADCC of these effector cells. KHYG-1/FcγRIIIA cells and Ramos cells that were treated with RTX, S-RTX, or A-RTX (0.1, 1, 10, or 100 nM) were incubated in the same way as in the ADCC assay. Sandwich ELISA was then used to determine the concentration of granzyme B or of perforin in medium from each culture. Elevations in granzyme B levels were dependent on antibody concentrations up to 10 nM of antibody (Fig. 4A). The granzyme B levels in cultures treated with RTX were much higher than those in cultures treated with S-RTX or A-RTX. Granzyme B levels in

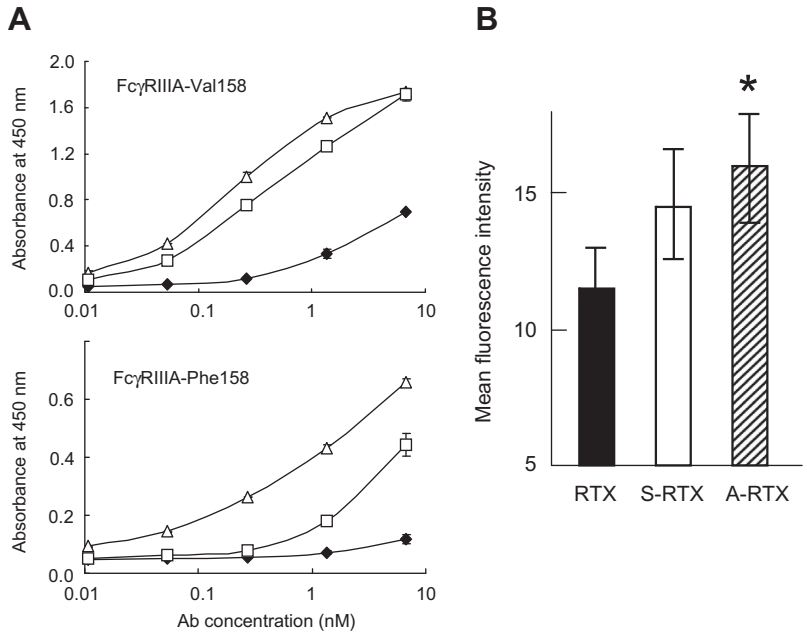

Fig. 2. Enhancement of FcγRIIIA-RTX binding affinity following cleavage of interchain disulfide bonds. (A) Extracellular fragments from human FcγRIIIA-Val158 or FcγRIIIA-Phe158 were used to coat ELISA plates, and HRP-conjugated anti-human IgG antibodies were used as the secondary antibody. RTX (♦), S-RTX (□), and A-RTX (△). (B) KHYG-1/FcγRIIIA-Val158 cells were incubated with RTXs on ice for 30 min. Bound RTXs were detected with FITC-conjugated goat anti-human IgG κ chain antibodies. The mean fluorescence intensities were measured by flow cytometry. RTX (■), S-RTX (□), and A-RTX (▨). The experiments were done in triplicate, and the values are expressed as means ± SD. *p value = 0.035.

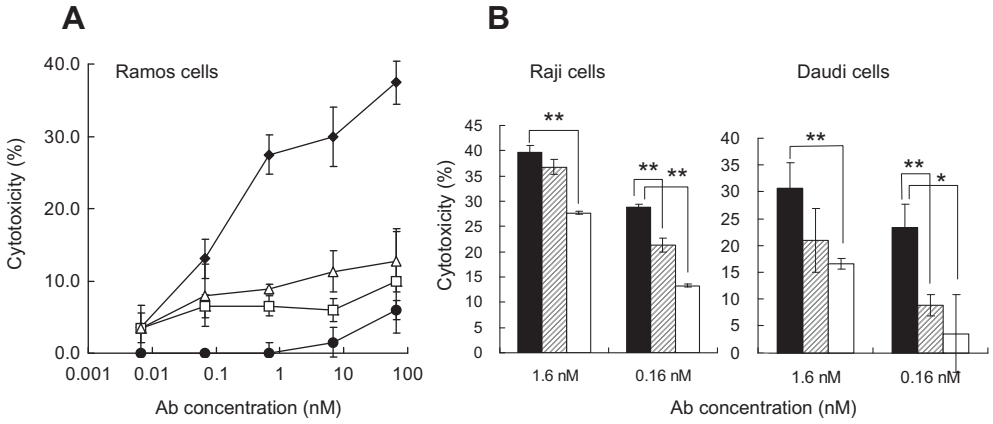

Fig. 3. Decrease of ADCC activity following interchain disulfide bond cleavage. (A) Calcein-AM-labeled Ramos cells were incubated with serially diluted RTX, S-RTX, or A-RTX at 37 °C for 30 min. Each suspension containing Ramos cells and KHYG-1/FcγRIIIA cells at an E/T ratio of 25/1 was incubated at 37 °C for 4 h. The fluorescence intensities of the calcein released from Ramos cells were measured with excitation at 485 nm and emission at 538 nm. RTX (♦), S-RTX (□), A-RTX (△), and non-specific IgG1 control (●). The experiments were done in triplicate, and the values are expressed as means ± SD. (B) ADCC activities of RTXs were determined using Raji or Daudi cells instead of Ramos cells. RTX (■), S-RTX (□), and A-RTX (▨). The p values <0.05 and <0.01 were indicated with symbols * and **, respectively.

A-RTX-treated cultures were slightly higher than those in S-RTX-treated cultures, as was the case with ADCC activity.

KHYG-1/FcγRIIIA cells secreted perforin, but Ramos cells did not (Fig. 4B). Although the perforin levels in RTX-treated cultures increased slightly with increases in RTX concentration, the levels were not significantly different from those in S-RTX-treated, A-RTX-treated, or the non-specific IgG1 control-treated cultures.

4. Discussion

The FcγRIIIA receptors are expressed on the surface of NK cells and bind to the Fc domains of IgG antibodies [3,4]. ADCC results from interactions between FcγRIIIA and the Fc domain of the antibodies that bind to target cells [2,4,16]. Previously, amino acid residues in the hinge and upper C_H2 region of IgG were mutated (1) to investigate the relationship between antibody structure and effector functions and (2) to develop therapeutic antibodies that are more effective than natural antibodies. Initially, the affinity for FcγRIIIA of each modified RTX mAb was assessed; next, the ADCC activity of each RTX mAb was assessed [9,10]. This research strategy was based on the idea that the ADCC activity of each modified mAb is proportional to its affinity for FcγRIIIA. Unlike previous modified mAbs, S-RTX and A-RTX were clearly less potent than RTX in the ADCC assay; however, their affinities for FcγRIIIA were much higher than that of RTX.

Mild S-sulfonation cleaves interchain, but not intrachain, disulfide bonds because the H and L chains of S-sulfonated IgG1 have 3 and 1 cysteine-S-SO_3Na residues, respectively [7]. Mild reduction

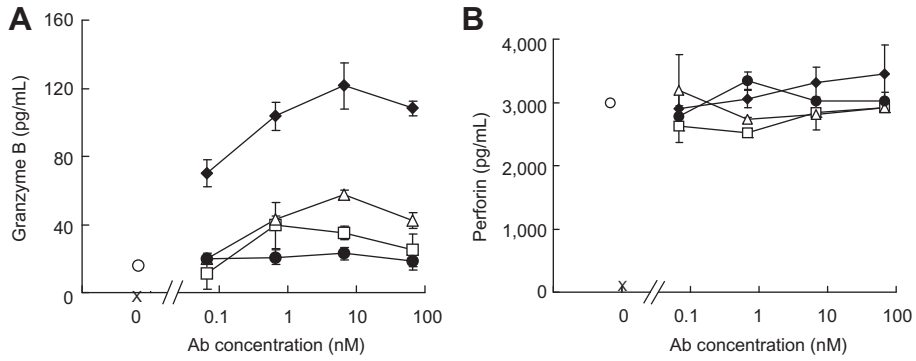

Fig. 4. Effects of the cleavage on secretion of granzyme B or perforin from KHYG-1/FcγRIIIA cells. Ramos cells were treated with RTX, S-RTX, or A-RTX and then incubated with KHYG-1/FcγRIIIA cells at an E/T ratio of 25/1 as described for the ADCC assay. The granzyme B (A) or the perforin (B) secreted from KHYG-1/FcγRIIIA cells were measured by ELISA. RTX (♦), S-RTX (□), A-RTX (△), non-specific IgG1 control (●), KHYG-1/FcγRIIIA cells alone (○) and Ramos cells alone (×).The experiments were done in triplicate, and the values are expressed as means ± SD.

with dithiothreitol also cleaves interchain disulfide bonds, and the bonds between each H chain and an L chain are more susceptible to reduction than are those between the H and H chains [8]. When subject to SDS–PAGE under non-reducing conditions, S-RTX and A-RTX showed predominantly H and L chain bands with a small amount of the H_2 band, suggesting that most of the interchain disulfide bonds would be cleaved by each of these two different methods (Fig. 1A). This disulfide bond cleavage did not affect or slightly increased the binding activity of RTX to the CD20 antigen on the cell surface (Fig. 1B and C).

S-RTX and A-RTX each had much higher affinity than the parental RTX for not only the recombinant extracellular fragments of FcγRIIIA-Val158 and of FcγRIIIA-Phe158 (Fig. 2A), but also KHYG-1/FcγRIIIA cells (Fig. 2B). Dall'Acqua and colleagues generated mutant versions of humanized mAb to study ADCC activity; their results indicated that mutants that lacked disulfide bonds between the H chains showed a significant decrease of not only antibody-FcγRIIIA binding, but also ADCC activity. Their results were not consistent with ours, but the reason for the discrepancy is unclear. Their variants not only lacked the disulfide bonds, but also carried a few mutant amino acids in the middle hinge region. Furthermore, they used a humanized mAb to the EphA2 receptor, while we used chimeric mAb to CD20. The amino acid sequence at the hinge and C_H2 region of RTX (DrugBank accession No. DB00073) [17] is identical to that of the naturally occurring IgG1 heavy chain (UniProtKB accession No. P01857). Therefore, the enhanced affinity of RTX for FcγRIIIA is not due to differences in the amino acid sequences of RTX and natural IgG1.

The KHYG-1 cell line, a human NK cell line, is highly cytotoxic, expressing FcRγ but not the FcγRIIIA chain on the cell surface [18,19]. FcRγ and FcγRIIIA are both essential for ADCC; therefore, we used transformed KHYG-1/FcγRIIIA cells to stably express FcγRIIIA-Val158 on the surface of effector cells in an ADCC assay. At antibody concentrations between 0.067 and 67 nM, treatment with either S-RTX or A-RTX resulted in much lower ADCC activity than did treatment with RTX (Fig. 3A). In general, ADCC occurs when antibodies bring target cells into contact with NK cells. S-RTX and A-RTX were as active as or slightly more active than RTX in binding to CD20 antigens on target cells (Fig. 1B and C) and more potent than RTX in binding to FcγRIIIA (Fig. 2); the higher affinity of S-RTX or A-RTX for FcγRIIIA indicated to us that these modified RTX antibodies should also have higher ADCC activities; however, the modified RTXs were less active than RTX in the ADCC assay. The reason for the lack of concordance between FcγRIIIA binding and ADCC activity is unknown and should be investigated.

The granzyme B and perforin pathway is important to NK cell-mediated cytotoxicity [16]. KHYG-1 cells reportedly express granzyme M at high levels constitutively, but they express granzyme B and perforin only at low levels in the absence of stimulation [19]. Whether those proteins are induced by ADCC is unknown. RTX induced the secretion of granzyme B from KHYG-1/FcγRIIIA cells in a concentration-dependent manner, but perforin secretion was not induced by RTX concentrations that resulted in ADCC (Fig. 4). S-RTX or A-RTX, like RTX, induced granzyme B secretion; however, the levels of secreted granzyme B were much lower following S-RTX or A-RTX treatment than following RTX treatment. Therefore, these results indicated that granzyme B might be secreted and work together with perforin to mediate ADCC and that S-RTX and A-RTX might be less potent inducers of granzyme B secretion than RTX and, therefore, cause less ADCC.

Modified IgG1s that lack interchain disulfide bonds may be useful antagonists of the FcγRIIIA receptor because S-RTX and A-RTX each had higher binding affinity for this receptor than did RTX and because ADCC activity of S-RTX or A-RTX was very weak relative to RTX. Reduced/alkylated human anti-Rh(D) antibodies exhibit enhanced inhibition of phagocytosis [20]; this finding, together with our results, indicates that IgG that lacks interchain disulfide bonds might be more effective than an unmodified IgG for treatment of immune thrombocytopenia.

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References

[1] L.M. Weiner, R. Surana, S. Wang, Monoclonal antibodies: versatile platforms for cancer immunotherapy, Nat. Rev. Immunol. 10 (2010) 317–327.
[2] O.H. Brekke, T.E. Michaelsen, I. Sandlie, The structural requirements for complement activation by IgG: does it hinge on the hinge?, Immunol Today 16 (1995) 85–90.
[3] F. Nimmerjahn, J.V. Ravetch, Antibodies, Fc receptors and cancer, Curr. Opin. Immunol. 19 (2007) 239–245.
[4] T. Takai, Roles of Fc receptors in autoimmunity, Nat. Rev. Immunol. 2 (2002) 580–592.
[5] P. Sondermann, R. Huber, V. Oosthuizen, U. Jacob, The 3.2-Å crystal structure of the human IgG1 Fc fragment-FcγRIII complex, Nature 406 (2000) 267–273.
[6] Y. Masuho, K. Tomibe, K. Matsuzawa, A. Ohtsu, Development of an intravenous g-globulin with Fc activities: I. Preparation and characterization of S-sulfonated human g-globulin, Vox Sang. 32 (1977) 175–181.
[7] H. Liu, C. Chumsae, G. Gaza-Bulseco, K. Hurkmans, C.H. Radziejewski, Ranking the susceptibility of disulfide bonds in human IgG1 antibodies by reduction, differential alkylation, and LC–MS analysis, Anal. Chem. 82 (2010) 5219–5226.
[8] T.E. Michaelsen, Alteration of the conformation of human IgG subclasses by reduction of the hinge S–S bonds, Mol. Immunol. 25 (1988) 639–646.

[9] W.F. Dall'Acqua, K.E. Cook, M.M. Damschroder, R.M. Woods, H. Wu, Modulation of the effector functions of a human IgG1 through engineering of its hinge region, J. Immunol. 177 (2006) 1129–1138.

[10] R.L. Shields, A.K. Namenuk, K. Hong, Y.G. Meng, J. Rae, J. Briggs, D. Xiei, J. Lai, A. Stadlen, B. Li, J.A. Foxi, L.G. Presta, High resolution mapping of the binding site on human IgG1 for FcγRI, FcγRII, FcγRIII, and FcRn and design of IgG1 variants with improved binding to the FcγR, J. Biol. Chem. 276 (2001) 65910–66601.

[11] T. Shinkawa, K. Nakamura, N. Yamane, E. Shoji-Hosaka, Y. Kanda, M. Sakurada, K. Uchida, H. Anazawa, M. Satoh, M. Yamasaki, N. Hanai, K. Shitara, The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity, J. Biol. Chem. 278 (2003) 3466–3473.

[12] D.G. Maloney, Anti-CD20 antibody therapy for B-cell lymphomas, N. Engl. J. Med. 366 (2012) 2008–2016.

[13] G. Cartron, L. Dacheux, G. Salles, P. Solal-Celigny, P. Bardos, P. Colombat, H. Watier, Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIIa gene, Blood 99 (2002) 754–758.

[14] Y. Machino, H. Ohta, E. Suzuki, S. Higurashi, T. Tezuka, H. Nagashima, J. Kohroki, Y. Masuho, Effect of immunoglobulin G (IgG) interchain disulfide bond cleavage on efficacy of intravenous immunoglobulin for immune thrombocytopenic purpura (ITP), Clin. Exp. Immunol. 162 (2010) 415–424.

[15] H. Nagashima, T. Tezuka, W. Tsuchida, H. Maeda, J. Kohroki, Y. Masuho, Tandemly repeated Fc domain augments binding avidities of antibodies for Fcgamma receptors, resulting in enhanced antibody-dependent cellular cytotoxicity, Mol. Immunol. 45 (2008) 2752–2763.

[16] K. Cheent, S.I. Khakoo, Natural killer cells: integrating diversity with function, Immunology 126 (2009) 449–457.

[17] D.S. Wishart, C. Knox, A.C. Guo, D. Cheng, S. Shrivastava, D. Tzur, B. Gautam, M. Hassanali, DrugBank: a knowledgebase for drugs, drug actions and drug targets, Nucleic Acids Res. 36 (2008) D901–D906.

[18] M. Yagita, C.L. Huang, H. Umehara, Y. Matsuo, R. Tabata, M. Miyake, Y. Konaka, K. Takatsuki, A novel natural killer cell line (KHYG-1) from a patient with aggressive natural killer cell leukemia carrying a p53 point mutation, Leukemia 14 (2000) 922–930.

[19] G. Suck, D.R. Branch, M.J. Smyth, R.G. Miller, J. Vergidis, S. Fahim, A. Keating, KHYG-1, a model for the study of enhanced natural killer cell cytotoxicity, Exp. Hematol. 33 (2005) 1160–1171.

[20] A.S. Kruspe, Y. Katsman, D. Sakac, C. Chagneau, A. Glistvain, R.F. Langler, D.R. Branch, Reduction of disulfide bonds within anti-D results in enhanced Fcgamma receptor blockade, Transfusion 49 (2009) 928–936.