

Effect of immunoglobulin G (IgG) interchain disulfide bond cleavage on efficacy of intravenous immunoglobulin for immune thrombocytopenic purpura (ITP)

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Introduction

Intravenous immunoglobulins (IVIG) are immunoglobulin (Ig) preparations fractionated from pooled plasma of thousands of healthy volunteers. Because the intravenous administration of immunoglobulin (Ig) at high doses sometimes causes anaphylactic adverse reactions, which have generally been ascribed to complement activation by polymeric IgG in the preparations, Igs are treated with polyethylene glycol and/or low pH solution to decrease the amount of polymeric IgG, or modified by S-sulfonation, S-alkylation or with β -propiolactone to reduce complement activation [1].

Imbach *et al.* [2] found that IVIG therapy was effective in treating immune thrombocytopenic purpura (ITP), an autoimmune disorder characterized by a low platelet count and mucocutaneous bleeding. The antibody-sensitized platelets are eliminated by Fc γ receptor (Fc γ R)-bearing phagocytes [3]. The mechanisms of IVIG efficacy have been studied extensively, but remain enigmatic. Although some findings suggest that the Fab domain of IgG works in IVIG

Summary

Intravenous immunoglobulin (IVIG) has been used widely to treat immune thrombocytopenic purpura (ITP), but the mechanisms of its action remain unclear. We investigated the affinity for Fc γ receptors (Fc γ Rs) and the thrombocytopenia-ameliorating effect of S-sulfonated gammaglobulin (SGG) and S-alkylated gammaglobulin (AGG), in comparison with unmodified gammaglobulin (GG), in a mouse ITP model. Cleavage of immunoglobulin (Ig)G interchain disulfide bonds by either S-sulfonation or S-alkylation did not decrease the affinity for Fc γ RIIA (CD32A) and Fc γ RIIB (CD32B), but did decrease the affinity for Fc γ RIA (CD64A) and Fc γ RIIIA (CD16A), presumably because of changes in H-chain configuration. The interchain disulfide bond cleavage decreased the affinity much more for mouse Fc γ RIV than for mouse Fc γ RIIB. The ability of AGG to ameliorate ITP was greatly diminished, while SGG, whose disulfide bonds are reconstituted *in vivo*, was as effective as GG. These results suggest that the interchain disulfide bonds are important for therapeutic effect. It is also suggested that the interaction of IVIG with the inhibitory receptor Fc γ RIIB is insufficient for effective amelioration of ITP and that, at least in this model, direct binding of IVIG to Fc γ RIIIA is also required.

Keywords: Fc receptors, idiopathic thrombocytopenia purpuria/thrombocytopenia, intravenous immunoglobulin therapy, platelets, protein structure/folding

therapy [4], the Fc fragments from IVIG have been found to be as effective as IVIG itself in clinical ITP therapy [5] and in a mouse ITP model [6], implying an essential role of the interaction of the Fc domain and Fc γ Rs.

There are two functionally different kinds of Fc γ Rs: activating receptors such as Fc γ RIA (CD64A), Fc γ RIIA (CD32A) and Fc γ RIIIA (CD16A), and an inhibitory receptor, Fc γ RIIB (CD32B) [7]. A simple explanation of the efficacy of IVIG for ITP seems to be blocking of the activating receptors, as autoantibody-bearing platelets do not bind to the activating receptors. It was reported that IgG dimers contained in IVIG, rather than monomers, were effective in the mouse ITP model [8], but another report claimed that large amounts of IgG monomers worked as an antagonist for these receptors [9]. The current understanding of the involvement of those receptors in IVIG efficacy for ITP was reviewed recently by Crow and Lazarus [10], but the mechanisms involved are not known fully.

Samuelsson *et al.* [6] found that efficacy of IVIG for ITP was abrogated in Fc γ RIIB-deficient mice and also in mice

pretreated with an Fc γ RIIB-blocking antibody, suggesting a pivotal role of this inhibitory receptor. Furthermore, individual IgG subclasses have different ratios of affinity for activating Fc γ RIIA to inhibitory Fc γ RIIB (A/I ratios), and the IgG subclasses with higher A/I ratios showed more potent cytotoxic activity via Fc γ Rs [11]. In addition, IVIG blocked both the down-regulation of Fc γ RIIB and the up-regulation of Fc γ RIIA by C5a [12]. These findings indicate that the interaction of IVIG with Fc γ RIIB is essential for its effect on ITP and that IVIG with a lower A/I ratio may be more effective for treatment of ITP.

S-sulfonation [13] or S-alkylation following reduction [14] under mild conditions cleaves the interchain, but not the intrachain, disulfide bonds of IgG, resulting in decreased activity of some effector functions, such as complement activation. Recently, Dall'Acqua *et al.* [15] showed that recombinant IgG molecules without inter-H chain disulfide bonds had decreased antibody-dependent cellular cytotoxicity. However, the effects of these chemical modifications on the affinity for individual activating and inhibitory receptors and on the efficacy for ITP amelioration have not been investigated.

In the present study, we studied comparatively the affinity for individual Fc γ Rs and the ITP-ameliorating abilities of S-sulfonated GG (SGG), unmodified gammaglobulin (GG) and reduced and S-alkylated GG (AGG). Unexpectedly, cleavage of interchain disulfide bonds did not change the affinity for Fc γ RIIA and Fc γ RIIB, although it decreased affinity for Fc γ RIIA and Fc γ RIA. Consequently, the A/I ratios of SGG and AGG were much lower than those of GG. However, AGG showed greatly reduced efficacy in the mouse ITP model, while the efficacy of SGG was equivalent to that of GG. SGG is known to revert to unmodified GG via reconstitution of disulfide bonds *in vivo* [16] and the affinity of SGG for Fc γ RIIA was actually restored by reduction and reoxidation. We also discuss the reason why disulfide bond cleavage resulted in a decrease of affinity for Fc γ RIIA, but not Fc γ RIIs, in terms of the amino acid residues of IgG that are involved in Fc γ Rs-binding.

Materials and methods

Reagents

Gammaglobulin fractions (GG) and S-sulfonated GG (SGG; Venilon™) were kindly provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). The contents of IgG1, IgG2, IgG3 and IgG4 in these preparations were 54–58%, 38–43%, 2.2–3.3% and 0.7–0.9%, respectively. There were no significant differences in these contents among the preparations. Polyethylene glycol 4000 (PEG4000) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and was added to all solutions of GG, SGG and AGG and their fractions at a final concentration of 0.2% to prevent IgG aggregation.

Biochemical analyses of IVIG preparations

The interchain disulfide bonds between H and L chains were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, using 10% acrylamide gel. Samples were applied at 5 μ g protein/lane and the protein bands were stained with Bio-Safe Coomassie. The compositions of polymers, dimers and monomers in GG, SGG and AGG were analysed by gel filtration high-performance liquid chromatography (HPLC) on a Protein Pak 300SW column (Waters Corporation, Milford, MA, USA) at a flow rate of 1 ml/min in 0.1 M phosphate buffer (pH 6.6) containing 0.14 M NaCl at 25°C. Samples containing 10 μ g of protein were injected into the column and the elution profiles were analysed using HPLC ChromNAV (Jasco Corporation, Tokyo, Japan). The contents of polymers and dimers decreased gradually when the preparations were stored in a warm environment, and thus samples were stored at –80°C, kept at 4°C for no longer than a week, and analysed by HPLC just after having been warmed to room temperature.

Fractionation of polymers, dimers and monomers from GG

For small-scale fractionation, 0.8 mg of GG was applied to Protein Pak 300SW at a flow rate of 1 ml/min in 0.1 M phosphate buffer (pH 6.6) containing 0.15 M NaCl, at 25°C. Protein concentrations were determined from the absorbance at 280 nm and components were then analysed by gel filtration HPLC as described above.

For large-scale fractionation, 250 mg of GG was applied to a column (ϕ 2.5 cm \times 110 cm) of Sephacryl S-200HR (Sigma-Aldrich Co., St Louis, MO, USA) at a flow rate of 0.2 ml/min in 20 mM phosphate buffer, pH 7.4, containing 0.14 M NaCl and 0.2% PEG4000 [phosphate-buffered saline (PBS)/PEG] to separate the polymer + dimer fraction from monomer. The polymer + dimer fraction used in the mouse ITP model experiments contained 6.3% polymer and 40% dimer, along with monomer, and the monomer fraction was composed of 1.7% dimer and 98% monomer. The starting material, GG, contained 1.3% polymer and 13.5% dimer.

S-sulfonation, S-alkylation and reconstitution of the interchain disulfide bonds of SGG

S-sulfonated GG (SGG) was prepared using the method of Masuho *et al.* [13], with some modifications. GG 0.5 mg/ml (final concentration), sodium sulfite 270 mM (final concentration; Wako Pure Chemical Industries Ltd) and sodium tetrathionate 70 mM (final concentration; Sigma-Aldrich Co.) 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% PEG4000 (modification buffer). The

reaction mixture was incubated at 37°C for 4 h to cleave the interchain disulfide bonds and then dialysed against PBS/PEG. S-alkylated GG (AGG) was prepared as follows. The same batch of GG at the final concentration of 0.5 mg/ml and dithiothreitol at a final concentration of 10 mM were mixed in modification buffer. The reaction mixture was incubated at 37°C for 1 h to reduce the interchain disulfide bonds, and then proteins were allowed to react with iodoacetamide at a final concentration of 50 mM for 30 min at 37°C. The solution was dialysed against PBS/PEG to remove residual reagents. As control, GG was incubated without reagents at 37°C for 2 h.

The disulfide bonds of SGG were reconstituted according to Masuho *et al.* [17]. 2-Mercaptoethanol at a final concentration of 0.1 M was added to SGG at a final concentration of 5 mg/ml in modification buffer. After reduction at room temperature for 60 min, 2-mercaptoethanol was removed by dialysis against modification buffer. Air oxidation was performed by aerating the outer buffer during dialysis. The samples were then incubated in a rotating tube device at 37°C overnight.

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 [18]. Microtitre plates (Becton-Dickinson Co., Franklin Lakes, NJ, USA) were coated with 4 µg/ml of the FcγR proteins in PBS at 4°C overnight and blocked with 25 mM Tris HCl at 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. GG, SGG and AGG samples diluted serially in ELISA buffer were 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, then 0.1 µg/ml horseradish peroxidase (HRP)-conjugated goat anti-human IgG (H + L) affinity-purified F(ab')₂ (Chemicon International, Inc, Temecula, CA, USA) in ELISA buffer was dispensed into the wells and the plates were incubated at 37°C for 1 h. The plates were again washed in the same manner, 0.1 ml of 0.04% *o*-phenylenediamine dihydrochloride and 0.01% hydrogen peroxide in 0.1 M citrate buffer at pH 5.0 was dispensed into the wells, and the plates were allowed to stand at room temperature for 10 min. Absorbance at 450 nm was measured using a microplate reader Model 550 (Bio-Rad Laboratories, Hercules, CA, USA). The effective concentration (EC₅₀) value was calculated by measuring the IgG concentration that gave 50% of the maximum absorbance in Fcγ receptor-binding ELISA. A/I ratios were calculated by dividing the EC₅₀ for FcγRIIB by the EC₅₀ for FcγRIIIA.

The binding activity for mouse FcγRs was measured using the same methods as those for human FcγRs. Recombinant proteins consisting of the extracellular domains of mouse FcγRs were purchased from R&D systems, Inc. (Minneapolis,

MN, USA). As the binding affinity for mouse FcγRs was relatively low, GG and AGG were slightly cross-linked with 2-iminothiolane (Sigma-Aldrich Co.) and *N*-(8-Maleimidocapryloxy)sulfosuccinimide (Dojindo Laboratories, Tokyo, Japan) [19] and their affinity for mouse FcγRIIB and FcγRIV was assessed by ELISA on the mouse FcγR-coated plates.

Analysis of the three-dimensional structure involved in the interaction between IgG and FcγRs

The three-dimensional structure of the hinge region and Fc domain of human IgG1 (PDB ID; 1E4K) was displayed in the Corey–Pauling–Koltun (CPK) representation using Discovery Studio™ (Accelrys Software Inc., San Diego, CA, USA). The amino acids of IgG involved in the interaction with FcγRIIB and FcγRIII and the cysteines that form inter-H chain disulfide bonds were depicted in different colours, based on reported data [20,21].

Induction and treatment of ITP model mouse

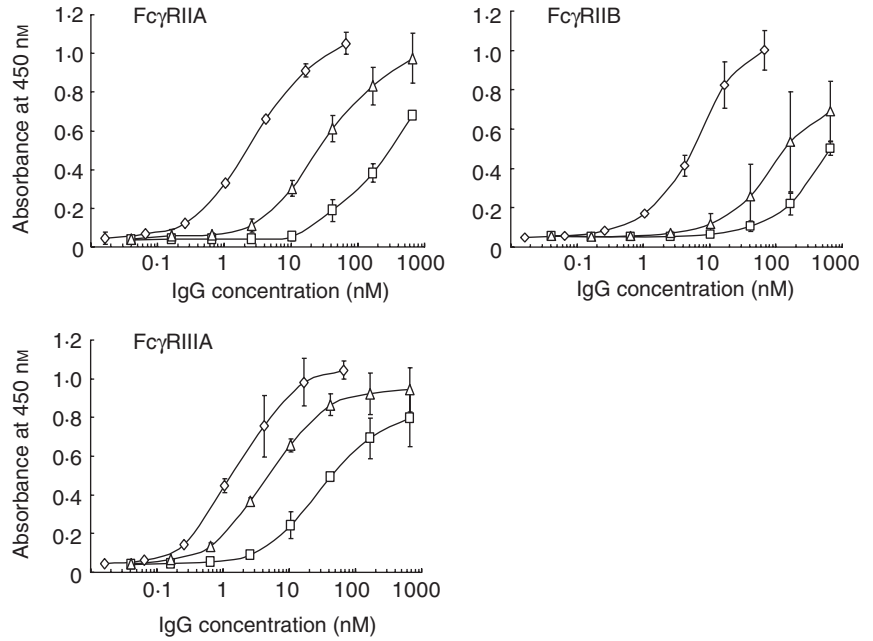
This study was approved by the Animal Experiment Committee of Tokyo University of Science. Male BALB/c mice, 5–6 weeks of age, were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). All mice were kept at the animal facility at Tokyo University of Science, and experiments were conducted while their body weight was in the range of 20–24 g. GG, SGG and AGG at various doses were administered intraperitoneally to the mice, and after 24 h the mice were rendered thrombocytopenic by intraperitoneal administration of 2 µg/mouse of mAb MWReg30 (rat IgG1 antibody against mouse integrin α_{IIb} or CD41; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Blood samples were collected from the tail vein 2 h before and 3, 6 and 24 h after the administration of MWReg30. Approximately 6 µl of blood was diluted with 56 µl of 1% ammonium oxalate and the platelet numbers were counted using an electronic cell counter (Celltac α; Nihon Kohden, Tokyo, Japan). Experiments were performed with groups of five mice, which were administered GG or SGG (five different batches) or AGG (single batch) intraperitoneally. The results shown here are representative of two or three separate experiments. The platelet counts of the five mice in each group were expressed as the mean ± standard deviation (s.d.). Statistical analysis was performed with Student's *t*-test. The criterion of significance was taken to be *P* < 0.05.

Results

Binding activity of IVIG for individual FcγRs

IVIg preparations contain non-covalent IgG polymer and dimer, as well as monomer. IVIg preparations containing IgG polymer were reported to be effective in the mouse ITP

Fig. 1. Fc γ receptor (Fc γ R)-binding activities of polymer, dimer and monomer fractions from gammaglobulin (GG). Those fractions were separated by gel filtration high-performance liquid chromatography (HPLC) and their binding activity was assessed by enzyme-linked immunosorbent assay (ELISA). The fractions were allowed to react with the extracellular domain of each Fc γ RIIA, Fc γ RIIB or Fc γ RIIIA coated on microtitre plates. The amounts of bound immunoglobulin G (IgG) were measured with horseradish peroxidase (HRP)-conjugated anti-human IgG (H + L) antibodies. Polymer (diamonds), dimer (triangles) and monomer (squares). Those fractions were separated into two independent experiments using two different batches of GG, and ELISA was performed in quadruplicate. The symbols indicate the means \pm standard deviation.



model, while monomer was not [8]. Thus, GG was separated into polymer, dimer and monomer fractions by gel filtration HPLC. The polymer fraction thus separated was composed of 81% polymer + dimer and 19% monomer. The dimer fraction was composed of 77% polymer + dimer and 23% monomer and the monomer fraction was composed of < 1% polymer + dimer and > 99% monomer (data not shown). The binding activity was examined by ELISA using the extracellular domains of Fc γ Rs (Fig. 1). The polymer fraction was much more active than the monomer fraction, i.e. it showed 140, 160 and 30 times greater affinity for Fc γ RIIA, Fc γ RIIB

and Fc γ RIIIA, respectively. The dimer fraction showed 10, three and nine times greater affinity than the monomer fraction for the three receptors, respectively.

The Fc γ R binding-activity of S-sulfonated GG (SGG) was compared with that of GG by means of Fc γ R ELISA (Fig. 2). SGG was found to be a little more active than GG in binding to Fc γ RIIB and as active as GG in binding to Fc γ RIIA, although it was only 1/40 to 1/50 as active as GG in binding to Fc γ RIA and Fc γ RIIIA. GG was composed of 1.3% polymer, 14% dimer and 85% monomer, while SGG was composed of 2.8% polymer, 16% dimer and 81% monomer.

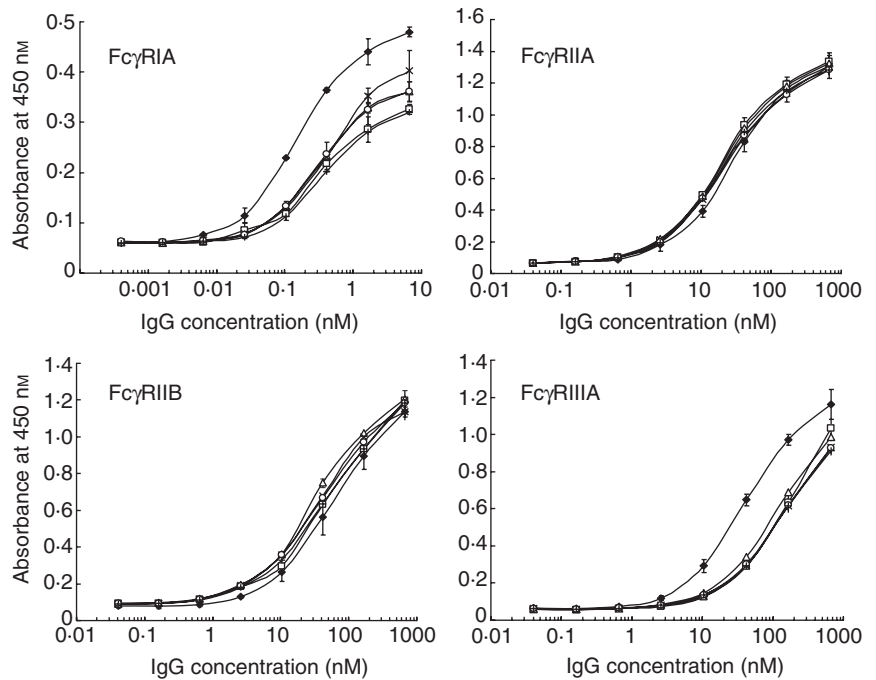


Fig. 2. Fc γ receptor (Fc γ R)-binding activity of S-sulfonated gammaglobulin (SGG) preparations. The binding activity of SGG and gammaglobulin (GG) was assessed by means of enzyme-linked immunosorbent assay (ELISA) as described in the legend of Fig. 1. Five different batches of SGG were assessed in triplicate and their individual binding curves are shown. Symbols indicate the means \pm standard deviation (s.d.). Three different batches of GG were also assessed; symbols (closed diamonds) indicate the means \pm s.d.

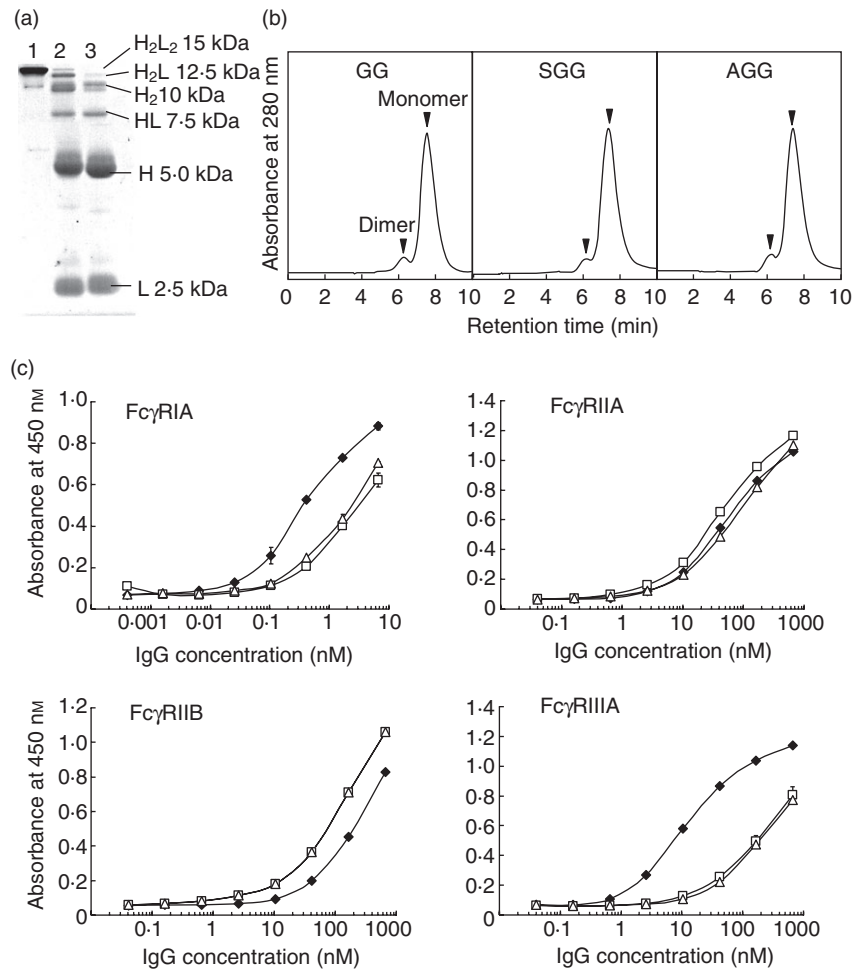


Fig. 3. Effect of interchain disulfide bond cleavage on Fc γ receptor (Fc γ)-binding activity. The interchain disulfide bonds of immunoglobulin G (IgG) were cleaved by S-sulfonation or S-alkylation with prior mild reduction, using the same batch of gammaglobulin (GG). (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Lanes 1, 2 and 3 are GG, S-sulfonated gammaglobulin (SGG) and S-alkylated gammaglobulin (AGG), respectively. (b) Profiles of gel filtration high-performance liquid chromatography (HPLC). (c) Fc γ R-binding activity of SGG (open squares) and AGG (open triangles) compared with GG (closed diamonds). Enzyme-linked immunosorbent assay (ELISA) was performed in triplicate. The symbols indicate the means \pm standard deviation.

The differences between GG and SGG in individual Fc γ R-binding activity cannot be explained by the differences in composition.

Effect of interchain disulfide bond cleavage on Fc γ R-binding activity

To compare the Fc γ R binding activity more precisely, SGG and AGG were prepared from the same batch of GG. S-sulfonation or S-alkylation with prior reduction under mild conditions cleaved the interchain disulfide bonds to give H and L chain bands with small amounts of H₂L, H₂ and HL in SDS-PAGE under non-reducing conditions (Fig. 3a). The polymer and dimer fractions of SGG and AGG showed very similar protein bands in SDS-PAGE, indicating that their interchain disulfide bonds had been cleaved to essentially the same degree (data not shown). There was no significant difference in gel filtration HPLC profiles among GG, SGG and AGG, indicating that their composition of polymers, dimers and monomers is also essentially the same (Fig. 3b).

SGG and AGG showed almost the same binding curves for all four Fc γ Rs (Fig. 3c). Both SGG and AGG were as potent as

the parental GG in binding to Fc γ RIIA and three times more potent than GG in binding to Fc γ RIIB, whereas their binding activity was decreased to 1/10 and 1/25 for Fc γ RIA and Fc γ RIIA, respectively. The A/I ratios were calculated by dividing the EC₅₀ for Fc γ RIIB by the EC₅₀ for Fc γ RIIA. The A/I ratios of these modified GGs were decreased to 1/70–1/80 compared with that of the parental GG (Table 1). Therefore, cleavage of the interchain disulfide bonds leads to decreased binding activity for Fc γ RIIA and a slight enhancement of binding to Fc γ RIIB.

Why does cleavage of the interchain disulfide bonds decrease the binding activity for Fc γ RIIA, but not for Fc γ RIIB? The amino acids in the Fc fragment involved in binding to Fc γ RIII were assigned by crystallographic study of the complex of Fc fragment and Fc γ RIII [22]. Fc γ RIII is in contact with Leu²³⁵, Gly²³⁶, Gly²³⁷, Ser²³⁹ and Asp²⁶⁵ on one H chain and with Leu²³⁵, Gly²³⁶, Pro³²⁹ and Ala³³⁰ on the other H chain (Fig. 4a). Fc γ RIIB, a peptide derived from the Fc domain, Thr²⁵⁶-Pro²⁷¹, was found to bind to Fc γ RIIB with an affinity close to that of IgG [20]. Those amino acids were superimposed upon the Fc tertiary structure (Fig. 4b). It appears that a single molecule of Fc γ RIIB binds to these amino acids on one H chain but not the other H chain.

Table 1. Comparative binding activities of gammaglobulin (GG), S-sulfonated gammaglobulin (SGG) and S-alkylated gammaglobulin (AGG) for different Fcγ receptors.

Preparation	EC ₅₀ (nM)				A/I ratio
	FcγRIA	FcγRIIA	FcγRIIB	FcγRIIIA	
GG	0.31	60	270	12	23
SGG	2.8	39	92	300	0.31
AGG	2.2	79	92	320	0.29

The activity [effective concentration (EC₅₀)] was calculated by measuring the immunoglobulin G (IgG) concentrations that gave 50% of the maximum binding values, based on the results shown in Fig. 3. A/I ratios were calculated by dividing the EC₅₀ for Fcγ receptor IIB (FcγRIIB) by the EC₅₀ for FcγRIIIA.

Cleavage of interchain disulfide bonds must change the configuration of the two H chains in the C_{H2} domain close to the hinge. Thus, it is suggested that the reason for the selective decrease of the affinity for FcγRIIIA by cleavage of the interchain disulfide bonds would be that FcγRIIIA recognizes both H chains, whereas FcγRIIB recognizes only one of them.

Ability of IVIG to ameliorate thrombocytopenia

The binding activity for mouse FcγRs was measured by the same methods as that for human FcγRs. The mouse orthologues of human FcγRIIB and FcγRIIIA are FcγRIIB and FcγRIV, respectively [11]. GG and AGG were dimerized with chemical linkers because their binding affinity for mouse FcγRs was relatively low. The dimerized GG showed a high

affinity for mouse FcγRIV (EC₅₀ 14 nM) compared with mouse FcγRIIB (EC₅₀ 230 nM), while the dimerized AGG showed EC₅₀ values of 240 nM and 380 nM for FcγRIV and FcγRIIB, respectively (Fig. 5). The A/I ratios were 16 and 1.6 for GG and AGG, respectively. Thus, cleavage of the interchain disulfide bonds resulted in decrease of the A/I ratio for mouse FcγRs, as with the ratio for human FcγRs.

The ability of IVIG to ameliorate thrombocytopenia was assessed in the mouse ITP model. IVIG was administered intraperitoneally to the mice, which were rendered thrombocytopenic 24 h later by intraperitoneal administration of rat IgG1 mAb specific for mouse integrin α_{IIB} (CD41). First, the polymer + dimer fraction and the monomer fraction separated from GG by gel filtration chromatography were compared with respect to amelioration of thrombocytopenia. The polymer + dimer fraction contain-

Fig. 4. The amino acid residues involved in binding to Fcγ receptor III (FcγRIII) and FcγRIIB. (a) FcγRIII-binding sites. (b) FcγRIIB-binding sites. The three-dimensional structure of Fc fragment is illustrated in the Corey–Pauling–Koltun (CPK) representation using Discovery Studio™ software. The two H chains are drawn in two different colours, grey and white, along with their carbohydrates in dark grey. The amino acid residues involved in binding to FcγRIII and FcγRIIB are depicted in red, and the cysteine residues of H–H interchain disulfide bonds are shown in yellow.

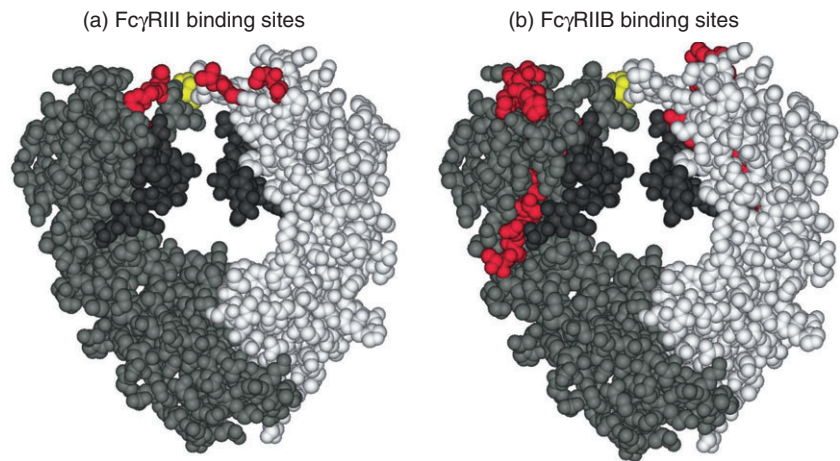
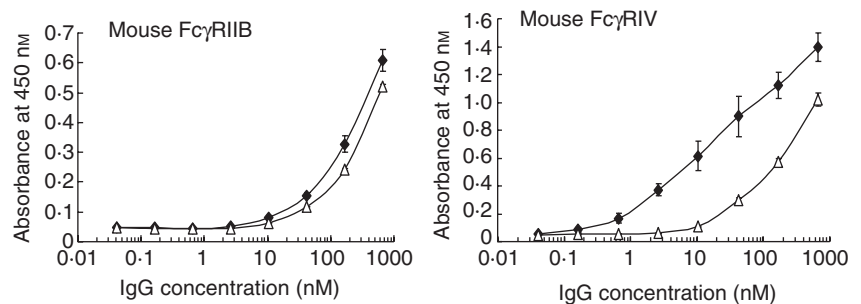


Fig. 5. Effect of the interchain disulfide bond cleavage on mouse Fcγ receptor (Fcγ)-binding activity. The activity of dimerized gammaglobulin (GG) (closed diamonds) and dimerized S-alkylated gammaglobulin (AGG) (open triangles) for mouse FcγRIIB and FcγRIV, respectively, was measured by enzyme-linked immunosorbent assay (ELISA) in triplicate and the symbols indicate the means ± standard deviation.



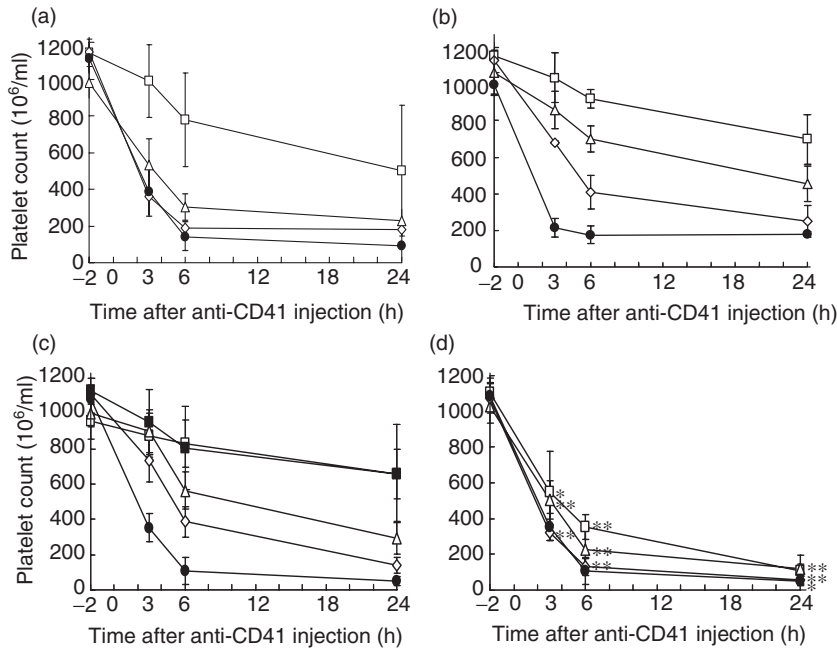


Fig. 6. Amelioration of thrombocytopenia with intravenous immunoglobulin (IVIG) preparations. IVIG was administered intraperitoneally to groups of five mice. After 24 h, 2 μ g of anti-CD41 monoclonal antibody (mAb) was administered intraperitoneally. Blood samples were obtained 2 h before and 3, 6 and 24 h after administration of anti-CD41 mAb. Platelets were counted by an electronic cell counter. The means \pm standard deviation (s.d.) are indicated by symbols with bars. (a) Thrombocytopenia amelioration with GG, polymer + dimer fraction and monomer fraction separated from gammaglobulin (GG). Polymer + dimer fraction (open squares), monomer fraction (open diamonds) or the parental GG (open triangles) was administered intraperitoneally at a dose of 0.2 g/kg. Phosphate-buffered saline (PBS) was used as the control (closed circles). (b) Amelioration of thrombocytopenia with GG at the dose of 2 g/kg (open squares), 1 g/kg (open triangles) or 0.5 g/kg (open diamonds). The control was PBS (closed circles). (c) Amelioration of thrombocytopenia with S-sulfonated gammaglobulin (SGG). The doses are the same as those in (b). In an additional experiment, 6 h before anti-CD41 mAb, 2 g/kg of SGG was administered intraperitoneally and the platelets were counted (closed squares). (d) Amelioration of thrombocytopenia with S-alkylated gammaglobulin (AGG). The doses are the same as those in (b). An asterisk and double asterisk indicate significant differences ($P < 0.01$ and $P < 0.001$, respectively) between the platelet counts with S-alkylated gammaglobulin (AGG) and GG. The platelet counts of the five mice in each group were expressed as the mean \pm s.d.

ing 6.3% polymer and 40% dimer ameliorated thrombocytopenia at a dose of 0.2 g/kg, whereas the monomer fraction containing only 1.7% dimer showed no efficacy (Fig. 6a). The parental GG was hardly effective at this dose in ITP. These results are consistent with the binding activity of these fractions for Fc γ R (Fig. 1). In addition, the polymer + dimer fraction was five to 10 times more effective than unfractionated GG in the amelioration of thrombocytopenia (Fig. 6b).

Various doses of GG, SGG and AGG were administered 24 h before induction of thrombocytopenia. GG and SGG induced amelioration in a dose-dependent manner (Fig. 6b and c). SGG was as effective as GG at all three doses tested and at all four time-points tested. Further, the platelet counts were higher than those with PBS even at the dose of 0.5 g/kg, with statistical significance ($P < 0.001$). There was no statistically significant difference ($P > 0.01$) between the platelet counts in mice treated with SGG and GG. Conversely, AGG was much less effective than either GG or SGG in terms of platelet counts, with statistical significance ($P < 0.001$ or < 0.01) at all the tested doses and at 3, 6 and 24 h (Fig. 6d). These results suggest that the cleavage of the interchain

disulfide bonds greatly decreases not only the binding activity for Fc γ R1IIIA, but also the ability to ameliorate thrombocytopenia.

Restoration of the Fc γ R1IIIA-binding activity of SGG by *in vitro* reconstitution of the disulfide bonds

In a clinical study of patients with agammaglobulinaemia it was found that the disulfide bonds of SGG were reconstituted [16]. Our finding that the ameliorating ability of SGG was equivalent to that of GG implies that the disulfide bonds are also reconstituted in mice.

To determine whether SGG recovers Fc γ R-binding activity following reconstitution of the disulfide bonds, three batches of SGG were reduced with 2-mercaptoethanol and then reoxidized by aeration. The reconstituted SGG (R-SGG) showed the H₂L₂ band with a very small amount of H₂L band and without either H or L chain band in SDS-PAGE under non-reducing conditions (data not shown). The Fc γ R-binding activity of R-SGG was compared with those of SGG and GG. The binding activity for Fc γ R1IIIA was completely

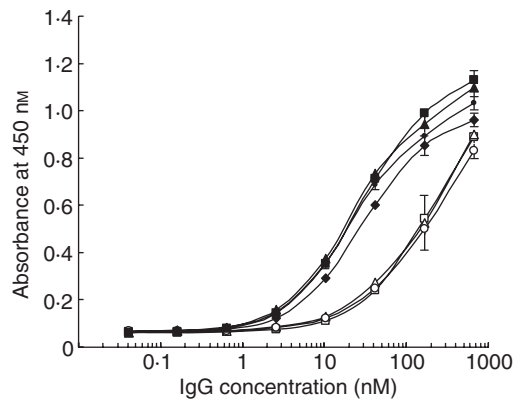


Fig. 7. Fc γ receptor IIIA (Fc γ RIIIA)-binding activity of R-SGG, prepared by *in vitro* reconstitution of the disulfide bonds of SGG. Three batches of S-sulfonated gammaglobulin (SGG) (open squares, triangles and circles) were reduced separately and re-oxidized to prepare R-SGG (closed squares, triangles and circles). Their Fc γ RIIIA-binding activity was compared with that of GG (closed diamonds).

recovered (Fig. 7), and the differences in binding to the other three Fc γ Rs between SGG and GG were also abrogated (data not shown). Administration of SGG at 6 h before injection of anti-CD41 mAb ameliorated thrombocytopenia as effectively as administration 24 h before injection, suggesting that the reconstitution of the disulfide bonds occurs within 6 h (Fig. 6c). These results suggest that SGG would recover its binding activity quickly for Fc γ RIIIA *in vivo*.

Discussion

We determined the binding activity of S-sulfonated GG (SGG) for activating and inhibitory Fc γ Rs compared with that of GG in order to elucidate the mechanism of amelioration of ITP by IVIG, especially in connection with the role of the interchain disulfide bonds in the hinge region of IgG. SGG retained binding activity for both Fc γ RIIA and Fc γ RIIB, but showed greatly decreased binding to Fc γ RIA and Fc γ RIIIA (Fig. 2). The binding activity would be influenced not only by S-sulfonation, but also by differences in the contents of polymer and dimer in the preparations (Fig. 1). Thus, SGG and mildly reduced and S-alkylated GG (AGG) were prepared from the same batch of GG and compared with the parental GG. SGG and AGG showed the same gel-filtration profiles, confirming that their compositions were the same as that of GG (Fig. 3b). Although the cysteine residues of GG were modified with different residues, $-\text{SO}_3\text{Na}$ for S-sulfonation and $-\text{CH}_2\text{CONH}_2$ for S-alkylation, SGG and AGG showed almost the same binding activity for all four Fc γ Rs. SGG and AGG were found not to show decreased affinity for Fc γ RIIA and Fc γ RIIB, but the affinity was decreased to 1/25 for Fc γ RIIIA and 1/10 for Fc γ RIA (Fig. 3c). Therefore, the cleavage of interchain disulfide bonds by S-sulfonation or S-alkylation leads to a

decrease in the affinity for Fc γ RIA and Fc γ RIIIA without any reduction in the affinity for Fc γ RIIA and Fc γ RIIB. Consequently, the A/I ratios of SGG and AGG were much lower than those of GG (Table 1).

To understand the different effects of disulfide bond cleavage on the binding activity of IVIG for the different Fc γ Rs, the tertiary structures of the sites that interact with the receptors were analysed by three-dimensional display of the Fc domain. Crystallographic study [22] of the complex of Fc and Fc γ RIII has revealed that Fc γ RIII interacts with amino acids of both H chains comprising the N-terminal region of the C_H2 domain (Fig. 4a). Conversely, a peptide from the Fc domain of human IgG1, Thr²⁵⁶-Pro²⁷¹, which bound to Fc γ RIIB as potently as IgG1 itself [20], was found to be located at a site on the H chain which is distant from the corresponding site of the other H chain (Fig. 4b). Thus, a single molecule of Fc γ RIIB seems to bind to only one of the two H chains, i.e. Fc γ RIII recognizes both H chains while Fc γ RIIB recognizes only one of them. It seems likely that cleavage of the interchain disulfide bonds would change the configuration between the two H chains in the hinge and the C_H2 domain [23], and this may be the reason why SGG and AGG retained binding activity for Fc γ RIIB but showed reduced binding activity for Fc γ RIIIA.

This explanation is also supported by stoichiometric analyses, indicating that IgG forms a complex with Fc γ RIII at a molar ratio of 1:1 [24] and a complex with Fc γ RII at a molar ratio of 1:2 [25]. In this connection, fucose-deficient IgG was much more active than fucosylated IgG in binding to Fc γ RIII, while fucose-deletion did not affect binding to Fc γ RII [26]. A comparison of the crystal structure of fucose-deficient IgG with that of the parental IgG revealed only a subtle conformational alteration in a limited region of the Fc domain [27]. It seems likely that modification of the sugar moiety would change the configuration of the two H chains in a similar manner to disulfide bond cleavage.

A mouse ITP model induced with anti-CD41 mAb was employed to evaluate the efficacy of GG, SGG and AGG for ameliorating thrombocytopenia. In connection with the mouse experiments, the binding affinity for mouse Fc γ Rs was assessed by ELISA with the Fc γ R extracellular domains. The cleavage of interchain disulfide bonds decreased the binding affinity for mouse Fc γ RIV much more than that for mouse Fc γ RIIB (Fig. 5) and consequently decreased the A/I ratio, as with that for human Fc γ Rs. In this ITP model, the polymer + dimer fraction apparently ameliorated thrombocytopenia, whereas the monomer fraction was ineffective (Fig. 6a). These results are consistent with previous findings using aged IVIG [8] and immune complexes [28]. SGG in which the disulfide bonds were cleaved reversibly was found to be as effective as GG for amelioration of thrombocytopenia in the mouse ITP model, whereas AGG in which the disulfide bonds were cleaved irreversibly was only one-quarter as effective as GG and SGG (Fig. 6). These results suggest that SGG reverted rapidly to the parental GG via

reconstitution of the disulfide bonds. We confirmed that R-SGG, prepared by *in vitro* reduction and reoxidation, recovered binding activity for Fc γ RIIIA, as well as the other Fc γ Rs (Fig. 7).

AGG showed a very low ratio of affinity for activating Fc γ RIIIA to inhibitory Fc γ RIIB (A/I ratio); its A/I ratio was only 1/80 of that of GG (Table 1). Thus, it was anticipated that AGG would stimulate Fc γ RIIB more than GG, inducing inhibitory signals, and that AGG would not stimulate Fc γ RIIIA as much as GG on the receptor-bearing phagocytes, leading to more efficient amelioration than in the case of GG. This view is supported by several reports. For example, the co-clustering of Fc γ RIIB with Fc γ RIIA on monocytes induced an inhibitory signal [29]. Mouse IgG subclasses of higher A/I ratio were more cytotoxic [11]. A Fc γ -Fc ϵ fusion protein co-clustered Fc γ RIIB and Fc ϵ RI to suppress the functions of Fc ϵ RI [30]. However, the present study revealed that AGG was much less effective than GG in the mouse ITP model. This may indicate that the interaction of polymeric IgG molecules with the inhibitory receptor Fc γ RIIB is insufficient, and that interaction with Fc γ RIIIA is necessary for full amelioration of ITP by IVIG.

There are several possible explanations of our result that the direct interaction of IVIG with Fc γ RIIB is insufficient for ITP amelioration, based on recent work. (i) The simplest explanation would be blocking of the binding of antibody-bearing platelets to Fc γ RIIIA in the reticuloendothelial system [31]. AGG was 1/25 as active as GG in binding to Fc γ RIIIA (Table 1). (ii) Boruchov *et al.* [32] showed that co-stimulation involving not only Fc γ RIIB, but also Fc γ RIIIA, was necessary for the inhibitory effect in dendritic cells. Their findings suggest that the interaction of the polymeric IgG molecules in IVIG with not only Fc γ RIIB, but also Fc γ RIIIA, would be necessary for ITP amelioration. (iii) The results of adoptive transfer of cells treated with IVIG suggest that IVIG stimulates the 'initiator' dendritic cells through Fc γ RIIIA, but not Fc γ RIIB, and that the initiator cells affect the secondary 'effector' cells, which require Fc γ RIIB for ITP amelioration [33]. This mechanism does not involve direct binding of IVIG to Fc γ RIIB. (iv) IVIG has been shown to up-regulate interferon γ receptor 2, suppressing macrophage activation. This mechanism is also dependent upon Fc γ RIIIA and independent of Fc γ RIIB [34]. Taken together, our results suggest a significant role of the interactions of IVIG with not only Fc γ RIIB, but also Fc γ RIIIA in ITP amelioration.

From the viewpoint of clinical ITP therapy, the results in the mouse ITP model indicate that SGG would be as effective as GG, because R-SGG, which was prepared by reconstituting the disulfide bonds of SGG *in vitro*, recovered binding activity for Fc γ RIIIA. Indeed, efficacy of SGG for ITP amelioration has been proved clinically [35]. In contrast to SGG, AGG, in which the interchain disulfide bonds are cleaved irreversibly, showed decreased ability to ameliorate thrombocytopenia in the mouse ITP model, suggesting that AGG would not be suitable for ITP therapy. Nachbaur *et al.* [36]

referred to AGG as one of the available IVIG preparations, but we could not find any literature on ITP therapy with AGG. Lastly, polymer and dimer contained in IVIG were much more active than monomer in terms of Fc γ R-binding and efficacy in the ITP model, suggesting that IVIG preparations with a higher content of polymeric IgG molecules would be more effective for ITP therapy, although possible adverse effects would need to be considered carefully.

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Disclosure

None.

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