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Chemically dimerized intravenous immunoglobulin has potent ameliorating activity in a mouse immune thrombocytopenic purpura model

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ABSTRACT

High-dose intravenous immunoglobulin (IVIG) preparations are currently used for the treatment of autoimmune diseases such as immune thrombocytopenic purpura (ITP). Although the mechanisms of IVIG efficacy remain enigmatic, some clinical and laboratory studies suggest that interaction of the Fc domain of IgG, especially the Fc domain of dimeric IgG, with its receptors (Fc gamma receptors; FcγRs) plays an essential role. In this study, IVIG was dimerized with chemical crosslinkers to augment its therapeutic efficacy. Dimerized IVIG was found to have a much higher affinity for FcγRs than monomeric IVIG. In a mouse ITP model, chemically dimerized IVIG abrogated the decrease in platelet numbers in the blood that was caused by an anti-platelet antibody at a dose that was one tenth of the required dose of IVIG. These results suggest that chemical dimerization of IVIG should greatly improve the efficacy of IVIG therapy of ITP.

1. Introduction

Intravenous immunoglobulins (IVIG) are preparations of gammaglobulin (GG) that are treated so that they do not cause untoward reactions when they are administered intravenously. IVIG was originally used as an antibody substitution therapy for agammaglobulinemic patients. Currently, IVIG is also used for therapy of autoimmune diseases such as Guillain–Barré syndrome and inflammatory polyneuropathy [1,2]. IVIG was first used for autoimmune disease by Imbach et al. [3], who found that IVIG therapy was effective for the treatment of immune thrombocytopenic purpura (ITP). ITP is an autoimmune disease that is characterized by a platelet decrease, which is mediated by pathogenic anti-platelet antibodies [4]. This platelet decrease was suggested to be mediated by Fcγ receptor (FcγR)-bearing macrophages in the reticuloendothelial system (RES) [5]. The mechanisms of IVIG efficacy have been extensively studied but remain to be elucidated. Although some reports suggest that the Fab domain of the IgG in IVIG neutralizes pathogenic factors such as complement and cytokines [6–8], the Fc fragments of IVIG have been found to be as effective as IVIG itself, not only in the mouse ITP model, but also in clinical ITP therapy [9,10]. These findings suggest that interaction of the Fc domain with FcγRs plays an essential role in IVIG effects.

Three different types of FcγRs are expressed on phagocytic cells [11]. FcγRI is a high affinity receptor for the Fc domain of IgG and binds monomeric IgG, whereas FcγRII and FcγRIII are receptors that preferentially bind immune complexes (IC) or polymeric IgG, which they bind with low affinity. These receptors are functionally divided into activating receptors such as FcγRI, FcγRIIA and FcγRIIIA, and an inhibitory receptor, FcγRIIB [12]. Regarding Fc receptor-mediated mechanisms that mediate IVIG efficacy for ITP therapy, a simple explanation is that IVIG blocks activating Fc receptors, thereby preventing the binding of autoantibody-bearing platelets to these activating receptors [13]. In the other report, immunosuppression that was mediated by activating FcγRs on dendritic cells was observed in an ITP model [14]. In addition, we recently found that cleavage of the interchain disulfide bonds of the IgG in IVIG decreased the binding of IVIG to FcγRIIIA but not to either FcγRIIA or FcγRIIB and reduced the ability of IVIG to ameliorate ITP in a model mouse [15]. These data suggest that IVIG binding to Fc receptors, especially activation receptors, is essential for therapeutic activity in mouse ITP model.

IVIG is clinically used at high dose (up to 2 g/kg) and its consumption is increasing. Materials of IVIG are prepared from blood donation, thus the supplied amount has limitations. In fact, product shortage occurred during the late 1990s [16]. Thus, further augmentation of therapeutic activity of IVIG is necessary to overcome these problems. Teeling et al. showed that a small amount of IgG dimers are present in IVIG preparations and that it is the IgG dimers and not the IgG monomers that are effective for amelioration of mouse ITP [17]. Actually, we showed IgG dimers in IVIG had potent binding activity to FcγRs [15]. These results suggest that a

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dimeric IgG fraction could potentially be used instead of IVIG for autoimmune disease therapy. However, the IgG dimers and monomers in IVIG exist in a dynamic equilibrium that depends on the IgG concentration and the temperature [18]. It is therefore difficult to make an IVIG preparation that is composed only of dimeric IgG. In this study, we prepared IVIGs that were stably dimerized by chemical crosslinking and evaluated their binding to FcγRs and their therapeutic activity in a mouse ITP model.

2. Materials and methods

2.1. IVIG

In this study, gammaglobulin fractions (GG) were used instead of IVIG. GG were kindly provided by The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan).

2.2. Gel filtration analysis

A gel filtration HPLC was done by using a Protein Pak 300SW column (Waters, Milford, MA) 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. The protein concentration in the eluate was determined by measurement of the absorbance at 280 nm and the chromatographic results were analyzed using the software package HPLC ChromNAV (JASCO Corporation, Tokyo, Japan).

2.3. Preparation of chemically dimerized and polymerized GG

We prepared highly purified chemically dimerized and polymerized IgG as described below. To separate monomeric IgG from GG, GG was applied to a Sephacryl S-200HR column (φ2.5 cm × 110 cm) at a flow rate of 0.2 ml/min in PBS containing 0.2% PEG4000. One half of the separated monomers were reacted with 2-iminothiolane (2-IT, Sigma–Aldrich Co.) in pH 7.5 and the other half were reacted with Sulfo-HMCS (Dojindo Lab., Kumamoto, Japan) in pH 7.0 at the ratio of IgG: crosslinker = 1:2.5 at 26 °C for 1 h. These modified IgGs were mixed in pH 7.0 at 26 °C for 1 h to form crosslinks. Free maleimide residues and SH groups were blocked by adding cysteine and iodoacetamide. The IgG was then purified by two gel-filtration chromatography steps and a highly purified chemically polymerized and dimerized IgG was obtained.

For *in vivo* use, a dimer-rich fraction was prepared using the above chemical method with a slight modification. Briefly, GG was directly reacted with 2-IT or Sulfo-HMCS without separation of monomers, and the dimers and polymers were separated from the monomers by one gel-filtration step.

2.4. Evaluation of FcγR-binding activities and antigen-binding activity by Enzyme-linked immunosorbent assay (ELISA)

FcγR-binding activities were measured by ELISA [15]. For measurement of antigen-binding activity, *Escherichia coli* (*E. coli*) DH-5α was used as an antigen. Microtiter plates (Becton–Dickinson Co., NJ, USA) were coated with *E. coli* that was diluted to an OD_{600nm} of 0.4 in 20 mM phosphate buffer (pH 7.0) containing 0.02% sodium azide at 4 °C overnight and subjected to the ELISA [15].

2.5. Induction and treatment of an 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. Mice were rendered thrombocytopenic by mAb MWReg30 (rat IgG1 antibody against mouse CD41; Santa Cruz Biotechnology Inc., CA, USA) and treated with GG or prepared dimers [15]. Blood samples were collected from the tail vein 6 h after MWReg30 administration. Groups of five mice were used for each experiment. The platelet counts of the five mice in each group are expressed as means ± SD. Statistical analysis was performed using Welch's *t*-test. The criterion of significance was taken to be $P < 0.05$.

3. Results

3.1. Preparation of chemically dimerized GG

For preparation of chemically dimerized GG, we used a two-step method with two chemical crosslinkers to avoid intramolecular crosslinking and to control formation of dimeric IgG. One crosslinker was 2-IT which react to primary amines to introduce sulfhydryl (SH) groups. Introduced 2-IT has 8.1 Å ethylene group spacer arm and confer flexibility. The other was Sulfo-HMCS which also react to primary amines to introduce maleimide residues. Introduced Sulfo-HMCS has 11.5 Å ethylene group spacer arm and confer flexibility. SH groups introduced molecules should react only to molecule with maleimide residues. As the result, intramolecular crosslinking does not occur, and dimerized GG has long flexible spacer between molecules. In practice, one half of the monomeric GG was reacted with 2-IT to introduce SH groups and the other half of the GG was reacted with Sulfo-HMCS to introduce maleimide residues. The two modified GGs were then mixed to form crosslinks between the introduced SH and maleimide groups, resulting in GG dimers and polymers (Fig. 1A).

The efficiency of introduction of SH groups and maleimide residues into IgG by 2-IT and Sulfo-HMCS depended on the IgG and crosslinker concentration, reaction time, temperature and the pH of the reaction buffer. Approximately 1 SH group and 1 maleimide residue were introduced per IgG molecule using the protocol described in the Methods section (data not shown). Since chemically linked IgG molecules contain not only dimers but also monomers or polymers (>trimer), the removal of polymers and monomers was required to obtain chemically linked dimers with high purity. Purification of the products of crosslinked IgG monomers by two sequential gel-filtration chromatography steps yielded highly purified chemically crosslinked dimers and polymers. Thus, gel-filtration HPLC analysis indicated that the highly purified crosslinked dimers did not contain polymers or monomers and that the crosslinked polymers were also a highly pure preparation (Fig. 1B). These crosslinked preparations were used for *in vitro* characterization. However, only a very small amount (about 2 mg) of highly purified dimers was obtained from an initial 250 mg GG using this procedure. Therefore, for *in vivo* use, a chemically crosslinked dimer-rich fraction of GG was obtained by modification of the protocol described in Section 2. The resulting chemically crosslinked dimer-rich fraction was composed of 36.1% polymer and 35.8% dimer (data not shown).

3.2. Receptor-binding and antigen-binding activities of chemically linked dimers

The binding of crosslinked IgG dimers and polymers to FcγRs was measured using ELISA. For comparative purposes, non-crosslinked polymer, dimer and monomer fractions that were obtained from GG by gel filtration were also assayed. The polymer, dimer and monomer composition of these non-crosslinked fractions were first analyzed by HPLC before their FcγR binding activity was assayed.

HPLC analysis of the composition of the GG fractions obtained by gel filtration indicated that the percent of IgG present as